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Activation of Mouse B Lymphocyte by Proteins Containing Hexahistidine

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Many recombinant proteins are produced as a fusion protein tagging hexahistidine, which has been used for studying their biological function both *in vitro* and *in vivo*. Unexpectedly, we observed activation of BALB/c mouse splenocytes when treated with hexahistidine-tagged recombinant proteins. This activation was hexahistidine-specific since the anti-pentahistidine antibody completely neutralized the effect, and the bovine serum albumin conjugated with the hexahistidine peptides also showed a similar activation effect. The B cells seemed to be the activated splenocytes, since the cell population stained with the anti-immunoglobulin antibody and anti-CD80 antibody was increased after the treatment. However, the activation signal by hexahistidine was insufficient to fully differentiate the B lymphocytes. This result suggests that caution must be taken in the use of hexahistidine-tagged recombinant proteins, due to their nonspecific activation of B lymphocyte.

Keywords: Activation; B lymphocytes; Hexahistidine.

Introduction

The hexahistidine (6× his) has been successfully employed as a fusion tag for the overexpression of proteins of interest in *E. coli* (Back *et al*., 2000; Oem *et al*., 2000; Stuber *et al*., 1990). This system also enables a single-step purification of recombinant proteins by using metal-affinity chromatography (Gu *et al*., 1994). Presently, a lot of recombinant proteins are expressed as hexahistidine-tagged proteins in various hosts, including yeast and mammalian cells (Feaver *et al*., 1993; Schmitt and Stunneberg, 1993). Most of these recombinant proteins could be further processed by proteolytic digestion to remove the 6× his-tag. However, fusion proteins retaining the hexahistidine-tag have also been used for studying their biological functions (Tian *et al*., 1999; Weining *et al*., 1998; Zhang *et al*., 1999). We identified the murine endogenous leukemia retroviral envelope protein as an autoantigen that is reactive with sera from prediabetic non-obese diabetic mice (Kang *et al*., 1998). To investigate the immunological role of this envelope protein, the recombinant envelope proteins expressed in fusion to a hexahistidine and S-protein (Kim *et al*., 1999) were mixed with mouse splenocytes. Interestingly, the activation of splenocytes demonstrated by the enlargement and aggregation of cells was observed.

Antigen-induced activation is a major source of the activation of immune cells. Proteins and small peptides serve as the major antigens for the activation of B- and T-lymphocytes, while carbohydrate polymers can be non-specific mitogenic activators. For example, concanavalin A or phytohemagglutinin is the well-known T cell mitogens; lipopolysaccharide is a strong mitogen for macro-

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Abbreviations: ABTS, 2-2′-azino-bis(3-ethylbenz)-thiazoline-6-sulfonic acid; BCR, B cell receptor; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HBSS, Hank's balanced salt solution; Ig, immunoglobulin; IPTG, isopropyl β-D-thiogalactopyranoside; LPS, lipopolysaccharide; PBS, phosphate buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; TCR, T cell receptor.

phage and B cells. To activate T lymphocytes, the T cell receptor (TCR)-mediated signal is necessary, which is given by the MHC-peptide complex on antigen presenting cells, such as macrophages, dendritic cells and B-lymphocytes (Jorgenson *et al*., 1992). However, the activation signal through TCR is insufficient to fully activate T cells, rather it induces an anergic state. The costimulatory signal transduced by the interaction between B7-1 and CD28, and the progression signal mediated by the interaction between cytokine receptors and their ligands should be added for a full activation (Allison, 1994). On the other hand, the signal through crosslinking of the B cell receptor (BCR) by antigen is the major stimulus to activate B cells. And also in this case, more stimuli such as the costimulatory signal through interaction between CD40 and the CD40 ligand, and the differentiation and progression signal through cytokines must be provided to fully activate B lymphocytes (Parker, 1993; Reth, 1992). In contrast to these observations, it was also reported that polymeric repetitive proteins like bacterial flagellin could activate B cells without the help of the T cell, albeit the activation signal was relatively weak (Garcia *et al*., 1998; Pike *et al*., 1987). It was reported that some lipoproteins also activate macrophage (Garcia *et al*., 1998). The *in vitro* activation of splenocyte is a relatively complex phenomenon since the help of each immunocyte in splenocyte was involved to activate each type of immunocyte. The full activation of immunocyte demonstrates DNA synthesis for cell proliferation, as well as protein synthesis standing for the production of soluble mediators like cytokines. In some case, the activation signals include the differentiation signal, resulting in immunoglobulin isotype switch or in the generation memory-cell (Or *et al*., 1994). However, partial activation was occasionally observed depending on the class and strength of signal (Armitage and Alderson, 1995). Many reports have recently been published about the intracellular signaling pathway transduced though the activation signal (Campbell, 1999; van Leeuwen *et al*., 1995).

In this study, we observed the activation effect of the hexahistidine-tagged recombinant envelope protein on mouse splenocytes and found that the hexahistidine peptide was the important determinant for splenocyte activation. Also, to identify the activated cell population, the phenotypic analysis of the activated splenocytes was carried out. To characterize the quality of this activation signal, the proliferation and differentiation of B lymphocyte was also studied. Only proteins containing the multivalent hexahistidine peptide motif could activate B lymphocytes, but the activation signal did not reach to proliferation and the differentiation level.

Materials and Methods

Reagents and animals The HBSS and RPMI 1640 medium for preparing and incubating splenocytes were obtained from Life Technologies Inc. (Germany). For staining macro-phage, T- or B-cells, fluorescein-conjugated anti-Mac1, anti-CD3 and anti-immunoglobulin antibodies were purchased from Pharmingen (USA). The fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD80 (B7-1) antibody (Pharmingen) was used as an activation marker of B lymphocyte. As the neutralization agent, an anti-pentahistidine antibody (Qiagen, Germany) was used. Quantitation of secreted immunoglobulins was carried out by a sandwich enzyme-linked immunosorbent assay (ELISA) with an anti-mouse IgM and an anti-mouse IgG antibody as coating antibodies, and the horseradish peroxidase-conjugated anti-immunoglobulin antibody (Serotech, Raleigh, NC) as a detecting antibody, respectively. Other chemicals were supplied from Sigma (USA). Female BALB/c (H-2^d) mice were purchased from the Daehan Laboratory Animal Research Center (Korea) and housed under specific pathogen-free conditions in the animal facility of Ajou University (Korea). Eight to twelve week old mice were used for the study.

Recombinant proteins Recombinant proteins fused to hexahistidine were expressed in *E. coli* and purified by Ni-NTA affinity chromatography (Quiagen). Endogenous murine leukemia virus envelope protein was expressed as a hexahistidine, and S-protein tagged protein using the expression vector pET-30b (Novagen, Madison, WI). Since the hexahistidine-tagged envelope protein (H-Env) was expressed in an insoluble form, the fusion protein was purified under denaturing conditions by using Ni-NTA affinity chromatography, according to the supplier's protocol. The purified H-Env protein was gradually dialyzed against the renaturation buffer (50 mM Tris.Cl, pH 7.5, 140 mM 2-mercaptoethanol, 250 mM cystamine). The soluble protein was re-dialyzed against the RPMI 1640 medium. To obtain the envelope protein itself, the fusion tag was excised by digestion with enterokinase (Novagen). After digestion, the envelope protein (Env) was purified by Ni-NTA chromatography under weakly denaturing conditions (1 M urea). The Env was dialyzed against the RPMI 1640 medium. The soluble hexahistidine-tagged envelope protein (H-Senv) was produced after removal of the hydrophobic transmembrane anchor domain (Kim *et al*., 1999). The hinge and CH_2 domain of murine immunoglobulin G (hCH2-H) fused to a hexahistidine tag at its C-terminal end was expressed using the pSW vector system, and purified by Ni-NTA chromatography (Kim *et al*., 1994). The hexahistidine-fused Hepatitis X protein (H-X) was expressed in the pQE vector (Qiagen), then isolated through Ni-NTA chromatography (Urban and Hildt, 1994). The purified hCH2-H and H-X proteins were also completely dialyzed against RPMI 1640.

Conjugation of oligopeptides to bovine serum albumin Hexahistidine (H-H), hexalysine (H-K), hexaglycine (H-G), hexaalanine (H-A), hexaaspartic acid (H-D) were synthesized by the solid phase method using Fmoc-chemistry, and purified by reverse phase high performance liquid chromatography (Peptron, Korea). The cross-linking between bovine serum albumin

(BSA) and the peptides was carried out by a chemical reaction between COOH and the NH_2 group through carbodiimide conjugation (Ciancialo *et al*., 1992). After conjugation, the reaction was extensively dialyzed against RPMI 1640. Successful coupling was confirmed by a shift of the molecular weight of BSA in a sodium dodecyl sulfate-polyacryl amide gel electrophoresis (SDS-PAGE).

Activation of splenocytes Spleens were aseptically removed from BALB/c mice, and single cell suspension was prepared by grinding the spleen with a plunger on a stainless-steel mesh and suspending it in HBSS. Red blood cells were removed by incubation in a Tris-ammonia buffer (0.85% NH_4Cl, 0.02 M Tris.Cl, pH 7.2) for 5 min at 37°C. The clean splenocytes were then harvested by differential centrifugation (300 × *g*, 5 min). The 2 × 10^5 splenocytes, suspended in 0.5 ml of 10% FBS-RPMI 1640, were plated in each well of a 24 well plate. To these cells, different concentrations of different proteins or peptides were added. One day later, the aggregation and enlargement of the splenocytes was observed under an inverted light microscope (Olympus CK2, Japan). To determine the blocking effects by neutralizing antibodies, the same activation experiment was carried out after premixing the hexahistidine-containing envelope protein (1 μM) and anti-pentahistidine antibodies (1 μM) in a 100 μl medium.

FACS analysis For analysis of the expression of the cell surface marker, the splenocytes were washed in a washing solution (1% BSA in PBS), blocked with 2% BSA for 30 minutes at 4°C, and then incubated with a 1:200 diluted solution of fluorescein-conjugated antibodies (FITC-anti immunoglobulin, FITC-anti Mac1, FITC-anti CD3, and FITC-anti CD80) for 30 min. A phenotypic analysis was then performed with a FACScan cytofluorometer (Becton-Dickinson, USA); the acquired data were then analyzed using the software program CellQuest™ (Becton-Dickinson, USA).

RT-PCR To confirm B cell activation by hexahistidine, the IgM mRNA level was analyzed by a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). After incubation with hexahistidine-bovine serum albumin (HH-BSA) for 24 h, total RNAs were extracted from the splenocytes with RNAzol B (TEL-TEST, USA) according to the supplier' s protocol. RT-PCR was carried out using the RNA-PCR kit supplied from Takara Shuzo (Japan). IgM genes were amplified by using the forward primer mix coding for the immunoglobulin heavy chain variable region 1, 2, and 3 (5′-ATGRRRWKSWGSTDDRDCWKSYTYTT), and the backward primer coding for the IgM heavy chain specific constant region (5′-ACCAGATTCTTATCAGACAGG) (Koefler *et al*., 1992). The PCR reaction was performed for 30 cycles under the following conditions: 95°C, 30 s; 60°C, 30 s; 72°C, 60 s. The amplified PCR products were analyzed by electrophoresis in a 1.2% agarose gel.

Proliferation assay To investigate the proliferation effect by hexahistidine, a thymidine incorporation assay was carried out. Briefly, 4 × 10^4 splenocytes suspended in 100 μl of 10% FBS-RPMI 1640 were plated on each well of a flat bottom 96 well plate. To these cells, HH-BSA was added to a final concentration of 1 μM (70 μg/ml). After incubation at 37°C in a 5% CO_2 atmosphere for 1 d, 5 μCi ^{3}H-thymidine was added, and the cells were further incubated for 18 h. The whole cells were harvested onto glass fiber filter mats using a cell harvester (Insel Co, UK); the degree of proliferation was determined by counting the incorporated ^{3}H in a liquid scintillation counter (Packard, Meriden, CT).

ELISA To measure secreted immunoglobulin, IgG- and IgM-specific sandwich ELISA was performed. First, 1 μg of an anti-mouse IgG1 antibody, or an anti-mouse IgM antibody, dissolved in 50 μl of PBS was coated onto polyvinyl chloride ELISA plates (Nunc, Denmark) by incubating at 4°C for 16 h. After washing three times with 0.05% Tween-20/PBS, non-specific binding sites were blocked by incubating with 3% BSA-PBS for 2 h. After washing three times with the Tween-20/PBS, an appropriate dilution of HH-BSA-treated splenocyte supernatant was delivered to each well and incubated for 2 h at room temperature. After washing three times with Tween-20/PBS, 1 μg of horseradish peroxidase-conjugated anti-immunoglobulin antibody (Sigma, USA), dissolved in 50 μl of 3% BSA-PBS, was added and incubated for 1 h at room temperature. After washing five times with Tween-20/PBS, the color was developed by adding 10 μg of ABTS [2-2' -azino-bis(3-ethylbenz)-thiazoline-6-sulfonic acid] dissolved in 50 μl of a 0.05 M citrate buffer (pH 4.0) and 0.02% H_2O_2. The optical density was determined at 405 nm using an automatic ELISA reader (Biorad, USA).

Results

Activation of splenocytes by the proteins harboring hexahistidine We observed the activation of BALB/c mouse splenocytes (enlargement and aggregation of cells) when treated with the recombinant endogenous murine leukemia virus envelope protein harboring the hexahistidine and S-protein as N-terminal fusion tags (H-Env). As shown in Fig. 1, 1 μM of the H-env protein was sufficient to induce this phenomenon within 24 h. The activation effect by the H-env protein was dose- and time-dependent (data not shown). To evaluate whether the activation effect was induced by the envelope protein, or otherwise by fusion hexahistidine and S-protein, the same experiment was carried out with the envelope protein alone (Env). Interestingly, the processed Env did not induce the aggregation of BALB/c splenocytes (Table 1). However, other hexahistidine-containing recombinant proteins, such as the hexahistidine-tagged hepatitis X protein (H-X) and the hexahistidine-tagged murine immunoglobulin hinge-constant heavy chain region (hCH2-H), induced similar activation effects on BALB/c mouse

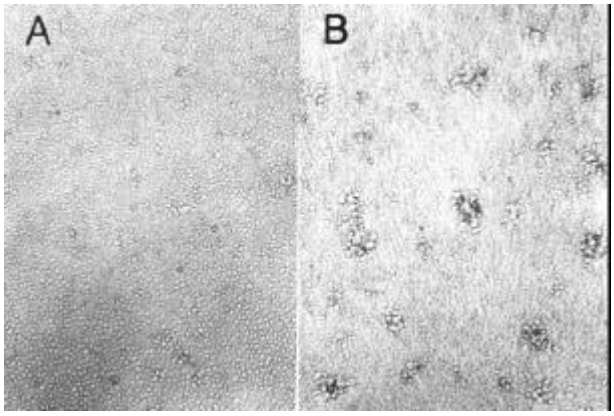


Fig. 1. Activation of splenocytes by treatment of the recombinant endogenous murine leukemia virus envelope protein with the N-terminal hexahistidine fusion tag. The 2×10^5 splenocytes isolated from BALB/c mice were plated in 0.5 ml on 24 well plates. Recombinant hexahistidine-tagged endogenous murine leukemia virus envelope protein was then added to 1 μM. After incubation under 5% CO_2 at 37°C for 1 d, cell morphology was examined under an inverted microscope. Activation of splenocytes was indicated by enlargement and aggregation of cells. **A.** splenocytes after treatment of bovine serum albumin (1 μM). **B.** splenocytes after treatment with 1 μM of the recombinant murine endogenous leukemia virus envelope protein containing hexahistidine residues (1 μM).

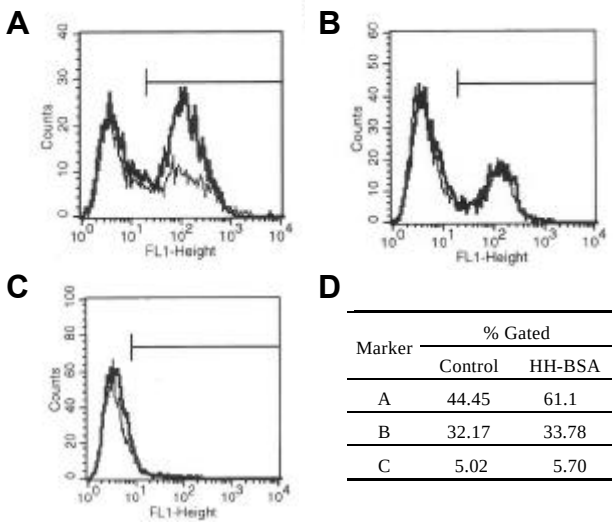
splenocytes. Unexpectedly, a soluble form of the envelope protein (H-Senv) that does not have a hydrophobic transmembrane anchor domain, but retains the hexahistidine residue, did not induce this activation effect. To pursue the mode of this effect, attempts were made to inhibit this effect by neutralizing agents, whereby an anti-pentahistidine antibody completely inhibited the splenocytes activation by the H-Env protein. These results suggest that the hexahistidine peptide might be an important determinant for activating BALB/c splenocytes. To determine whether a hexahistidine peptide can independently activate the mouse splenocytes, we treated BALB/c splenocytes with a synthetic hexahistidine peptide, or with bovine serum albumin chemically conjugated with various kinds of hexapeptide. It was shown that the hexahistidine peptide itself (H-H) could not activate mouse splenocytes, but the hexahistidine-conjugated bovine serum albumin (HH-BSA) was able to induce this activation effect. However, other hexapeptides conjugated to BSA (hexalysine-BSA, hexaglycine-BSA, hexaalanine-BSA, and hexaaspartic acid-BSA) did not activate mouse splenocytes (Table 1).

Selective activation of B cells by hexahistidine To identify the target of activation in mouse splenocytes, phenotypic differentiation was analyzed by FACS. After incubation with HH-BSA or BSA for 1 d, the activated splenocytes were stained with FITC-conjugated anti-Ig antibody, anti-CD3, and anti-Mac1 antibody, respectively. As shown in Fig. 2A, anti-Ig-stained cells were major cells (61.1%), and the signal intensity by the FITC-Ig antibody staining was significantly increased in HH-BSA-treated splenocytes compared to BSA-treated cells. This indicates that only the B cell population, designated by the Ig antibody staining, was increased by the HH-BSA treatment. On the other hand, the T cell and macrophage population was only slightly changed. This result showed that the HH-BSA could activate the B cell population of the splenocytes, and that the hexahistidine peptide could increase the surface immunoglobulin on B lymphocytes. To confirm the activation of B cells by hexahistidine, the enhance-

Table 1. Activation of BALB/c splenocytes by the proteins containing hexahistidine.

Proteins	Activation
H-Env	+[a]
Env	–[b]
H-Senv	–
hCH2-H	+
H-X	+
H-Env + anti-His5	–
BSA	–
HH	–
HH-BSA	+
HK-BSA	–
HG-BSA	–
HA-BSA	–
HD-BSA	–

BALB/c splenocytes (2×10^5 cells in 0.5 ml) were mixed with the corresponding hexahistidine-containing proteins (1 μM). After incubation in a 5% CO_2 atmosphere at 37°C for 24 h, cell activation was determined by observation of cell enlargement and aggregation under an inverted microscope (Fig. 1). +[a] means the activation of splenocytes while –[b] denotes no effect on splenocytes. H-Env, endogenous murine leukemia virus envelope protein containing hexahistidine as an N-terminal tag; Env, endogenous murine leukemia virus envelope protein after removing the fusion tag; H-Senv, soluble endogenous murine leukemia virus envelope protein containing hexahistidine after removal of the hydrophobic transmembrane anchor domain; hCH2-H, second constant region of heavy chain immunoglobulin containing hexahistidine as a C-terminal fusion tag; H-X, Hepatitis virus X protein containing hexahistidine as N-terminal tag; anti-His5, monoclonal antibodies against pentahistidine; BSA, bovine serum albumin; HH, hexahistidine peptide; HH-BSA, bovine serum albumin conjugated with hexahistidine; HK-BSA, bovine serum albumin conjugated with hexalysine; HG-BSA, bovine serum albumin conjugated with hexaglycine; HA-BSA, bovine serum albumin conjugated with hexaalanine; HD-BSA, bovine serum albumin conjugated with hexaaspartic acid.



Marker	% Gated	
	Control	HH-BSA
A	44.45	61.1
B	32.17	33.78
C	5.02	5.70

Fig. 2. Selective activation of B lymphocytes by treatment of proteins containing hexahistidine. BALB/c splenocytes (2×10^5 cells in 0.5 ml) were mixed with 1 μM bovine serum albumin conjugated with hexahistidine peptides (HH-BSA) or bovine serum albumin (BSA), then incubated in a 5% CO_2 atmosphere at 37°C for 24 h. Single cells were stained with a FITC-labeled anti-immunoglobulin antibody (A), anti-CD3 antibody (B) or anti-Mac 1 antibody (C). The stained cells were analyzed by using a FACScan cytofluorometer. To determine the quantitative change of each cell type, live cells were gated and analyzed by the CellQuest program. The thin lines indicate the staining profile of the BSA-treated cells (control), while the thick lines indicate the staining profile of cells after treatment of HH-BSA. The ratio of each cell population was listed on D.

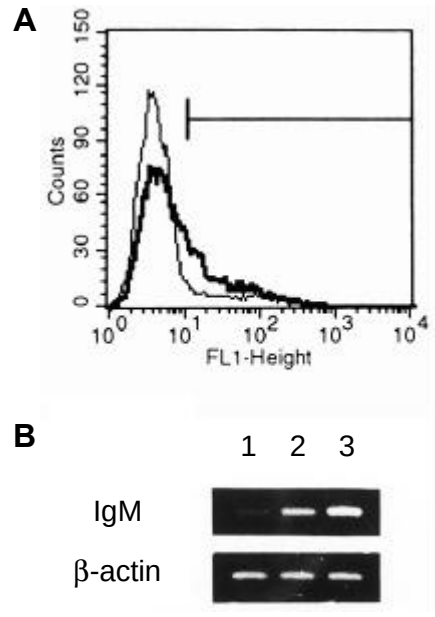


Fig. 3. Activation of B cells by treatment of the bovine serum albumin conjugated with hexahistidine (HH-BSA). Activation of B cells was proven by an increase of the costimulatory marker and expression of immunoglobulin M. After treatment of BALB/c splenocytes with HH-BSA or BSA, the cells were stained with the FITC-labeled anti-CD80 antibody. The stained cells were analyzed by a FASCscan fluorometer. The thin line designates the scan of BSA-treated cells and the thick line designates the scan of HH-BSA-treated cells (A). After treatment of 0.5 ml of 2×10^5 splenocytes with BSA (1 μM), HH-BSA (1 μM), or 5 μg of lipopolysaccharide (LPS) for 24 h, total RNAs were isolated with a RNAzol B solution. The cDNAs were synthesized with AMV reverse transcriptase and random nine-mer. PCR was carried out with immunoglobulin M-specific promoter sets. The expression of IgM was analyzed by comparing the degree of the amplified DNAs (B). Lane 1, amplified DNAs after treatment of BSA; lane 2, amplified DNAs after treatment of HH-BSA; lane 3, amplified DNAs after treatment of LPS.

ment of the costimulatory B7-1 (CD80) levels and IgM levels was investigated. As shown in Fig. 3A, the CD80-positive cell population was increased from 3.4 to 16.4% and the transcription level of IgM was also enhanced after treatment with HH-BSA (Fig. 3B).

Competence signal for B cell activation by hexahistidine residues Since the proteins containing hexahistidine activate the B cell population, we questioned whether activation by hexahistidine included the proliferation and/or differentiation of lymphocytes. To investigate whether splenic B lymphocytes were proliferated after HH-BSA treatment, a ^{3}H-thymidine incorporation assay was carried out. As shown in Fig. 4A, HH-BSA treatment did not enhance the DNA synthesis, while the LPS dramatically increased the DNA synthesis in mouse splenocytes. In addition, to monitor B cell differentiation, the levels of secreted IgG and IgM were measured. The IgG level was not increased by treatment of HH-BSA, while the IgM level was slightly increased (Fig. 4B). These results show that the activation of mouse B lymphocytes by a hexahistidine dose not include the prolix-feration or differentiation signals. For full activation of B lymphocytes, the progression signal, as well as a competence signal, was essentially required. Since the hexahistidine-mediated activation showed only an increase of IgM levels, the activation through hexahistidine appears to contain only the competence signal.

Discussion

Through this study, we showed that His-tagged fusion proteins, mainly produced from heterologous expressions in *E. coli*, selectively activate mouse B lymphocytes, and that the activation signal through hexahistidine include

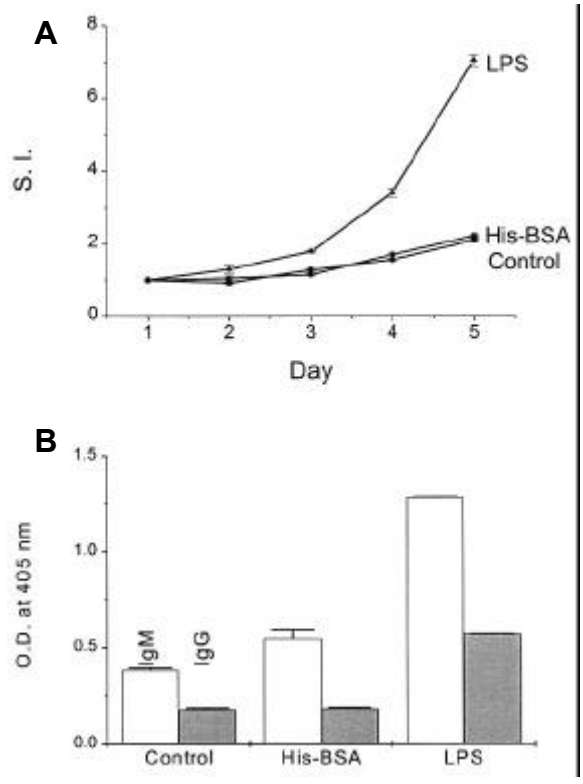


Fig. 4. Proliferation and differentiation of B cells after treatment of bovine serum albumin conjugated hexahistidine (HH-BSA). Cellular proliferation was determined by measuring thymidine incorporation (A). 0.1 ml of 4×10^4 splenocytes were mixed with 1 μM of BSA (control), HH-BSA, or 1 μg of LPS. After incubation under 37°C at 5% CO_2 for 1 d, 5 μCi of ^3H thymidine was added. After further incubation for 18 h, the whole cells are harvested. The incorporated ^3H thymidine was analyzed by counting the radioactivity of the harvested cells in a scintillation counter. S.I (stimulation index) was defined as the value of the radioactivity after stimulation divided by the radioactivity without stimulation. Cellular differentiation was determined by the production of immunoglobulin G (B). After treatment of 2×10^5 BALB/c splenocytes with 1 μM of BSA (control), 1 μM of HH-BSA or 5 μg of LPS for 3 d, the culture medium was collected. Then, the IgG was quantitated by sandwich ELISA with an anti-mouse immunoglobulin G antibody and peroxidase-conjugated anti-immunoglobulin antibody, as a coating and detecting antibody, respectively. After developing, the relative amount of IgG was determined by a comparison of the optical density at 405 nm.

only a competence signal without a progression signal. This activation effect was hexahistidine-specific since an anti-pentahistidine antibody completely neutralized the activation effect, and BSA cross-linked to hexahistidine peptide activated mouse splenocytes. In addition, the consecutive histidine oligopeptide seems to be important for splenocyte activation since the hexapeptide, consisting of three histidine and three aspartic acids, did not exhibit the same activation effect (data not shown). The activation effect appears to be mediated by hexahistidine peptide regardless of position, either to the N-terminal or C-terminal end of protein. BALB/c splenocytes were activated by both the recombinant hepatitis X protein tagged with hexahistidine at the N-terminal, and the recombinant immunoglobulin hinge-constant heavy chain tagged with hexahistidine at the C-terminal. Furthermore, an artificial protein, chemically cross-linked with the synthetic hexahistidine peptides, also showed a similar activation effect.

The activation of B lymphocytes by hexahistidine seemed to be mediated by its multivalency, since both the soluble retroviral envelope protein (H-Senv) and free hexahistidine peptides could not activate splenocytes. In fact, it was reported that the retroviral envelope protein existed as a trimer, and the transmembrane anchor region and leucine zipper region was involved in its trimerization (Ramsdale *et al*., 1996). It was also reported that the hepatitis X protein, and the constant region of immunoglobulin (h-CH2), existed as a dimer (Kim *et al*., 1994; Lin and Lo, 1989). The HH-BSA used in this study seems to have several hexahistidine motifs per BSA, since the molecular weight of HH-BSA was several thousand daltons higher than that of unconjugated BSA, as determined by a SDS-polyacrylamide gel electrophoresis (data not shown). Therefore, it appears that the proteins containing at least two hexahistidine motifs are required for mouse B cell activation. These results are consistent with the observation that the capacity for cross-linkage of the B cell receptor (BCR) molecule is pivotal in the induction of an activation signal. Therefore, it is plausible that hexahistidine peptide interacts with BCR, and then the multivalency of hexahistidine can cross-link the BCR molecule on the B cell surface, resulting in the transduction of activation signal.

Although it is unclear why the proteins containing hexahistidine initiate B cell activation, there seems to be an interaction between the hexahistide residue and the B cell receptor. It is possible that hexahistidine is a general ligand for certain subtypes of surface immunoglobulins in B lymphocytes. For example, hexahistidine might be a kind of superantigen that is reactive with certain types of variable regions of membrane immunoglobulin. This hypothesis is consistent with the observed activation of a relatively large subpopulation of B lymphocytes, and the weakness of this activation signal. Several reports showed that proteins, such as protein A, the gp120 envelope protein of the human immunodeficiency virus, staphylococcal enterotoxin B, and D served as superantigens for B cells (Domiati-Saad *et al*., 1996; Silverman, 1997). These superantigens had a relatively broad specificity to B cell activation, while the activation signal was relatively weak. However, further study needs to be done regarding the specific types of variable-gene segments of immu-

noglobulin that are required for B cell activation, in order to confirm whether or not hexahistidine is a real B cell superantigen.

In the stimulation of B-lymphocytes, the binding of antigen to the BCR is the first step. However, additional signals transduced by costimulatory ligands and cytokines are required for a full activation, such as cell proliferation and cell differentiation. In fact, there are two kinds of B cell antigen according to the T-cell dependency in the stimulation. Most soluble proteins are thymus-dependent antigens, due to their requirement for a direct contact between B cell and helper T cells. In contrast, it was reported that some polymeric proteins, such as bacterial flagellin or polysaccharide, were thymus-independent antigens (Schneider *et al*., 1992). Earlier reports showed that the response to thymus-independent antigen was weak, and did not have the capability of isotype switching (Chan *et al*., 1993; Reth, 1992). The hexahistidine-tagged proteins seem to be thymus-independent antigens since they initiate the B cell activation, but the activation signal is very weak. In fact, we observed that HH-BSA was able to induce the weak aggregation of isolated B lymphocytes (data not shown).

In conclusion, our study showed that multivalent hexahistidine selectively activated mouse B lymphocytes, but did not supply proliferation or differentiation signals to fully activate the B cells. This study suggests that caution must be taken in the use of multimeric recombinant proteins containing hexahistidine tag for studying their biological role *in vivo* and *in vitro*, due to their nonspecific activation capability to the mouse B-lymphocytes.

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