การพัฒนาเทคนิคทางอิมมูโนเอนไซม์สำหรับตรวจหาแอนติเจนบนผิวของไวรัสตับอักเสบชนิด บี ด้วยสเตรบตาวิดิน-ไบโอติน

นางสาววันเพ็ญ บุญวานิช

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DEVELOPMENT OF IMMUNOENZYMATIC TECHNIQUE FOR DETECTION OF HEPATITIS B SURFACE ANTIGEN USING STREPTAVIDIN-BIOTIN

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วันเพ็ญ บุญวานิช : การพัฒนาเทคนิคทางอิมมูโนเอนไซม์สำหรับตรวจหาแอนติเจนบนผิวของไวรัสตับอักเสบ บี ด้วยสเตรบตาวิดิน - ไบโอติน (DEVELOPMENT OF IMMUNOENZYMATIC TECHNIQUE FOR DETECTION OF HEPATITIS B SURFACE ANTIGEN USING STREPTAVIDIN-BIOTIN) อ. ที่ปรึกษา ผศ. ดร. อมร เพชรสม, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : นางสาววิยะดา เจริญศิริวัฒน์, 97 หน้า ISBN 974-583-844-6

Enzyme-linked Immunosorbent Assay (ELISA) เป็นเทคนิคที่นิยมใช้ในการวิจัย และการตรวจวินิจฉัยโรคต่างๆ ปัจจุบันมีการค้นพบปฏิกิริยาจำเพาะของโปรตีนอะวิดินหรือสเตรบตาวิดิน และวิตามินไบโอตินที่จับตัวกันอย่างแน่นหนา และสามารถใช้พัฒนาเทคนิค enzyme immunoassay ได้ ดังนั้น การวิจัยนี้จึงมีวัตถุประสงค์เพื่อพัฒนาเทคนิค ELISA ในการตรวจหาแอนติเจนบนผิวของไวรัสตับอักเสบชนิด บี โดยการใช้เซลไฮบริโดมาจำนวน 2 โคลน หมายเลข 129 และ 1C5 ผลิตโมโนโคลนัลแอนติบอดี และนำโมโนโคลนัลแอนติบอดีที่ได้ มาทดลองติดฉลากกับไบโอติน และกับเอนไซม์อัลคาไลน์ฟอสฟาเตส จากนั้นทดลองเคลือบสเตรบตาวิดิน ที่ความเข้มข้นต่างๆ บนผิวของไมโครไตเตอร์เพลท นำน้ำยาที่เตรียมขึ้นมาทดสอบหาสภาวะที่เหมาะสมในการตรวจหาแอนติเจน พบว่า โมโนโคลนัลแอนบอดีที่ผลิตจากเซลไฮบริโดมาหมายเลข 129 มีความเหมาะสมในการติดฉลากกับไบโอติน ส่วนหมายเลข 1C5 เหมาะสมในการติดฉลากกับ เอนไซม์อัลคาไลน์ฟอสฟาเตส ความเข้มข้นของสเตรบตาวิดินที่ 1 ug/mL เหมาะสมในการเคลือบผิวหลุมของเพลท เปรียบเทียบประสิทธิภาพของชุดน้ำยาที่ใช้และไม่ใช้สเตรบตาวิดิน-ไบโอติน ตรวจหาแอนติเจนบนผิวของไวรัสตับอักเสบชนิด บี ที่ความเข้มข้นต่างๆ พบว่า ชุดน้ำยาที่ใช้สเตรบตาวิดิน-ไบโอติน สามารถตรวจหาได้ที่ปริมาณต่ำสุด 12 ng/mL ขณะที่น้ำยาที่ไม่ใช้สเตรบตาวิดิน-ไบโอติน ตรวจหาได้เพียง 25 ng/mL เมื่อเปรียบเทียบประสิทธิภาพของชุดน้ำยา ELISA ที่ใช้สเตรบตาวิดิน-ไบโอตินกับชุดน้ำยา ELISA ของบริษัท Abbott ในการตรวจหาแอนติเจนบนผิวของไวรัสตับอักเสบชนิด บี ในซีรั่มจำนวน 213 ราย พบว่าชุดน้ำยา ELISA ที่ใช้สเตรบตาวิดิน-ไบโอติน มีความไวร้อยละ 100 ความจำเพาะร้อยละ 98.29 และมีประสิทธภาพร้อยละ 99.06 ดังนั้น เทคนิค ELISA ที่ใช้ในการตรวจหาแอนติเจนบนผิวของไวรัสตับอักเสบชนิด บี สามารถจะพัฒนาให้มีประสิทธิภาพดีขึ้นด้วยการใช้ เสตรบตาวิดินและไบโอติน

ภาควิชา เทคโนโลยีทางชีวภาพ ลายมือชื่อนิสิต วันเพ็ญ บุญวานิช

สาขาวิชา - ลายมือชื่ออาจารย์ที่ปรึกษา

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Enzyme-linked immunosorbent assay (ELISA) is one of the versatile technique used in research study and diagnosis. Nowadays there is a discovery of a specific reaction between avidin protein or streptavidin and vitamin biotin forming a strong binding which could be used to develop the ELISA technique for the detection of Hepatitis B surface antigen by using 2 clones of hybridoma cells numbered as 129 and 1C5 for the production of the monoclonal antibody. These two monoclonal antibodies were used to label with biotin and alkaline phosphatase enzyme. Followed by the coating of streptavidin at various concentrations on microtiter plates and the prepared reagents were tested for optimal conditions for the detection of HBsAg. It was found that the monoclonal antibody produced from the hybridoma cell no. 129 was suitable to label with biotin and the hybridoma cell no. 1C5 was suitable to label with alkaline phosphatase enzyme with the concentration of streptavidin at 1 ug/mL was suitable for coating the microtiter plate. By comparing the efficiency of the ELISA incorporating streptavidin and biotin to the conventional ELISA, it was found that the reagent of the streptavidin-biotin ELISA could detect Hepatitis B surface antigen (HBsAg) at the minimum concentration of 12 ng/mL while the conventional ELISA could detect at 25 ng/mL. In addition, the comparison of the efficiency in detection of HBsAg utilizing the Streptavidin-Biotin ELISA to ELISA commercial reagent kit obtained from Abbott company in 213 specimens, it was found that the sensitivity, specificity and efficiency were 100%, 98.29% and 99.06% respectively. Therefore, the efficiency of the ELISA technique for the detection of the Hepatitis B surface antigen could be improved by using streptavidin and biotin.

ภาควิชา เทคโนโลยีทางชีวภาพ ลายมือชื่อนิสิต .....
สาขาวิชา - ลายมือชื่ออาจารย์ที่ปรึกษา .....
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List of Abbreviations

anti-HBs	=	antibody against hepatitis B surface antigen
anti-MS	=	antibody against mouse γ-globulin
AP-129	=	enzyme alkaline phosphatase-anti-HBs clone 129 conjugate
AP-1C5	=	enzyme alkaline phosphatase-anti-HBs clone 1C5 conjugate
BNHS	=	biotinyl-N-hydroxysuccinimide ester
B-129	=	monoclonal anti-HBs clone 129 label with biotin
B-1C5	=	monoclonal anti-HBs clone 1C5 label with biotin
BSA	=	bovine serum albumin fraction V
HRP	=	enzyme horseradish peroxidase
hr	=	hour
L	=	litre
MAb	=	monoclonal antibody
MAb 129	=	monoclonal antibody against HBsAg clone 129
MAb 1C5	=	monoclonal antibody against HBsAg clone 1C5
μg	=	microgram
M	=	molarity
min	=	minute
mL/hr	=	millilitre per hour
mg	=	milligram
N	=	normality
ng	=	nanogram

List of Abbrevations (cont.)

nm	=	nanometer
NPP	=	p-nitrophenyl phosphate
OPD	=	o-phenylenediamine
EDAC	=	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
PBS	=	phosphate buffer saline
PBG	=	0.15 M phosphate buffer pH 7.4 containing 0.001 M EDTA and 0.1 % gelatin

CHAPTER I

INTRODUCTION

The serological methods have played important roles in the diagnosis and assessment of diseases. The ability to elicit an immune response to an antigen, and more particularly the advent of monoclonal antibody technology has led to a proliferation of immunological techniques and diagnostic tests. The application of immunoglobulins in basic research, diagnostic or immunotherapy, rely on the ability of the labelling of antibody to a reporter molecule i.e. enzymes,radiolabels, fluorochromes, biotin and toxins. With the advent of the quantitative enzyme-linked immunosorbent assay described by Engvall&Pearlman (1), the development of the enzyme-linked immunosorbent assay rests on advance in immunochemistry, protein coupling reagents that allow both enzyme and antibody to retain biological activity when conjugated to each other(2). Enzyme-linked immunosorbent assay (ELISA) is interested in the application of enzyme immunoassay as patient-oriented.

Since the discovery of the relationships between Australia antigen,now called hepatitis B surface antigen (HBsAg), a number of immunochemical with different specificity,sensitivity, and practicability for the detection of HBsAg have been described. ELISA for the detection of HBsAg has claimed that such reactivity in the hepatitis B virus(HBV) hyperendemic area allows the possibility of non-specific reaction, the basis of false-positive ELISA reactivity(3). In recent years, the avidin-biotin complex has been discovered and based on the strong interaction, it has also

been used as a tool for universal mediator in biological sciences. Antibodies can be labelled with biotin without significantly influencing their immunological activity. Realizing that the avidin-biotin complex could be effectively used for immunoassay was first demonstrated by the work of Avrameas and collegues in which they suggested several related methods for use to increase the sensitivity and specificity of ELISA(4). In this study, attempt is made of the product local-made ELISA reagent and improve ELISA sensitivity for the detection of HBsAg via the incorporation of streptavidin (avidin from bacteria) and biotin into ELISA system.

CHAPTER II

LITERATURE REVIEW

1. The Hepatitis B Virus

It has long been recognized that the so-called viral hepatitis consists of at least two clinically distinct human diseases, formerly designated as "infectious hepatitis" and "serum hepatitis". MacCallum proposed in 1947 that the name hepatitis B was used to designate the viral-associated liver diseases. Since that time, a third type of viral hepatitis, known as non-A, non-B hepatitis, has emerged as a significant cause of human diseases. The number of reported case of hepatitis B and non-A, non-B hepatitis is increasing. Although a huge worldwide reservoir of hepatitis B virus (HBV) exists, hepatitis B is usually a relatively mild disease. Most individuals do not require hospitalization. In those individuals in whom complication occur, however,the consequences can be serious. Potential complications include arthritis, acute polyneuritis, and vasculitis. The carriers can be expected to develop chronic active hepatitis, which progress to cirrhosis or HBV-related cancer of the liver.

Hepatitis A and Hepatitis B are clearly distinct from one another but do have some characteristic in common, jaundice, malaise,increasing of serum transaminases level. As information on non-A, non-B hepatitis emerges, it appears that it may also be endermic and responsible for both acute and chronic state disease. The onset of initial acute hepatitis B is usually insidious and occurs 60 to 160 days

after exposure to HBV. Although the hall-mark of hepatitis is often jaundice, most initial infections caused by HBV product non icteric disease. The time of incubation is related to the dosage of viruses. Higher doses are associated with shorter incubation times. During the icteric phase of the disease, patients frequently complain of extreme fatique. Chronic infections of HBV are usually asymptomatic, but hepatomegaly and splenomegaly could be detected. Transient elevations in serum glutamic-oxaloacetic transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT) can persist for years. Chronic infection is frequent in infants and elderly. Progression to more serious diseases rarely occurs, but if it does, the entity would be active hepatitis.

In active chronic hepatitis, jaundice may or may not occured. Elevations in SGPT and SGPT accompany the necrosis that follows an inflammatory response. In children, the onset of hepatitis is abrupt, abdominal pain and vomiting, accompanied by fever are often present. The duration of jaundice and fever tends to be shorter in children than in adults. Immune complex disease is manifested more often as dermatitis or glomerulonephritis in children than as the arthritis complications seen in adults. Although there is no recognized for HBV infections(5).

1.1 Etiology

The discovery by Blumberg and his colleagues of Australia (Au) antigen, at first thought to occur only in Australian Aborigins, turned out to be an important step in understanding the etiology of hepatitis B. When the Au antigen was found associated with 20% of serum of individuals with hepatitis, it then became

know as the hepatitis-associated antigen (HAA) (6). The antigen was also found to occur in 10 to 14% of patients with leukemia and also in some apparently healthy people of South East Asia (7).

In 1968, HAA was detected in the blood of a patient during the incubation period of post-transfusion hepatitis (8). It was apparent in subsequent studies that the antigen can persist in blood of persons who have had hepatitis B for 1 to 2 years (9). Three virus-like particles have been identified in the blood of individuals who have or have had hepatitis B (10) (Figure 1) spherical particles that are 20 nm in diameter; filamentous particles the measure up to 50-230 nm in longer and 20 nm in diameter; and spherical Dane particles that measure 42 nm in diameter (11-15). The core component of the Dane particle contains the antigens HBsAg and HBeAg, DNA polymerase and double strand DNA.

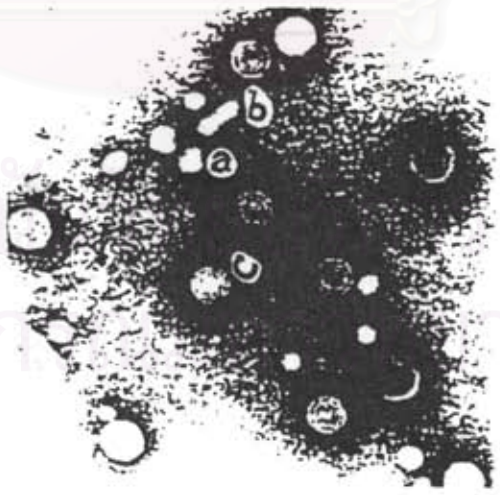

Fig.1 Electron micrograph of serum showing the presence of three distinct morphological entities:(a) 20 nm pleomorphic spherical particles;(b) tubular or filamentous forms with adiameter of 20 nm; and (c) 42 nm spherical particle.

Surface antigen (HBsAg) is the antigen once called HAA. The HBsAg of all three particles are identical. Purified preparation of HBsAg contain 4 polypeptides and 4 glycoproteins, with molecular masses ranging from 22,000 to 68,000 daltons. The Dane particle, now consider to be the HBV, can be differentiated from core particles by physical properties.

At least nine antigenically distinct major subtypes and seven minors of HBsAg have been recognized (Table 1). The antigenic type adw2 is found more frequently in the United States, whereas antigenic type ayw2 and ayw3 are more common in Africa and the Middle East and antigenic type ad is found in Thailand and East Asia(16). Determination of subtypes may be useful in epidemiologic studies and can explain partial identity in immunodiffusion tests.

Table 1 Major and minor subtypes of HBsAg

Major	Minor
ayw1	q
ayw2	x
ayw3	f
ayr	t
adw2	j
adw4	n
adr	g
adwy	
adyr	

HBsAg appears in the blood approximately 4 weeks after exposure to HBV. In another week, HBeAg and DNA polymerase activity are the most important

markers for viral replication. Viral replication was related in part to hepatic cell necrosis but did not seem to be responsible for progression to liver cirrhosis (10). Understanding remission of progression of HBV associated disease awaits the clarification of the long-term host-parasite relationship.

1.2 Epidemiology

Hepatitis B is typically an endemic disease in which about 5-10 % of persons who are infected in adulthood become persistant carriers of the virus in Asia including Thailand, there are 10% of carrier having HBsAg and 31 % among neonates were infected from carrier mothers (surface antigen positive) and 65-90% from carrier mothers(e antigen positive) (17).

In an important study published in 1981, Shafriz et al.(18) reported integration of hepatitis B virus DNA into the genome of human liver cells and provided an explaination for the well-recognized association of hepatitis B virus carrier state with liver cancer. Additional support for the casual relationship of HBV with cancer has been given by the findings in the comparative pathology of HBV-caused liver cancer of woodchuck (19). HBV infection can result from close contact with individuals who are viremic with HBV, they provide a source of HBV for the spread of infection in the community (20). The family would be a setting where this type of HBV transmission frequently occurs and hepatitis B relate to cancer in man becomes very important that the World Health Organization has chosen hepatitis B as one of its imperatives for disease control and has set the objective of substantial reduction in hepatitis B.

1.3 Serological Tests

Serological Technique is available for detection of HBV markers and antibodies. Interpretation of serological markers require familiarity with the time of appearence of specific antigens and antibodies.

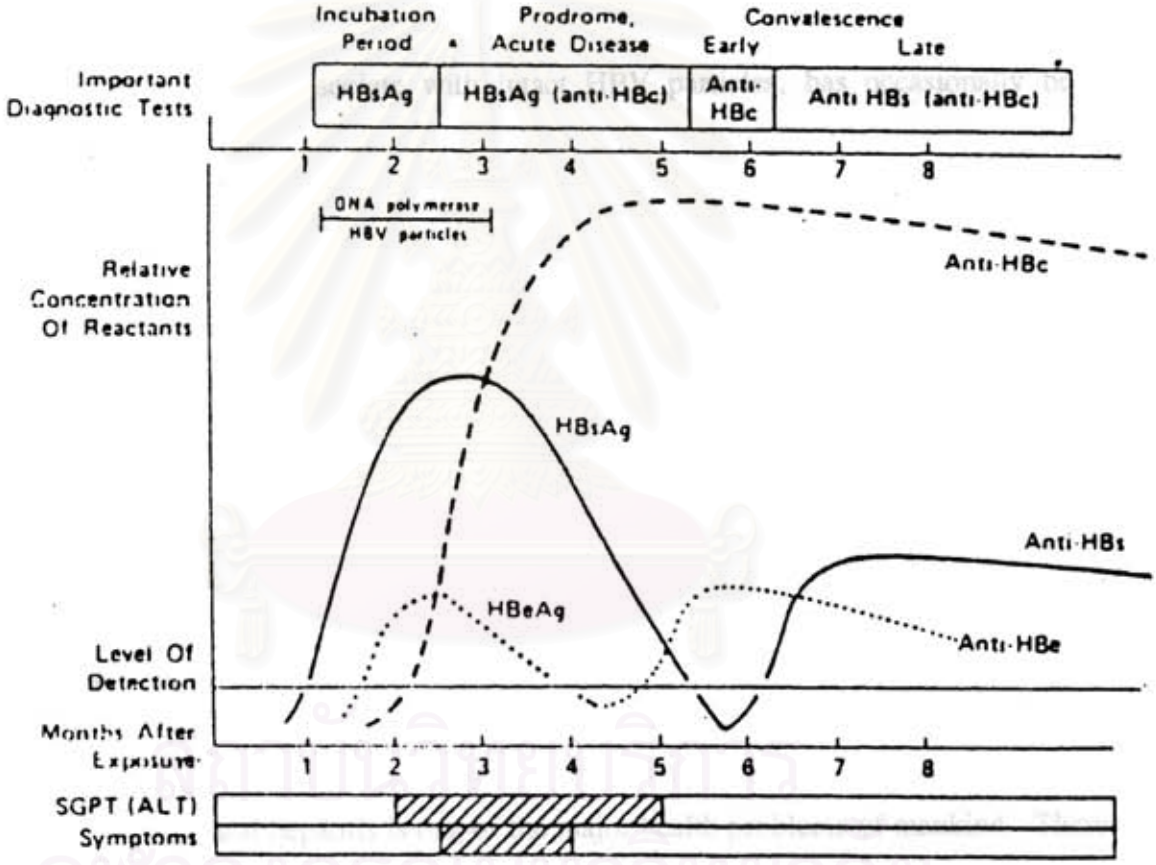

Fig. 2 Serological and clinical patterns observed during acute hepatitis B virus infection.

Serological changes that occur after exposure to HBV represent antigens and antibodies. HBsAg typically appears 1 to 2 months prior to the

development of liver abnormalities, reaches a peak which declines within 4 to 6 months to undetectable level by current methods (figure 2). As the aminotransferase [serum glutamic pyruvic transaminase (SGPT)] or alanine aminotransferase (ALT) levels rise, various HBV markers can be detected in the sera of many, but not all patients. These serological markers include HBeAg, the 42 nm HBV (Dane) particles, specific endogenous DNA polymerase activity, and HBV associated HBcAg. Free HBcAg do not associate with intact HBV particles, has occasionally been detected in icteric hepatitis patients prior to the development of anti-HBc when ALT levels are at their highest. Anti-HBc is the first antibody appeared and may be detected for several years. It is of no value, however, if HBsAg is present in detectable amounts. Anti-HBe appears 10 or more days after anti-HBc,but does not reach the levels of anti-HBc. Anti-HBs is the last antibody appeared and is associated with immunity to HBV infectious(21).(Table 2)

2. Laboratory Diagnosis of Hepatitis B Surface Antigen

Viral hepatitis is one of the major health problems of mankind. There are at least three viruses that mainly cause of hepatitis in man. The hepatitis B is the most important because of a large proportion of cases that present distressing late sequel are resulting from persistent retention of the virus in the liver. The present of HBsAg in serum will mark the acute and chronic infection. So, laboratory diagnosis of HBsAg is needed to certainly indicate the hepatitis symptom in patient caused by HBV and to screen people who are carriers.

Table 2 Time of appearance and interpretation of HBV serological markers in hepatitis

Marker	Time of appearance	Interpretation
HBsAg	~ 40 to 60 days after exposure	Active hepatitis B infection acute or chronic
HBeAg	~ 50 to 60 days after exposure	Active hepatitis B infection acute or chronic possibly enhanced infectivity
Anti-HBc	~ 60 to 90 days after exposure	Active hepatitis B infection acute or chronic* or carrier state
Anti-HBe	~ 100 to 120 days after exposure	Active hepatitis B infection acute or chronic** or carrier atate
Anti-HBs	~ 30 to 60 days after disappearing of HBsAg	Immunity to HBV infection

* A test for anti-HBc is of value only if HBsAg levels are not present in detectable levels.

** A fourfold or greater rise in titer between acute and convalescent sera occurs in active HBV infections.

Various techniques have been developed to detect HBsAg in serum and the three commonly methods used are as follows:

2.1 Reverse Passive Heamagglutination (RPHA)

RPHA is mainly consisting of sensitized red blood cells which are fixed with purified anti-HBs. Anti-HBs antibody is agglutinated specially in the presence of HBsAg in serum. Presence or absence of HBsAg can be read with this test system, using microplate, in comparison with haemagglutination. The advantages of this method are sensitized cells (lyophilized) are stable for a year if store strictly at

this method are sensitized cells (lyophilized) are stable for a year if store strictly at 2-10°C, a clear evidence of agglutination makes it easy to read and a simple determination method permits to judge the evidence obtained within one hour at 37°C. However RPHA still have a disadvantage like many other agglutination tests, the presence of rheumatoid factor, lipemia, autoimmune or heterophil antibodies and albumin globulin imbalance may cause false-positive reaction (22-23).

2.2 The Radioimmunoassay (RIA)

Radioimmunoassays have been developed for virtually every type of biological molecule and their overall strategy is similar. Individual techniques vary mainly in the way in which free antigen is separated from antibody-bound antigen and in whether the reaction is carried out in liquid phase or with antibody coupled to a solid support(solid phase radioimmunoassay). RIA continues to be the most sensitive and specific method for testing of all HBV markers. The amount of HBsAg presented in a sample is determined by a fixed amount of radiolabeled HBsAg competes for a limiting amount of specific anti-HBsAg. A standard curve is constructed using known amounts of unlabeled HBsAg concentration in samples can be determined. The RIA tests do required safe handling and disposal of hazardous material (24).

2.3 The Enzyme-linked Immunosorbent Assay (ELISA)

In 1971, Engvall and pearlman (1) described that an enzyme-linked immunosorbent assay (ELISA) for quantitation of rabbit-IgG appeared to be as

sensitive as the corresponding RIA. Its applications for a sensitive diagnostic were immediately evident. Detection methods for HBsAg require optimum sensitivity, specificity, practicability, speed and simplicity. An ELISA has the potency to fulfil these requirements. The method uses enzyme-conjugated anti-HBs for the quantitative determination of antigen in solution. The principle is to coupling the anti-HBs on a solid phase which is allowed to react with antigen. After washing of excess protein, enzyme-conjugates anti-HBs is added. The uptake of labeling to the solid phase gives a measure of the intensity of digested substrate by enzyme that corresponds with the amount of antigen in serum. By comparing to RIA, the question of whether enzyme immunoassay would replace RIA was araised. Wolters et al.(1976) reported that, with respect to subtype ad, the sensitivity of ELISA is lower than RIA but with respect to subtype ay,the sensitivity of ELISA is about three times higher(25). The difference in sensitivity towards the two subtypes is rather a characteristic of the immune response than of the test procedure (17). Generally, ELISA sensitivity has been reported less than or equal to RIA but the advantages of ELISA over RIA are evident. Radio-iodinated reagents have a short shelf life and require radioactive waste disposal. The ELISA technique has been chosen by the World Health Organization as the preferred method for the detection of HBsAg in developing countries of the world (26).

3. The Application of Avidin-Biotin Interaction in Immunoenzymatic Technique

The recent explosive usage of the avidin-biotin complex as a universal mediator in the biological sciences has underscored the necessity for sensitive and efficiency of ELISA (27-33). Bayer et. al.(34) researched on a sensitivity enzyme assay for biotin,avidin and streptavidin in 1986,the research mentioned that the assays were sensitive enough for the immunized system and provided more sensitive or rapid assays. Physicochemical studies on the nature of the avidin-biotin complex have heretofore been difficult to perform the high-affinity interaction rendered unreliable spectrophotometric methods. The first prototype study which demonstrated that the avidin-biotin system could be used in immunoassays was a study in which it was shown that biotinylated bacteriophage could be inactivated by avidin. The inactivation could be reversed by biotin and derivatives in solution, similar to the interaction of phages using antibodies and antigens (35). The work of Gesdon and collegue on the avidin biotin complex (36) suggested several related methods for its use in increasing the sensitivity and specificity of ELISA. It will be very interesting to see whether sensitivity would be further increased if two different monoclonal antibody preparations (each specific for a different non overlapping determinant on the same antigen) would be used. In immunoassay studies, the avidin biotin complex has several advantages, biotin can be attached to antibody under mild conditions, the stability and the introduction of biotin groups into antibodies lead to amplify detectability of the antigen

4. History of Avidin-Biotin Interaction

Avidin, a heat labile protein found in raw egg white. When animals are fed on a diet containing raw egg white as their sole source of protein, they develop a condition know as "egg-white injury", characterized by neuromuscular disorders, dermatitis and loss of hair. In 1930, there was investigations that egg white injury was due to deficiency of the water-soluble vitamin biotin,and subsequently it was shown that egg-white contains a protein which binds biotin with extremely high affinity. The high affinity constant between the glycoprotein avidin and the vitamin biotin prompted early attention to the nature of this complex. In 1952 Fraenkel-Conrat and co-workers (37) purified avidin and studied the effect of chemical modification on its activity. Since 1963,Green (38) has been the leading figure in the efforts to understand this unique interaction by various biophysical and biochemical methods. However the innate reason for strong interaction between biotin and avidin is not yet known. Judging from the structure of biotin, it is difficult to understand why such a simple molecule should possess such an unprecedented affinity for a given protein. In this respect the avidin biotin complex represents a complementary approach to be a tool in molecular biology.

5. Biotin Binding to Avidin

Avidin is a basic glycoprotein having a molecular mass of about 67,000 daltons with its isoelectric point at pH 10. It is very soluble in water and salt

solutions, and it is stable over a wide range of pH and temperature. It crystallizes from strong salt solutions between pH 5 and pH 7 and has an acid isoelectric region. Avidin molecule contains four essentially identical subunits, each of which consists of a single polypeptide chain bearing 128 amino acids and possessing one biotin binding site. The carbohydrate moiety (about 10% of the total molecular mass) which contains four or five residues of mannose and three residues of N-acetyl-glucosamine per subunit. The composition and structural arrangement of the oligosaccharide residues, one avidin have been shown to exhibit a high degree of heterogeneousity. The biotin-binding activity is not lost between pH value of 2 and 13. Extensive interest in avidin has persisted, mainly due to its unusually strong interaction with the vitamin biotin. The complex formed between these two biomolecules has a remarkably low dissociation constant ($K_D = 10^{-15}$ M)

Biotin was first isolated in 1935 by the Dutch biochemist, F. Kogl, from a liver concentrate known to contain growth factors for yeast. Its structure was solved by V. du Vigneaud and his colleagues. Biotin is a vitamin having a molecular weight of 230 daltons and contains fused imidazole and thiophenyl rings(39). It was found that even antibody molecules was extensive substitution of amino groups by biotin, antigen binding capacity was not modified. In this respect the avidin-biotin complex can be exploited in quantitative enzyme immunoassay and immunohistochemical staining (34).

Biotin must be derivatized before coupling to protein; the most convenient method of coupling uses the N-hydroxysuccinimide ester of biotin, which is commercially available from numerous suppliers. Succinimide esters are extremely useful compounds for derivatization of antibodies because they allow formation of a stable amide bond under very mild conditions. The structure of biotin and its N-hydroxysuccinimide ester are shown in Figure 3.

Physicochemical studies on the nature of the avidin-biotin complex have heretofore been difficult to perform. But the demand for developing rapid, simple assay systems that can detect quantitative minute amounts of biologic substances such as peptides, hormones, vitamins and drugs are needed.

6. Replacement of Streptavidin to Avidin in ELISA

The main problem usually involves the presence of high levels of non-specific binding, generally attributed to the basicity of the avidin molecule. The carbohydrate moieties of avidin might increase non-specific association in various diagnostic assays. These limitations can be eliminated by replacing egg-white avidin with bacterial streptavidin, which is a neutral nonglycosylated protein (31,37). Streptavidin is avidin like protein. It was discovered in culture filtrates of several species of *Streptomyces avidinii* and was in most other respects remarkably similar to avidin from eggs inspite of the fact that there is considerable differences in composition (Table 3). The molecular weight of streptavidin is about 60,000. Each

of the four subunits binds biotin with extremely high affinity that is essentially irreversable. In addition, the neutral isoelectric point of streptavidin at physiological pH results in less non-specificity binding than the positively charge avidin with an isoelectric point near 10. In contrast to avidin which is a glycoprotein (7% carbohydrate), streptavidin has no carbohydrate moieties which cause non-specific binding to protein (36,40).

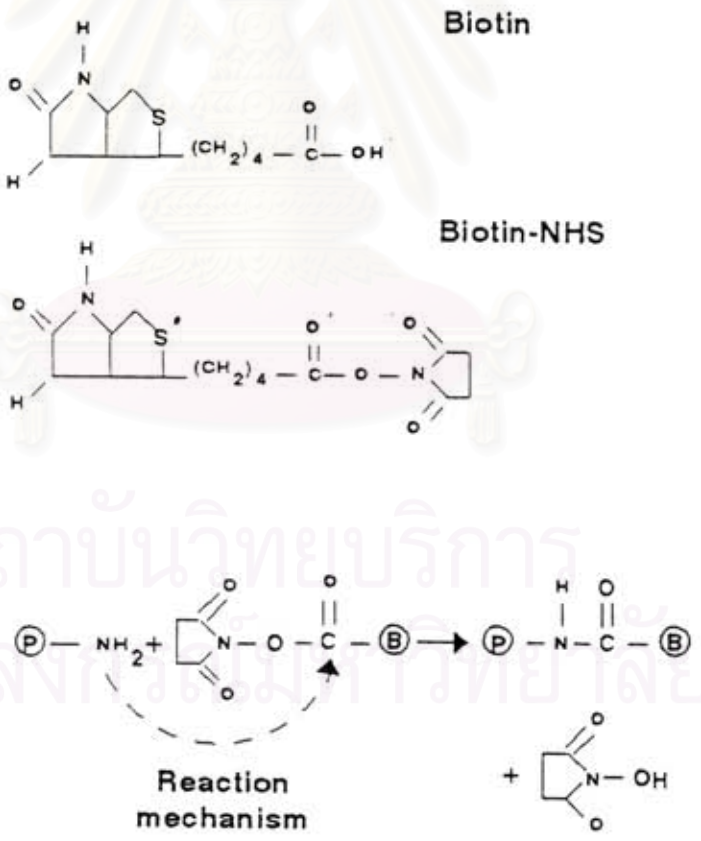

Fig. 3 Structure of biotin and its succinimide ester, and mechanism of coupling to proteins.

7. Exploitation of Avidin/Streptavidin-Biotin Complex in Enzymic Immunoassay(EIA)

Liu V. et. al.(1985) applied avidin-biotin system to detect HBsAg, labelled three monoclonal anti-HBs with biotin and linked covalently horseradish peroxidase to avidin. The assay is reactive with adw2, adw4, adr, ayw2 and ayr subtypes and can detect viral determinants in HBsAg-anti-HBs immune complex form(41). Zrein M. et. al.(1986) used biotin avidin system in direct and indirect ELISA for detecting a broad range of serological related tobacoviruses. The assay increased the sensitivity and allowed a detection of wider range of related viral serotype (42). Followed by Teng X.C.,et. al. incorporated biotin-avidin system into ELISA passive haemagglutination assay (McAB RPHA), standard ELISA and solid phase radioimmunoassay (SPRIA). This study indicated that the enhanced sensitivity of the biotin-avidin ELISA system significantly increased the correctness of diagnosis of HBV infection(43). Hao L.J.(1987) used the labeled avidin biotin dot ELISA technique as a simple and rapid method for detection of HBsAg in serum(44) and Mushahwas I.K. (1987) detected and quantitated antibody against HBsAg (anti-HBs) by utilizing HBsAg as a solid phase capture reagent and a mixture of biotinylated HBsAg and avidin-conjugated horseradish peroxidase as a probe detector reagent. The assay could measure the same molecules and the result correlated well to anti-HBs titers(45). Korce E.et. al. (1989) detected antibodies against hepatitis B core antigen using the avidin-biotin system. The assay was found to be specific and was compared to a commercial RIA kit. Both assays gave identical results(46).

Table 3 Amino Acid Composition of Avidin and Streptavidin

Amino acid	Avidin residues/subunit	Streptavidin residue/subunit
Lysine	9	4
Histidine	1	2
Arginine	8	4
Aspartic acid	15(14)	12
Threonine	20.5(19)	19
Serine	9	10
Glutamic acid	10	9
Proline	2	2
Glycine	11	17
Alanine	5	17
Half-cystine	2	0
Valine	7	7
Methionine	2	0
Isoleucine	7.5(8)	3
Leucine	7	8
Tyrosine	1	6
Phenylalanine	7	2
Tryptophan	4	8
Total residues	128	ca.130
Amide	16	-
Mannose	4(5)	0
Glucosamine	3	0
Subunit weight	15,600	14,000
Molecular weight	67,000	60,000
Biotin-bound/subunit 1%	0.97	0.95
$E_{280}^{(1\%)}$	15.4	34 47
$^{\cdot}282^{(subunit)}$	24,000	56,000

CHAPTER III

MATERIALS and METHODS

Materials

1. Hybridoma cell

Two hybridoma cells were obtained from Health Science Research Institute, Department of Medical Sciences, clone 129 and 1C5 were selected to produce monoclonal antibodies to HBsAg after fusion with myeloma cell P3-X63-Ag8.653 line and HBsAg immunized spleen cell. These hybridoma cells were kept in complete RPMI 1640 containing 10 % dimethyl sulfoxide and 20 % fetal bovine serum at -70 °c.

2. Purified hepatitis B surface antigen

Hepatitis B surface antigen (HBsAg) was purified from pooled serum of donors who are HBsAg positive.

3. Tested samples

Two hundred and thirteen human sera were collected for testing the efficiency of the local-made reagent ELISA. Eighty six samples were collected from blood donors at the Thai Red Cross, Chulalongkorn Hospital. Another one hundred and twenty seven sera were specimens from routine hepatitis B service check up at Virus Research Institute, Department of Medical Sciences, Ministry of Public Health.

4. The commercial kit

4.1 Zymed's Mouse MonoAb ID kit (HRP) was utilized to determine the class and subclass of mouse monoclonal antibodies.

4.1 AUZYME EIA reagent kit from Abbott Laboratories was utilized for detection of HBsAg in human sera.

4.2 AUSAB EIA reagent kit from Abbott Laboratories was utilized for detection of anti-HBS

5. Chemical reagents

Acrylamide (Sigma , USA.)

Alkaline phosphatase (Sigma,USA.)

Ammonium persulphate (Sigma, USA.)

Ammonium sulphate (MERCK, W. Germany)

Bovine serum albumin fraction V (Sigma, USA.)

D-Biotinyl-N-hydroxysuccinimide ester (Boehringer Mannheim Co., W.Germany)

N,N-Methyl-bis-acrylamide (Sigma,USA.)

Cell culture flask and plate (Nunc, Denmark)

Citric acid anhydrous (MERCK , W. Germany)

Dimethyl Sulfoxide (Sigma, USA.)

Dimethyl Formamide (Sigma, USA.)

Diethanolamine (Sigma, USA.)

Fetal bovine serum (GIBCO, USA.)

D-glucose (MERCK, W. Germany)

L-glutamine (MERCK, W. Germany)

Glutaraldehyde (Sigma,USA.)

Gelatin (MERCK, W. Germany)

HEPES (Sigma, USA.)

Magnesium chloride (MERCK, W. Germany)

Molecularporous membrane tubing (Spectrum Medical Industries Inc.,USA.)

p-nitrophenyl phosphate (Sigma, USA.)

Pristane (Sigma, USA.)

Recombinant protein A-sepharose 4 B (Zymed, USA.)

RPMI 1640 (GIBCO, USA.)

Sodium bicarbonate (MERCK, W. Germany)

Sodium pyruvate (Sigma, USA.)

di-Sodium hydrogen phosphate (MERCK, W. Germany)

Sodium dihydrogen phosphate (MERCK, W. Germany)

Sodium chloride (MERCK, W. Germany)

Sodium azide (MERCK, W. Germany)

Streptavidin (Sigma, USA.)

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) (Sigma, USA.)

Sodium hydroxide (MERCK, W. Germany)

Trisma base (Sigma, USA.)

Tween 20 (Sigma, USA.)

6. Instrument

CO_2 Incubator model 3164 (Forma Scientific, USA.)

Safety cabinets model Nu 425-300 (Nuaire, USA.)

High speed centrifuge model RS-20 IV (Tomy Seiko Co.,Ltd.)

Ultra centrifuge model 55P-72 (Hitachi,Japan)

Column chromatography (Pharmacia, Sweden)

Spectrophotometer Hewlett model 84524 A (Packard, USA.)

Power supply model 200/2.0 (Biorad Laboratories,Inc.,USA.)

Vortex mixer (Scientific industries, USA.)

Microtiter plate reader model EL 311 (BIO-TEX instrument microplate autoreader, USA)

pH meter model F-8L (Horiba, Japan)

Top pan balance model LY-300 (Yamato, Japan)

Analytical balance model Chyo jupiter SD-300 (Yamato, Japan)

Magnetic stirrer model Nuova II (Thermolyne co.,USA.)

Multiwasher (Nunc,Denmark)

Methods

1. Preparation of monoclonal antibodies

1.1 Recloning and limiting dilution of hybridoma

Two hybridoma cells ,clone 129 and 1C5 were frozen at -70°c. The cell lines were thawed quickly in a 37°c water bath. As soon as the medium was liquified, the cells were pelleted at 2,000 rpm for 5 min and washed with 1 mL of complete RPMI 1640. The cells were then cultured in 25 cm^2 cell culture bottles containing 5 mL of complete RPMI 1640 and incubated at 37°C in 5 % CO_2 atmosphere incubator. After the cells were propagated, they were recloned by the technique of limiting dilution, as followed(47-49):

Limiting dilution

- Counted the concentration of cell suspension
- Diluted the cell suspension with a complete RPMI 1640 to give 0.5 and 1 cell/mL
- Each diluted cell suspension was plated out in 96 well cell culture plate containing 100 μL of $1x10^4$ cell/mL peritoneal macrophage as a feeder cell by seeding 200 μL per well. Generally, plates were incubated in a 5 % CO_2 incubator at 37°C
- After 7 days from the initial seeding, microscopic colonies were visible. At this stage, the colonies were examined microscopically. Any wells containing more than one colony, or a colony that appeared asymetric, i.e. consisting of two adjacent

colonies, was rejected. Usually, the monoclonal colony was circular.

- Supernatants were tested by passive haemagglutination (PHA) test, the method of detection of anti-HBs from Virus Reseach Institute, Department of Medical Sciences, Ministry of Public Health. The strongest positive wells were selected and expanded for the production of mouse ascites.

1.2 Ascites production of monoclonal antibodies

After providing monoclonal hybridoma cultures, antibodies were produced from ascites tumors in mice. Hybridoma cells clone 129 and 1C5 were injected ($1x10^7$ cell per 0.5 mL) intraperitoneal into 7-14 days pristane-treated BALB/C mice. Ten to forteen days later, the ascites fluid was collected when the peritoneal cavity became distended. The ascites fluid was removed from the peritoneal cavity by the use of No. 20 hypodermic needle, allowing it to flow through and dropped into the small tube. The fluid was then pooled and the cells were removed by centrifugation; the oil layer was aspirated from the surface of the fluid, and the supernatant liquid was transferred to another tube. The ascites fluid was frozen in aliquots.

1.3 Characterization of monoclonal antibodies to HBsAg (anti-HBs)

The interesting characteristics of monoclonal antibodies are specificity and isotyping which can be determined as follows:

1.3.1 Determination of specificity

To determine the specificity of monoclonal antibodies, the AUSAB EIA reagent kit from Abbott company was used to detect anti-HBs in each culture supernatant using 200 μL of culture supernatant or controls (three negative and two positive controls) and pipetted to assigned wells. One bead was added to each well, then applied cover seal and tapped tray gently to soak the beads thoroughly. The tray was incubated at 40 ± 1^{0}C for 2 hours and the cover seal was removed. After washing, 200 μL of a mixture of HBsAg:biotin and anti-biotin:peroxidase (horseradish) conjugate was pipetted into each reaction well. The tray was then tapped gently, sealed and incubated at 40 ± 1^{0}C for 2 hours. Then the cover seal was removed and the beads were washed by the bead washer machine and transfered immediately to properly identified assay tubes. 300 μL of freshly prepared OPD substrate solution was pipetted into two empty tubes as substrate blank and then into each tube containing the bead. The tubes were incubated at room temperature for 30 minutes and the reaction was stopped by adding 1 mL of 1 N sulfuric acid.

1.3.2 Determination of isotyping

To determine isotype or immunoglobulin class and subclass of monoclonal antibodies, Zymed's Mouse Mono Ab ID kit (HRP), a complete set of ELISA reagent, was provided and tested: 50 μL of each culture supernatant was added to each well in the first row of HBsAg-coated plate. One additional culture supernatant was added to each subsequent row of microtiter plate. Then the plate was incubated at 37°C for 30 minutes. After washing, 50 μL of PBS was added to each

well of the first column as a blank, 50 μL of normal rabbit serum was added to each well of the second column as negative control and 50 μL of subclass specific rabbit anti-Mouse IgG1 was added to each well of the third column. 50 μL of the rest of the subclass specific antibodies to columns 4 through 10 as was done for rabbit anti-Mouse IgG1. The plate was incubated at 37°C for 30 minutes. After washing, 50 μL of diluted HRP-Goat Anti-Rabbit IgG was added to all wells and incubated at room temperature for 30 minutes. Followed by washing and 100 μL of ABTS substrate was added to all wells and then incubated at room temperature for 30 minutes. The result was identified by visual inspection or by a spectrophotometer at 405 nm.

1.4 Purification of monoclonal antibodies using protein A-sepharose 4B affinity chromatography

The swollen gel, protein A-sepharose CL-4B, was packed into a column and washed with 2 mL of 0.1 M citric acid. The column was then equilibrated with 5-10 mL of 0.1 M phosphate buffer (pH 8.0) as binding buffer. After the ascites was adjusted pH to 8.0 with 1 M Tris HCL pH9.0, it was applied to the column with the flow rate approximately 5 mL/h. The unbounded protein was washed with 30-40 mL of 0.1 M phosphate buffer (pH 8.0). The column was eluted with 5 mL citrate buffer pH 6.0 as one millilitre fractions to obtain IgG1, then monitored by the absorbance at 280 nm. 5 mL citrate buffer pH 4.5 was used as an eluant for IgG2a and 5 mL of citrate buffer pH 3.5 for IgG2b respectively. The eluate was collected in the tube containing 60 μL of 2 M Tris. Then, the column was

washed with 2 mL citric acid and followed by 5 mL 0.1 M phosphate buffer. Each peak of the eluted fractions was pooled and dialysed against PBS.

2. Preparation of enzyme-antibody conjugate

2.1 Enzyme-monoclonal anti-HBS coupling

The procedure for coupling the enzyme to monoclonal anti-HBs antibodies clone 1C5 and 129, is as follows: The enzyme selected was alkaline phosphatase (AP),Sigma type VII-N as a suspension in 34 M ammonium sulfate with the specific activity of 2,000 unit/mg protein and the concentration of protein was 10 mg/mL. Various amount of alkaline phosphatase in suspension were centrifuged at 4°C for 10 minutes at 10,000 rpm. The supernatants were discarded and the pellets were suspended with 0.5 mL of the solution of monoclonal anti-HBs containing 0.5 mg. After dialysis overnight against PBS, 4 μl of 25 % glutaraldehyde were added. The solution was left at room temperature and a portion of 0.15 mL was pipetted into a dialysis bag at an interval of 30,60,90,120 minutes respectively for the study of optimal incubation period. These resulted conjugates were then dialyzed. After dialysis, it was diluted to 10 ml with 5 % bovine serum albumin in 0.05 M Tris - HCl pH 8.0 containing 1 mM $MgCl_2$ and 0.02 % Na N_3 until tested.(1,50)

2.2 Confirmation of enzyme-antibody conjugate

Disposible polystyrene microtiter plates were coated with purified MAb 129 for testing AP-1C5 conjugate and with purified MAb 1C5 for testing

AP-129 conjugate as follows: 5 μl/ml of purified anti-HBs in 0.1 M sodium carbonate buffer (pH9.6) containing 0.02 % NaN_3 was added to each well of the microtiter plates. The plates were incubated at 4°C overnight and washed with PBS-Tween. Then, 5 μg/mL of HBsAg in PBS-BSA were added into each well and incubated at 37°C for 2 hours and followed by washing. 100 μL of various diluted conjugates were added to the wells and incubated at 37°C for 1 hour. After washing, 100 μL of 15 mM nitrophenyl phosphate (NPP) was added to each well and incubated at 37°C for 1 hour. 50 μL 3M NaOH was added to each well to stop the reaction. The colour developed from each well was then measured by ELISA microtiter plate reader at 405 nm.

3. Preparation of biotinylated monoclonal antibody.

3.1 Labelling biotin to monoclonal anti-HBs (Biotinylation)

Biotinyl-N-hydroxysuccinimide ester (BNHS) was used for incorporation of biotin moieties into protein(36,51,52). This procedure of biotinylation was performed for two monoclonal anti-HBs antibodies, clone 1C5 and 129 as the following: 1 mg of monoclonal antibody in 1 mL of 0.1 M $NaHCO_3$ pH 8.3 was mixed with 4 μL of various concentration of BNHS in dimethyl formamide. The reaction mixture was incubated at room temperature for a specific period of time (i.e.: 2, 3 and 4 hours respectively) and then dialyzed for 24 hours at 4°C against several changes of PBS. After dialysis, an equal volumn of glycerol was added and the preparations were kept at -20°C until used. A fresh solution of BNHS was

prepared each time immediately before used.

3.2 Confirmation of biotin-antibody coupling (biotinylated Ab)

1 μg/mL of purified HBsAg was coated in each well of a disposible polystyrene microtiter plate. After incubating at 4°C overnight and washing with PBS-Tween, 100 μL of various dilution of biotinylated MAb 129 (B-129) or biotinylated MAb 1C5 (B-1C5) from 3.1 was added into each well. Then the plate was incubated at 37°C for 1 hour. After washing, 100 μL of anti-biotin alkaline phosphatase conjugate was added into each well and incubated at 37°C for another hour. Followed by washing, then 100 μL of 15 mM p-nitrophenyl phosphate (NPP) was added into each well and reaction was incubated at 37°C for 1 hour. The reaction was then stopped by adding 50 μL of 3M NaOH into each well. The developed colour was measured at 405 nm by the ELISA plate reader.

4. Preparation of streptavidin plate

4.1 Coupling of streptavidin to polystyrene micro-titer plate

A series of streptavidin solution at concentration of 0.1, 1, 5, 10, 15, 20, 25 μg/mL in 3 mM phosphate buffer (pH 6.8) was prepared as the coating medium. 200 μL was pipetted to each well of a polystyrene microtiter plat. The plate was incubated at 4°C for 1 hour, then 67 μL of 0.2 M EDAC in 3 mM phosphate buffer was added to each well, mixed thoroughly and left for another 3 hours at 4°C. The plate was washed twice with 0.01 M phosphate buffer saline (pH 7.2), then

twice with high-salt buffer (pH 7.2) and followed by 0.01 M phosphate buffer saline (pH 7.2). 300 μL of PBS-BSA (2%) was added into each well, incubated at 4°C for 1 hour and washed twice with PBG. Finally, 300 μL of PBG containing 0.01% sodium azide was added into each well and lyophilized. The lyophilyzed coated plate was then stored at 4°C until used.(53,54)

4.2 Determination of optimal concentration of streptavidin for plate coating

The checkerboard experiment was designed to optimize the concentration of streptavidin for coating on polystyrene plate and reacting with various dilution of B-129 as follows:

Coating 0.1 , 1.0 , 5 , 10 ,15 and 20 μL/mL of streptavidin onto the wells of 1st-2nd column,3rd-4th column, 5th-6th column , 7th-8th column, 9th-10th column and 11th-12th column,respectively. The procedure was described in 4.1.

The B-129 antibody were diluted into 1:1,000 , 1:1,500 , 1:2,000 and 1:2,500 of B-129 with diluent I

Then 100 μL of 1:1,000 B-129 was added to each well in row A-B, 1:1,500 in row C-D, 1:2,000 in row E-F and 1:2,500 in row G-H respectively.

The plate was then covered and incubated at 37°C for 1 hour in a moisture chamber. Then, aspirated and the wells were washed 4 times with PBS-Tween

Each 100 μL of 1.0 μg/mL of HBsAg in PBS-BSA was added into each wells of column 1st, 3rd, 5th, 7th, 9th and 11th and 100 μL of 1.0 μg/ml of HBsAg into

wells of column 2^{nd} , 4^{th} ,6^{th} ,8^{th} ,10^{th} and 12^{th} . The plate was then incubated at 37°C for 2 hours in a moisture chamber and washed with 5 times PBS-Tween.

Then 100 μL of 1:100 dilution of AP-1C5 in diluent II was pipetted to all wells. The plate was incubated at 37°C for 1 hour in a moist chamber and washed 5 times with PBS-Tween.

100 μL of 15 mM nitrophenyl phosphate was added and incubated at 37°C for 1 hour in a moisture chamber.

Finally, 50 μL of 3M NaOH was added and developed colour was measured at 405 nm by an ELISA plate reader.

5. Determination of affecting factors of the ELISA system

The indirect ELISA was used in this study. In order to establish the assay, checker board titration was carried out to determine the optimal conditions of the reagents in this assay.

5.1 Determination of optimal antibody labelling to biotin or to enzyme

1:1,000, 1:1,500, 1:2,000 and 1:2,500 dilution of biotinylated MAb 129 (B-129) were added into streptavidin microtiter plate column 1^{st} and 2^{nd} and coordinatedly with row A-B, C-D, E-F and G-H respectively. Similarly, 1:1,000, 1:1,500, 1:2,000 and 1:2,500 dilution of biotinylated MAb 1C5 were added into column 3^{rd}-4^{th} and row A-B, C-D, E-F and G-H, respectively. The plate was incubated at 37°C for 2 hours and washed out. 100 μL of 1:200 dilution of AP-1C5

and AP-129 conjugate were added into the plate column 1st-2nd and 3rd-4th, respectively. The plate was incubated at 37°C for 1 hour and washed. 100 μL of 15 mM nitrophenyl phosphate was added into all wells and incubated at 37°C another hour. 50 μL of 3M of NaOH was added to stop the reaction and the developed colour was measured at 405 nm.

5.2 Determination for optimal dilution factor of labelled antibody

The B-129 was diluted to 1:1,000,1:2,000 and 1:3,000 with diluent I

100 μL of each dilution was added into wells of the first three column, second three column and third three column, respectively.

The plate was then covered and incubated at 37°C for 1 hours in a moisture chamber. Then, aspirated and the wells were washed 4 times with PBS-Tween.

100 μL of PBS-BSA and each diluted purified HBsAg in PBS-BSA (ie. 10, 12.5, 25, 50, 100 ng/mL and 1 μg/mL) was added into row B, C, D, E, F, G and H, respectively. The plate was then incubated at 37°C for 2 hours in a moisture chamber and washed with 5 times PBS-Tween.

Then 100 μL of 1:100 , 1:200 and 1:300 dilution of AP-1C5 in diluent II. was pipetted to the first column, second column and third column, respectively. The procedure was identically repeated in the other sections. The plate was incubated at 37°C for 1 hour in a moisture chamber and washed plate 5 times with PBS-Tween.

100 μL of 15 mM nitrophenyl phoshate was added and incubated at 37°C for 1 hour in a moisture chamber.

Finally, 50 μL of 3M NaOH was added and the developed colour was measured at 405 nm by an ELISA plate reader.

5.3 Determination of optimal temperature and reaction time for:

For every reaction as designate, 100 μL of 1:2,000 of the diluted B-129 with diluent I was pipetted into all wells. 100 μL of 0 , 5 , 10 , 12.5 , 25 , 50 and 100 ng/mL of HBsAg suspension were applied to each well in row B, C, D, E, F, G and H, respectively. Then, 100 μL of 1:250 fold dilution of AP-1C5 in diluent II was pipetted into all wells. The plates were tested by the protocol outline in table as follows:

5.3.1 Reaction of streptavidin plate and biotinylated monoclonal anti-HBs 129

Well contents	Temperature(°C)	Incubation time
(1) B-129	37	30, 60 min (wash)
	RT	30, 60 min (wash)
(2) HBsAg suspension	37	120 min(wash)
(3) AP-1C5	37	60 min(wash)
(4) Chromogenic substrate	37	60 min
(5) 3 M NaOH	RT	-

5.3.2 Reaction of biotinylated monoclonal anti-HBs 129 (B-129) and HBsAg

Well contents	Temperature (°C)	Incubation time
(1) B-129	37	60 min (wash)
(2) HBsAg suspension	37	1,2,3,4 hrs (wash)
	45	1,2,3,4 hrs (wash)
	RT	1,2,3,4,20 hrs (wash)
(3) AP-1C5	37	60 min (wash)
(4) Chromogenic substrate	37	60 min (wash)
(5) 3 M NaOH	RT	-

5.3.3 Reaction of HBsAg and alkaline phosphatase-anti-HBs 1C5 conjugate (AP-1C5)

Well contents	Temperature (°C)	Incubation time
(1) B-129	37	60 min (wash)
(2) HBsAg suspension	45	60 min (wash)
(3) AP-1C5	37	30,60,90 min (wash)
	RT	30,60,90 min (wash)
(4) Chromogenic substrate	37	60 min
(5) 3 M NaOH	RT	-

5.3.4 Colour development

Well contents	Temperature (°C)	Incubation time
(1) B-129	37	60 min (wash)
(2) HBsAg suspension	45	60 min (wash)
(3) AP-1C5	37	60 min (wash)
(4) Chromogenic substrate	37	45,60,90 min (wash)
	RT	45,60,90 min (wash)
(5) 3 M NaOH	RT	-

6. Standardization of the Streptavidin-Biotin ELISA test

6.1 Precision study

A within-plate precision was determined by using three sera; HBsAg strong positive serum , HBsAg positive and negative control. Precision for each serum was calculated by running 15 replicates in a microtiter plate.

A between plate precision (reproducibility) was determined by the same controls, analysed on 20 separate microtiter plates. All plates were processed by using optimal conditions.

6.2 Interpretation of results

6.2.1 Sensitivity of the assay

The sensitivity of the assay was determined by either visual inspection

or by calculation of a cutoff value based upon 3 fold of standard deviations above the mean obtained from twenty HBsAg negative sera. This value corresponds to at least the 99 % confidence level for the upper limit of negative sera absorbance using a one-tailed test. Twenty negative sera were repeatedly for five different assays in order to obtain the means absorbance for further calculations of the cutoff value. The plates were tested by using optimal conditions and the tests were repeatedly for five times.(26)

6.2.2 Specification of the assay

Normal human sera, pretested and found to be negative for HBsAg by AUZYME (Abbott Laboratories,USA.) were assayed by local-made ELISA. These values were used to calculate mean and standard deviation for the N value (absorbance of negative control). Sera with an absorbance value greater than 3 SD above the mean were considerd false positive.

7. Comparative study of detection of HBsAg by Streptavidin-Biotin ELISA and commercial ELISA kit

The developed Streptavidin-Biotin ELISA technique was applied to study HBsAg in the serum of 220 subjects, compared to the commercial ELISA kit , AUZYME (Abbott Laboratories, USA.).

7.1 Procedure of streptavidin-biotin ELISA

Polystyrene microtiter plate was coated with 200 μL of streptavidin (1 μg/mL) using protocol in 4.1. 100 μL of 1:2000 dilution of B-129 with diluent I was added to all wells. The plate was incubated at optimal temperature and time, washed five times. For each time of washing, the washing solution was left in the well for 3 minutes. Then, 100 μl of each serum was added at the selected optimal condition. After washing, 100 μL of 1:250 dilution of AP-1C5 with diluent II was added to all wells. The plate was incubated at optimal condition and washed. Chromogenic substrate, 100 μL of 15 mM nitrophenyl phosphate was added to all wells and incubated at optimal condition. The reaction was stopped by the addition of 50 μL of 3M NaOH.

7.2 Procedure of commercial ELISA kit , AUZYME EIA

200 μL of each control or specimen was dispensed into bottom of wells of reaction tray (usually, 3 Negatives controls and 2 Positive controls). 50 μL of conjugate was added into each well containing a specimen or control. Gently tapped the tray to enhance mixing of conjugate with controls or specimens. Anti-HBs coated bead was added carefully to each well containing a specimen or control. Cover seal was applied and gently tapped tray to cover beads and removed any trapped air bubbles. The tray was incubated at 40°C for 3 hours, then the cover seal was removed and the liquid was aspirated, The bead was then washed three to five times with 4 to 6 mL of distilled or deionized water. Beads were transfered immediately to properly identified assay tubes. 300 μL of freshly prepared OPD substrate solution

was pipetted into two empty tubes (substrate blank) and then into each tube containing a reacted bead. The reaction was incubated at room temperature for 30 minutes and 1 mL of 1 N sulfuric acid was added to each tube. The developed colour was determined by measuring the absorbance at 492 nm within 2 hours after addition of acid.

7.3 Statistical Analysis

$$Mean\ (\bar{X}) = \Sigma\ \frac{X}{N}$$

$$Standard\ deviation = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$$

$$\%\ Coefficient\ of\ variation(CV) = \frac{SD}{\bar{x}} \times 100$$

$$\%\ Sensitivity = \frac{TP}{TP+FN} \times 100$$

$$\%\ Specificity = \frac{TN}{TN+FP} \times 100$$

$$\%\ Efficiency = \frac{TP+TN}{Total\ number\ of\ samples} \times 100$$

$$\%\ Positive\ predictive\ value = \frac{TP}{TP+FP} \times 100$$

$$\%\ Negative\ predictive\ value = \frac{TN}{TN+FN} \times 100$$

TP = true positive, TN = true negative

FP = false positive, FN = false negative

8. Study of the stability of the Streptavidin-Biotin ELISA reagent kit in various conditions

The ELISA reagent kit consisted of streptavidin plate, diluted B-129, diluted AP-1C5 , Chromogenic substrate and 3M NaOH was stored at 4°C , 37°C and room temperature and was tested for stability. The assay was considerd to be valid if the following criteria were fulfilled(55):

(1) The median negative control absorbance was less than 0.1

(2) The mean absorbance of the weak positive control was at least 0.2 greather than the median negative absorbance

(3) The mean of TNR of the weak positive controls was at least 10

The TNR was culculated for each weak positive controls by expressing the absorbance in terms of the median negative controls.

$$TNR = \frac{A}{M} \; ratio$$

TNR = test/negative absorption ratio

A = absorbance of test

M = median of negative absorbance value

CHAPTER IV

RESULTS

1. Preparation of monoclonal anti-HBs antibodies.

1.1 Hybridoma cell line from limiting dilution

After limiting dilution process, the two retrieved hybridomas clones 129 and 1C5, were obtained. It was found that both still secreted the monoclonal antibodies to HBsAg when they were tested by PHA method. The PHA titers of both monoclonal antibodies detected in cell culture medium were more than 10^4. Their characteristic was determined and found to be both specific to HBsAg after confirming with the commercial AUSAB EIA reagent kit which was utilized to detect anti-HBs and the isotype of monoclonal anti-HBs clone 129 and 1C5 were IgG2a and IgG1, respectively.

1.2 Purification of monoclonal anti-HBs antibodies using protein A-sepharose CL-4B chromatography

By using three different pHs of citrate buffer for elution, MAb 129 was collected when eluted with citrate buffer pH 4.5 while MAb 1C5 was eluted with citrate buffer pH 6.0 as shown in Figure 4 (see page 62).

2. Coupling of enzyme alkaline phosphatase to monoclonal anti-HBs

In order to determine the degree of conjugation of enzyme alkaline phosphatase (AP) to monoclonal anti-HBs which would yield the highest level of reactivity, various weight ratio of AP to MAb 129 or MAb 1C5 were tested, For conjugation of MAb 129, where 3:1 of AP:MAb 129 produced a satisfactory result when the reaction was incubated for 60 minutes while 1:1, 2:1 and 4:1 of AP gave a lower absorbance than the 3:1 ratio as presents in Table 4 (see page 50). For the conjugation of MAb 1C5, the ratio of 2:1 and 3:1 of AP:MAb 1C5 for 90 minutes produced higher dose response than 1:1 and 4:1 as presented in Table 5 (see page 51)

To determine a suitable clone of MAb which was conjugated to enzyme alkaline phosphatase, reactivity level of each conjugate was compared with various dilutions of each AP-MAb conjugate. Table 6 indicated that AP-1C5 yielded higher dose response than AP-129 for detection of HBsAg (see page 52).

3. Labeling biotin to monoclonal anti-HBs (Biotinylation)

10, 20 and 40 mg/mL of biotinyl-N-hydroxysuccinimide ester (BNHS) were labeled to 1 mg/mL monoclonal anti-HBs 129 and incubated at room temperature for 2, 3 and 4 hours. Four hours incubation time gave higher dose response than 2 and 3 hours as presented in Figure 5, 6 and 7, respectively (see page 63,64 and 65). When each concentration of BNHS reacted with 1 mg/mL of MAb

129 was made, it was found that 20 and 40 mg/mL of BNHS gave the same dose response but higher than 10 mg/mL as presented in Figure 8 (see page 66). Similarly, Figure 9, 10 and 11 showed the activity of biotinylated MAb 1C5 (B-1C5) obtained from the reaction of using 10, 20 and 40 mg/mL of BNHS labeled to 1 mg/mL of MAb 1C5, respectively (see page 67,68 and 69). Although each of them yielded the highest dose response at 4 hours incubation period, it was noticible that 20 mg/mL of BNHS yielded the highest dose response as shown in Figure 12 (see page 70).

For comparing the binding activity of monoclonal anti-HBs to HBsAg after labeling antibody with BNHS, it was found that biotinylated MAb 1C5 (B-1C5) gave higher absorbance than biotinylated MAb 129 (B-129) as presented in Figure 13 (see page 71).

4. Coupling of streptavidin to polystyrene microtiter plate

In order to determine the optimal concentration of streptavidin for coating onto the polystyrene microtiter plate, various concentrations of streptavidin were designed for the futher experiment. In Table 7, the polystyrene microtiter plate coated with 0.1 μg/mL of streptavidin gave lower absorbance than the others whereas the streptavidin concentration from 1, 5, 10, 20 and 25 μg/mL yielded almost the same absorbance at 405 nm (see page 53).

5. Determination of factors affecting the ELISA system for the detection HBsAg

5.1 Determination of the optimal clone of antibody for the biotin labeling or the enzyme labeling

Preliminary experiment were carried out in order to compare the activity of the labeled MAbs when they were used in ELISA system. Figure 14 illustrated that the pair of B-129 and AP-1C5 conjugate gave the higher yield than the pair of B-1C5 and AP-129 for detecting 100 ng/mL of HBsAg (see page 72). Therefore, B-129 and AP-1C5 conjugate was chosen for further work.

5.2 Determination of optimal dilution factor of labeled antibodies

As presented in Table 8, for detecting 0.1 μg/mL of HBsAg, 1:2,000 dilution of B-129 gave the higher absorbance than the others. It was obviously seen that the dilution of AP-1C5 at 1:100, 1:150, 1:200 and 1:250 yielded the similar amount of absorbance although the different dilution of B-129 were used. However, 1:2,000 dilution of B-129 gave the slightly higher (see page 54). So, 1:2,000 dilution of B-129 and 1:250 dilution of AP-1C5 conjugate were chosen as the appropriate dilutions for further work.

5.3 Determination of optimal temperature and reaction time

Using the selected conditions of each reagent, the effects of time and temperature on the reaction in ELISA procedure were investigated for the maximal binding.

5.3.1 Reaction of streptavidin plate and biotinylated monoclonal anti-HBs 129 (B-129)

Figure 15 indicated that a 60 minutes incubation time at 37°C was the best condition for biotin/streptavidin reaction in detection of HBsAg by ELISA (see page 73).

5.3.2 Reaction of biotinylated monoclonal anti-HBs 129 (B-129) and HBsAg

Maximal binding of previous experiment in 5.3.1 is essential to attain high levels of sensitivity. The reaction of B-129 and HBsAg was done at 3 incubation conditions i.e. 37°C, 45°C, and room temperature. Figure 16 showed that at 37°C, the reaction time longer than 2 hours did not yield the higher significant absorbance. At 45°C, 1 hour reaction time yielded the highest absorbance while the time longer than 1 hour decreased the absorbance. And at room temperature, the optimal incubation time was 20 hours(see page 74). When the absorbance of each incubation temperature was compared, it could be seen that each reaction time did not give any significant difference. Thus, the reaction condition can be selected at any above incubation temperature.

5.3.3 Reaction of HBsAg and alkaline phosphatase-monoclonal anti-HBs 1C5 conjugate (AP-1C5)

From the previous studies, the assay of 1:250 dilution of AP-1C5 was selected as the optimal dilution to determind the optimal incubation time

and temperature. Figure 17 showed that a 60 minute incubation time at 37°C gave higher absorbance than the one at room temperature. Thus, it was selected as the incubation condition (see page 75).

5.3.4 Colour development

The intense of colour was depended upon both the quantity of enzyme presented and the length of the reaction-period. Figure 18 presented the effect on reaction-times that nitrophenyl phosphate as the chromogenic substrate was hydrolysed by enzyme alkaline phosphatase. The results indicated that the optimal incubation time to develop colour should be 60 minutes at 37°C (see page 76).

6. Standardization of the Streptavidin-Biotin ELISA

6.1 Precision analysis of ELISA test

A within-plate precision was determined by using three sera; HBsAg strong positive serum, HBsAg positive and negative control. Precision for each serum was calculated by running 15 replicates in one streptavidin plate. The coefficient of variation (CV) of the within-plate precision were ranging from 5.7 % to 14.89 %.

A between-plate precision (reproducibility) was determined by the same controls and analysed on separated streptavidin plates. All plates were processed by using optimal conditions. The reproducibility varied with the coefficient of variation (CV) ranging from 10.87 % to 18.24 % as shown in Table 10 (see page 56).

6.2 Calculation of cutoff value

number of negative sera	=	20
Mean of absorbance value	=	0.04
SD of absorbance value	=	0.021
cutoff value	=	0.105

Since the Streptavidin-Biotin ELISA could determine detectable concentration of HBsAg at 12 ng/mL,the comparison of the detectable minimum concentration of HBsAg were made by carrying out the experiment using the local-made reagent of the Streptavidin-Biotin ELISA, conventional ELISA and AUZYME EIA kit from Abbott company. The lowest concentration of HBsAg that could be detected by the conventional ELISA, the Streptavidin-Biotin ELISA and AUZYME EIA kit were 25, 12 and 1 ng/mL, respectively. As shown in Table 11 (see page 57).

7. Comparative study on detection of HBsAg by the Streptavidin-Biotin ELISA and commercial ELISA kit

After standardization of various ELISA parameters, 213 sera were tested to detect HBsAg by Streptavidin-Biotin ELISA. These results were then compared to commercial ELISA kit as presented in Table 12. Of the 213 sera tested, 96 sera were positive for HBsAg by AUZYME EIA kit but 2 sera of 117 negative

sera were positive by Streptavidin-Biotin ELISA. Consequently, the sensitivity and specificity of the Streptavidin-Biotin ELISA were 100% and 98.29%, respectively. The efficiency, positive predictive value and negative predictive value were 99.06%, 97.96% and 100%, respectively as compared with commercial ELISA kit (see page 58).

8. The stability of the Streptavidin-Biotin ELISA reagent kit in various condition

To study the stability of the Streptavidin-Biotin ELISA reagent kit, the kit consisted of streptavidin plate, diluted B-129, diluted AP-1C5, chromogenic substrate, substrate buffer and 3M NaOH were stored at 37°C, room temperature and 4°C. The stability of reagent kit was study from the duration of 1 day to 3 months. The result showed that at 37°C, the reagent kit was invalid quickly after keeping for one day as shown in Table 13 (see page 59). While the storage at room temperature, the duration of time could be extended to two days because criteria for considering valid was still fulfilled as presented in Table 14 (see page 60). When the reagent kit was stored at 4°C, the validity was extended to more than three months as presented in Table 15 (see page 61).

Table 4 Effect of weight ratios and incubation time on conjugating enzyme to MAb 129 for the detection of 5 μg/mL of HBsAg by ELISA.

Weight ratios AP:MAb 129	Absorbance at 405 nm				Incubation time (min)
	Dilution of AP-129 conjugate				
	1:100	1:150	1:200	1:250	
1:1	0.174	0.121	0.095	0.090	30
	0.393	0.368	0.297	0.234	60
	0.321	0.236	0.219	0.200	90
	0.318	0.223	0.200	0.198	120
2:1	0.200	0.212	0.098	0.092	30
	0.615	0.586	0.579	0.425	60
	0.492	0.439	0.330	0.312	90
	0.404	0.359	0.284	0.221	120
3:1	0.905	0.994	0.660	0.575	30
	2.042	1.936	1.486	1.012	60
	1.506	1.113	0.983	0.736	90
	0.912	0.590	0.439	0.417	120
4:1	0.349	0.585	0.613	0.830	30
	0.586	0.845	1.080	1.167	60
	0.430	0.535	0.885	1.049	90
	0.144	0.287	0.301	0.286	120

Table 5 Effect of weight ratios and incubation time on conjugating enzyme to MAb 1C5 used to detect 5 μg/mL of HBsAg by ELISA.

Weight ratios AP:MAb 1C5	Absorbance at 405 nm				Incubation time (min)
	Dilution of AP-1C5 conjugate				
	1:100	1:150	1:200	1:250	
1:1	0.782	0.670	0.448	0.242	30
	1.293	1.024	0.830	0.581	60
	1.792	1.348	1.057	0.921	90
	2.171	1.448	1.393	0.951	120
2:1	0.879	0.683	0.445	0.238	30
	1.980	1.517	1.392	1.018	60
	>2.5	2.481	2.240	1.625	90
	2.309	1.9 5	1.452	0.948	120
3:1	1.244	0.968	0.850	0.742	30
	2.402	2.016	1.594	1.318	60
	>2.5	>2.5	2.413	2.021	90
	>2.5	2.148	1.915	1.559	120
4:1	0.801	0.867	0.943	1.239	30
	0.591	0.682	0.693	0.711	60
	0.342	0.448	0.601	0.693	90
	0.170	0.176	0.181	0.148	120

Table 6 Comparison of dose response between AP-129 and AP-1C5 for detection of HBsAg.

Dilution of conjugate	Absorbance at 405 nm	
	AP-129	AP-1C5
1:100	2.042	>2.5
1:150	1.936	>2.5
1:200	1.486	2.413
1:250	1.102	2.021

Table 7 Effect of concentration of streptavidin on coating to polystyrene microtiter plate and used to detect HBsAg by ELISA.

Conc. of streptavidin (μg/mL)	Absorbance at 405 nm		
	Conc. of HBsAg (μg/mL)		
	0	**0.1**	**1.0**
0.1	0.003	0.102	0.929
1.0	0.001	0.776	2.113
5.0	0.003	0.951	2.171
10.0	0.017	0.692	2.142
15.0	0.014	0.780	2.122
20.0	0.016	0.830	2.233
25.0	0.014	0.758	2.269

Table 8 Checkerboard titration to determine optimal dilution of labeled MAb by ELISA.

Dilution of B-129	Absorbance at 405 nm			Dilution of AP-1C5
	Conc. of HBsAg (ng/mL)			
	0	0.1	1.0	
1:1,000	0	0.776	>2.5	1:100
	0.004	0.392	>2.5	1:150
	0.001	0.357	2.293	1:200
	0.002	0.508	2.443	1:250
1:1,500	0.002	0.624	>2.5	1:100
	0.005	0.438	>2.5	1:150
	0.006	0.455	2.246	1:200
	0.001	0.433	2.364	1:250
1:2,000	0	1.069	>2.5	1:100
	0.025	0.630	2.442	1:150
	0.010	0.602	2.367	1:200
	0.012	0.688	2.261	1:250
1:2,500	0	0.915	>2.5	1:100
	0.014	0.424	1.669	1:150
	0.001	0.430	1.917	1:200
	0	0.560	1.929	1:250

Table 9 Optimal conditions of the Streptavidin-Biotin ELISA test for detection of HBsAg

Well contents	Temperature (°C)	Incubation time
(1) B-129	37	60 min
(2) HBsAg suspension	37	2 hours
	45	1 hour
	RT	20 hours
(3) AP-1C5	37	60 min
(4) Chromogenic substrate	37	60 min
(5) 3M NaOH	RT	-

RT = room temperature

Table 10 Precision analysis of the Streptavidin-Biotin ELISA for detection of HBsAg

	Absorbance of tested sera		
	Strong positive	**Weak positive**	**Negative**
Within-plate (n=15)			
Mean	1.994	0.435	0.047
SD	0.144	0.025	0.007
% CV	7.220	5.700	14.89
Between-plate (n=20)			
Mean	1.819	0.416	0.036
SD	0.332	0.057	0.004
% CV	18.240	13.790	10.870

Table 11 Comparison of sensitivity of the S-B ELISA, conventional ELISA and Abbott EIA kit

Reagent kit	Absorbance at 405 nm					
	Conc. of HBsAg (ng/mL)					
	0	1	10	12	25	50
Abbott	0.005	**0.102**	0.960	1.451	>2.0	>2.0
S-B ELISA	0.026	0.043	0.074	**0.122**	0.260	0.452
conventional ELISA (local made)	0.010	0.011	0.024	0.079	**0.134**	0.213

* Absorbance at 492 nm

Table 12 Comparison of the Streptavidin-Biotin ELISA and Abbott EIA kit

S-B ELISA	Number of sera		
	Abbott EIA kit		
	Positive	**Negative**	**Total**
Positive	96	2	98
Negative	0	115	115
Total	96	117	213

Sensitivity = 100 %

Specificity = 98.29 %

Efficiency = 99.06 %

Positive predictive value = 97.96 %

Negative predictive value = 100 %

Table 13 Stability of the Streptavidin-Biotin ELISA reagent kit stored at 37°C

Period of time (days)	Valid criteria of reagent		
	Median of negative control absorbance	Mean of weak positive control absorbance	Mean of TNR
0	0.014	0.600	42.85
1	0.024	0.205	8.54
2	0.024	0.149	6.23
3	0.026	0.150	5.68

(validity : mean of TNR > 10)

Table 14 Stability of the Streptavidin-Biotin ELISA reagent kit stored at room temperature.

Period of time (days)	Valid criteria of reagent		
	Median of negative control absorbance	Mean of weak positive control absorbance	Mean of TNR
0	0.014	0.600	42.85
1	0.013	0.348	26.77
2	0.014	0.200	13.75
3	0.017	0.160	9.38
4	0.014	0.140	9.86

(validity : mean of TNR > 10)

Table 15 Stability of the Streptavidin-Biotin ELISA reagent kit HBsAg stored at 4°C.

Period of time (weeks)	Valid criteria of reagent		
	Median of negative control absorbance	Mean of weak positive control absorbance	Mean of TNR
0	0.014	0.600	42.86
2	0.019	0.520	27.37
4	0.020	0.401	20.05
6	0.024	0.447	18.62
8	0.025	0.465	18.62
10	0.020	0.394	19.72
12	0.019	0.312	16.42

(validity : mean of TNR >10)

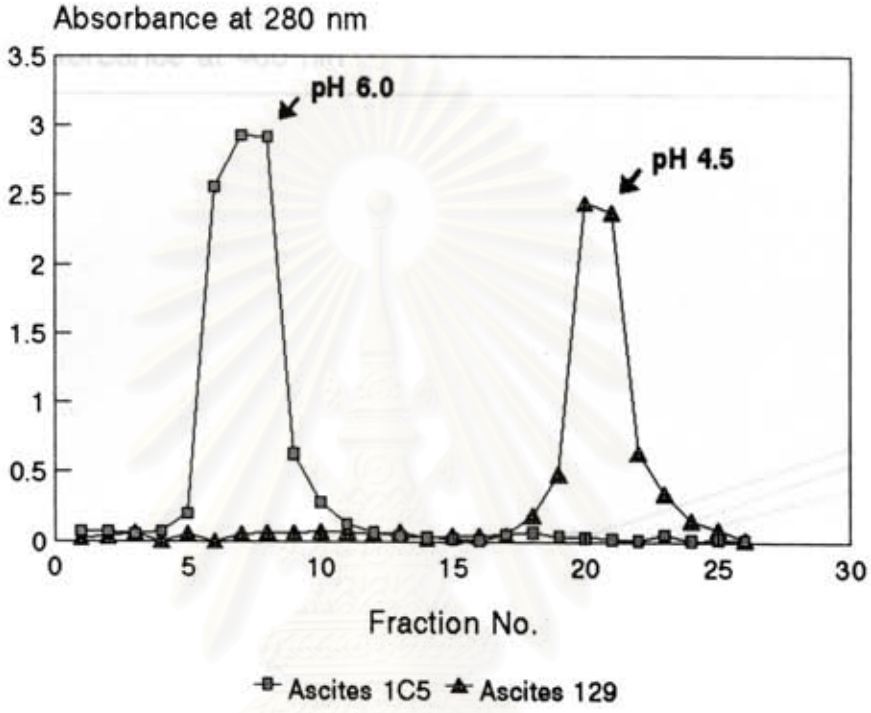

Figure 4 Chromatogram of mouse ascites on protein A-sepharose CL-4B column

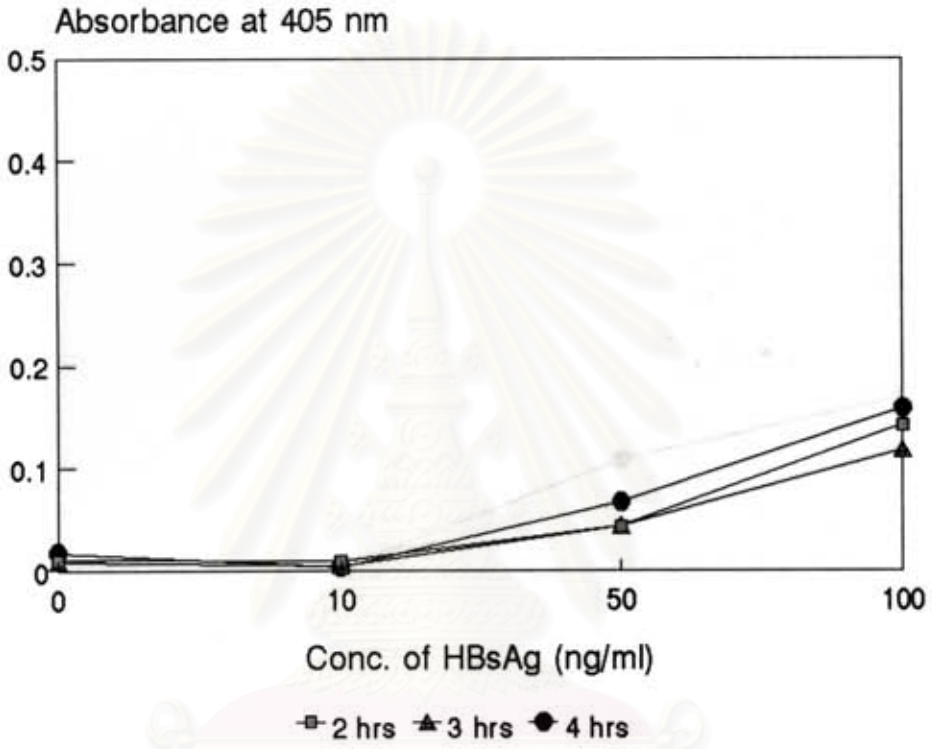

Figure 5 Dose response obtained by ELISA using B-129; label with 10 mg/mL of BNHS to 1 mg/mL of MAb 129

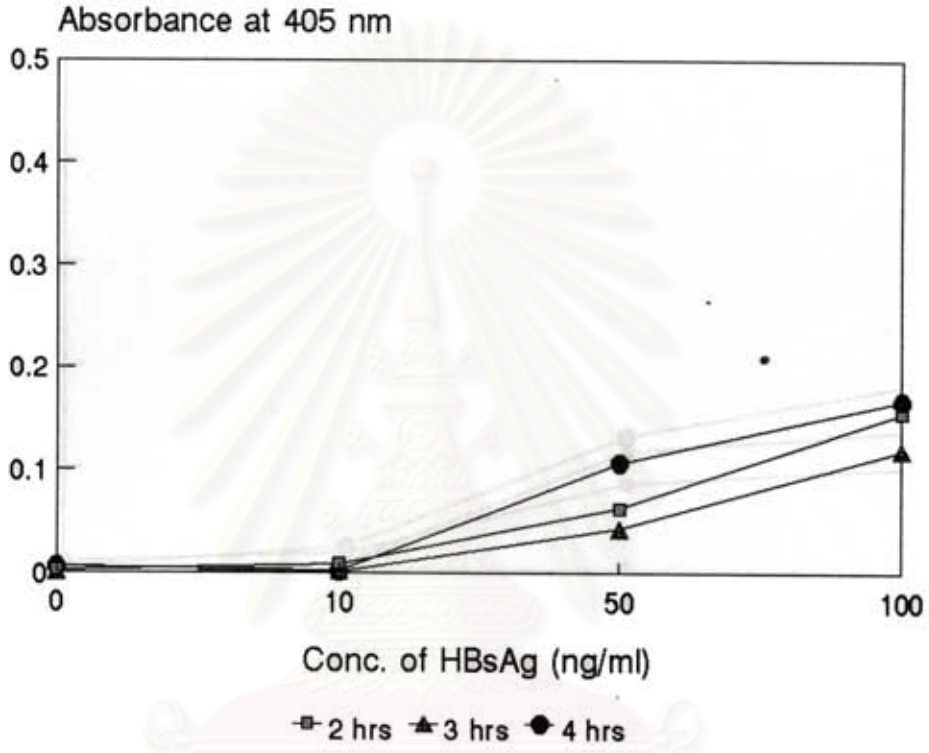

Figure 6 Dose response obtained by ELISA using B-129; label with 20 mg/mL of BNHS to 1 mg/mL of MAb 129

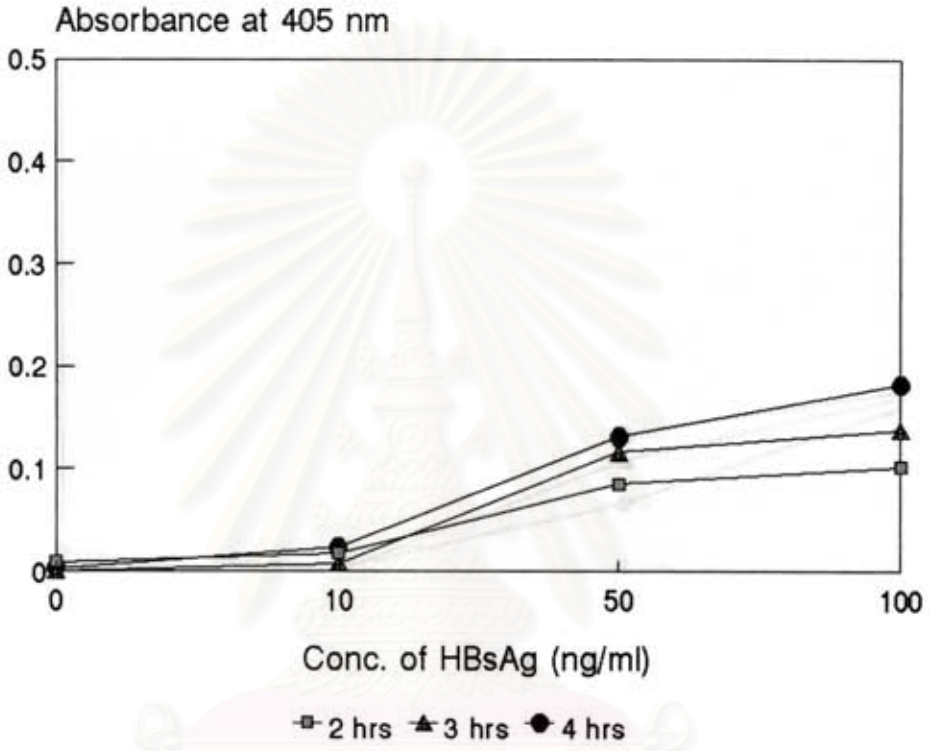

Figure 7 Dose response obtained by ELISA using B-129; label with 40 mg/mL of BNHS to 1 mg/mL of MAb 129

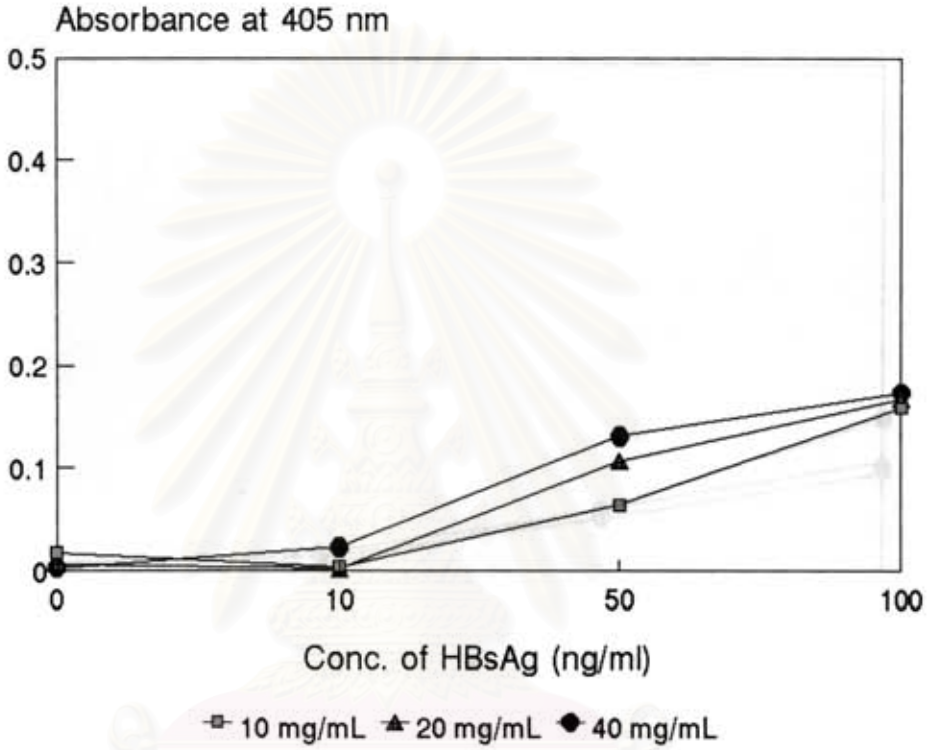

Figure 8 Comparison of B-129 obtained by labeling various concentration of BNHS to 1 mg/mL of MAb 129

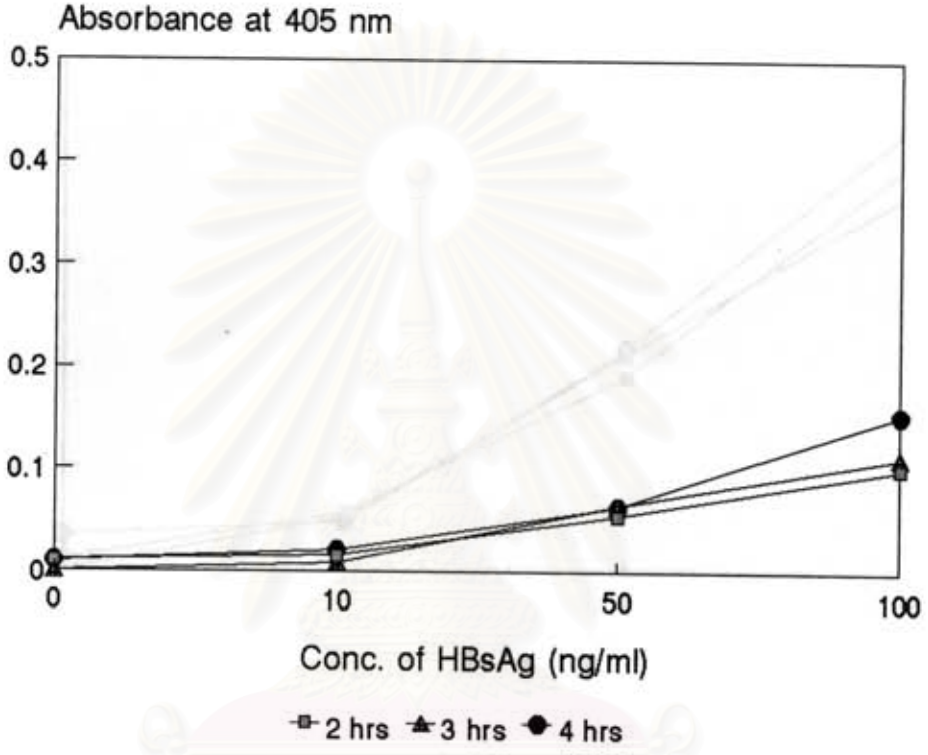

Figure 9 Dose response obtained by ELISA using B-1C5; label with 10 mg/mL of BNHS to 1 mg/mL of MAb 1C5

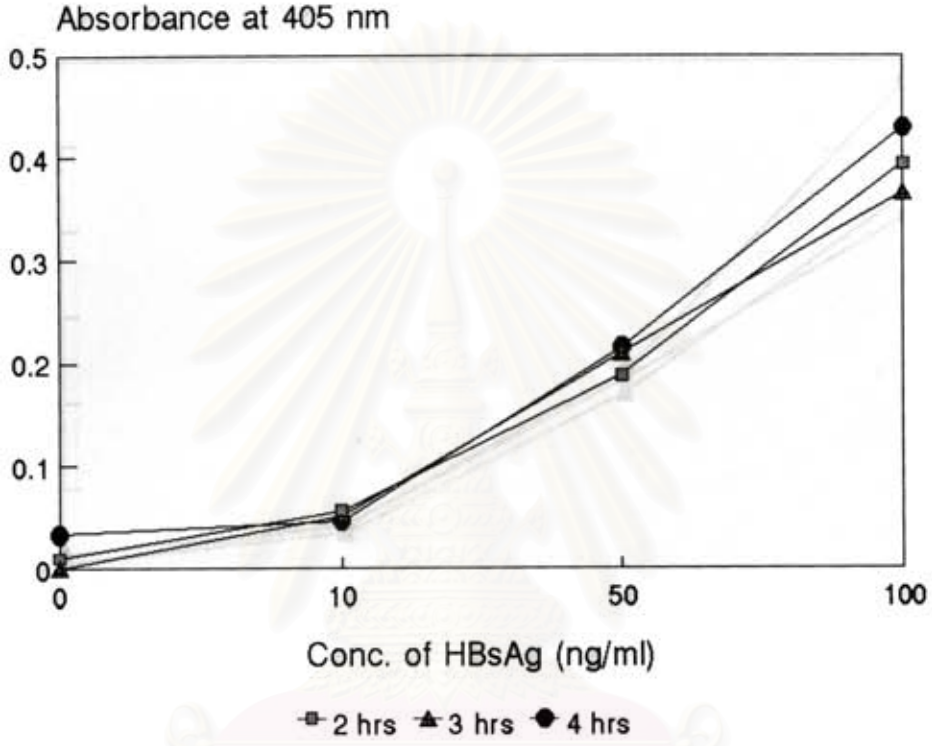

Figure 10 Dose response obtained by ELISA using B-1C5; label with 20 mg/mL of BNHS to 1 mg/mL of MAb 1C5

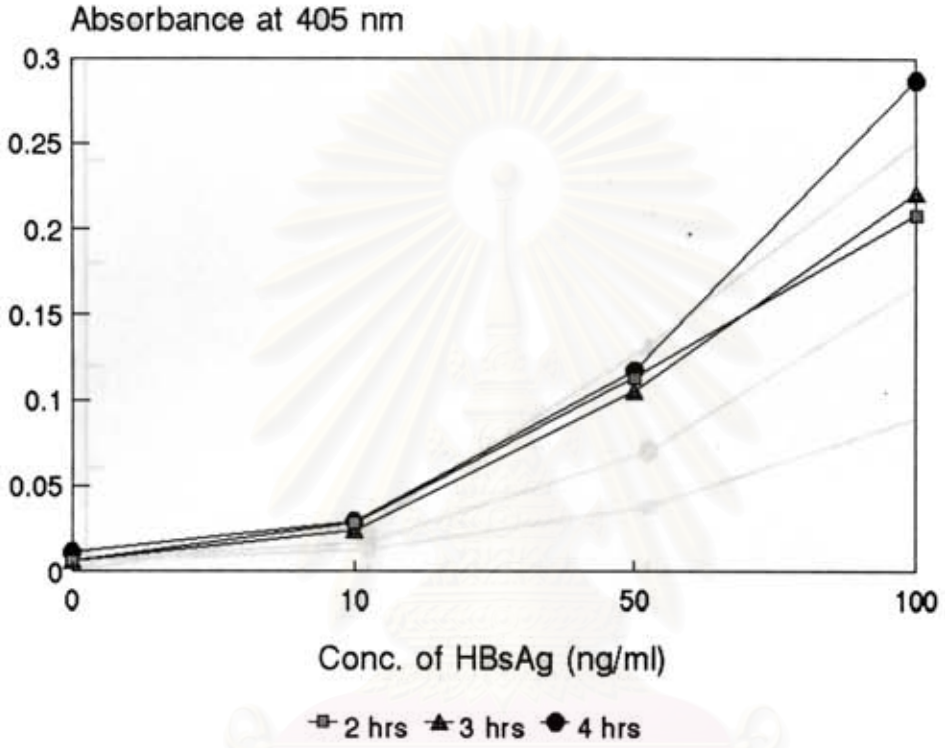

Figure 11 Dose response obtained by ELISA using B-1C5; label with 40 mg/mL of BNHS to 1 mg/mL of MAb 1C5

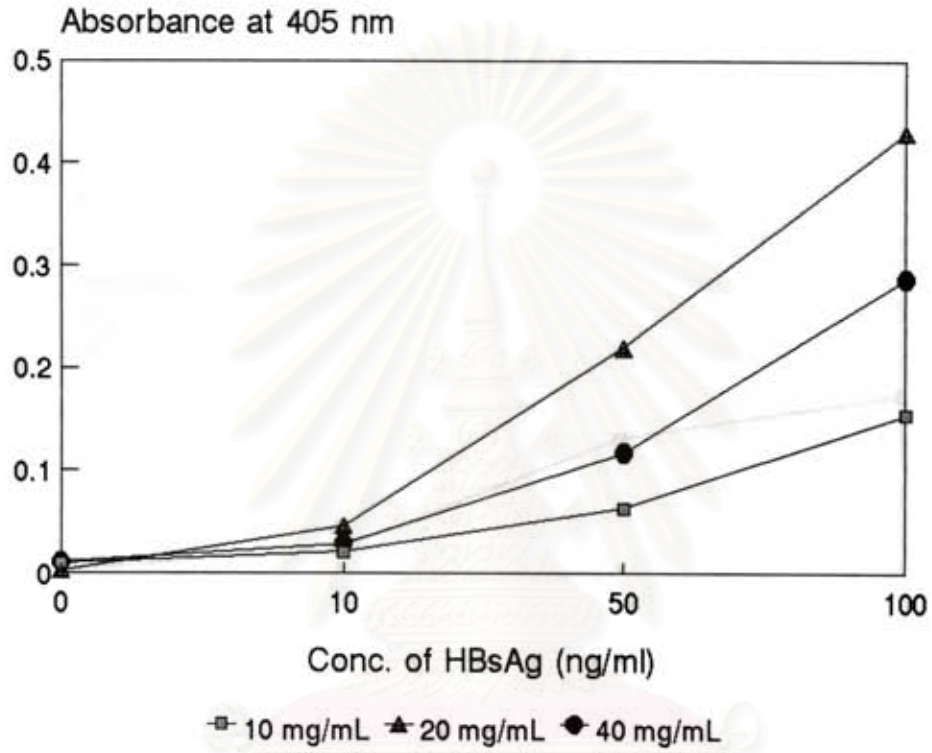

Figure 12 The comparison of B-1C5 obtained by labeling various concentrations of BNHS to 1 mg/mL of MAb 1C5

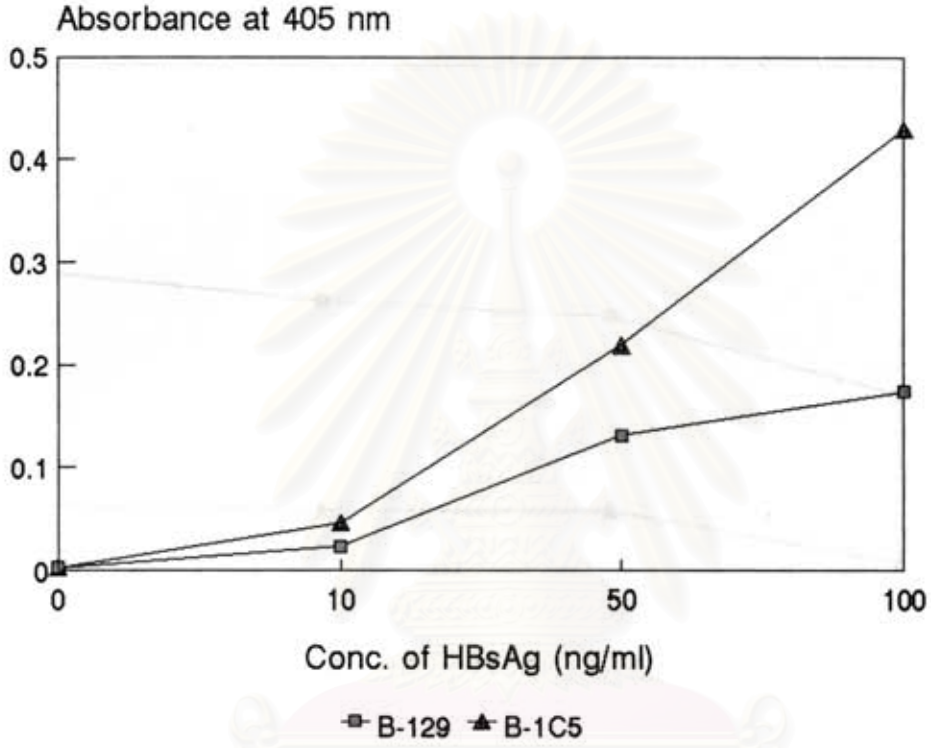

Figure 13 The Comparison of the binding activity to HBsAg between B-129 and B-1C5 by ELISA

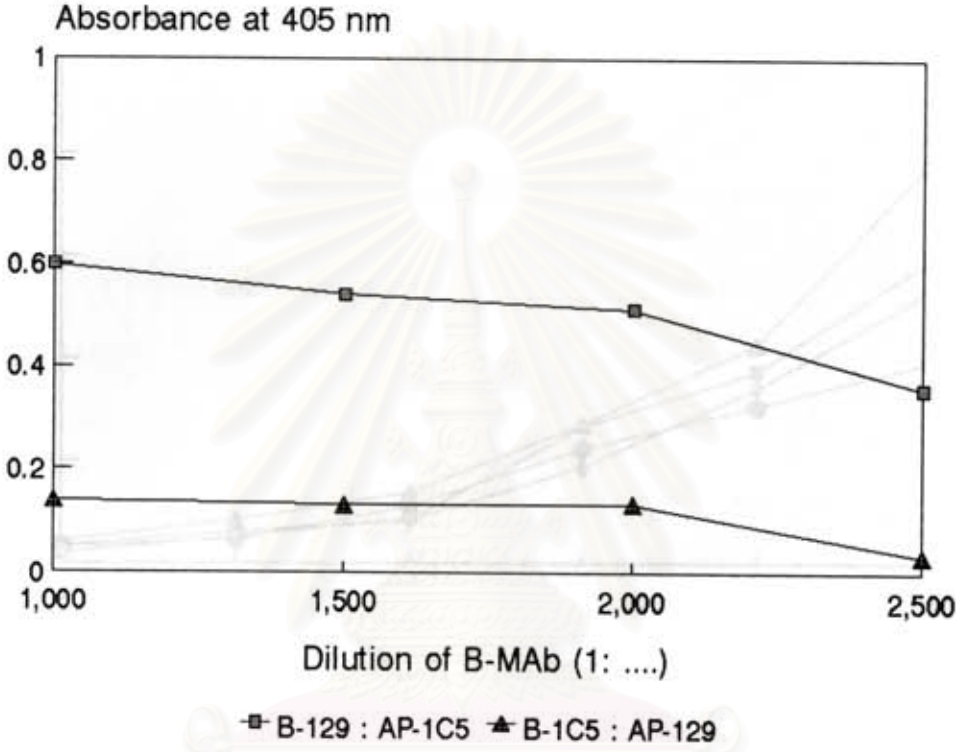

Figure 14 Determination of appropiate pairingr between B-129 : AP-1C5 and B-1C5 : AP-129 for the detection of HBsAg by Streptavidin-Biotin ELISA

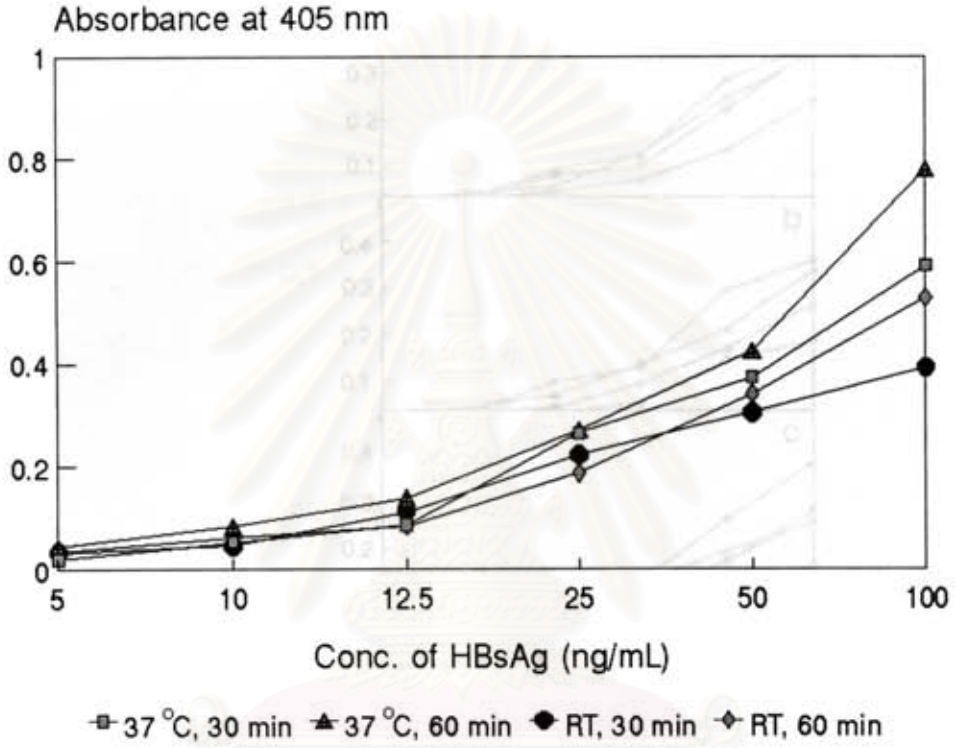

Figure 15 Determination of optimal temperature and reaction time of streptavidin plate and B-129

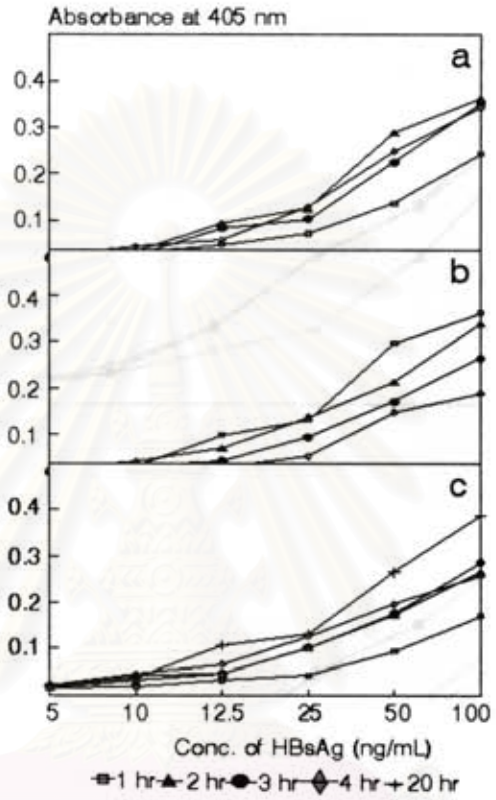

Figure 16 Determination of optimal temperature and reaction time of B-129 and HBsAg
a) 37 °C; b) 45 °C; c) room temperature

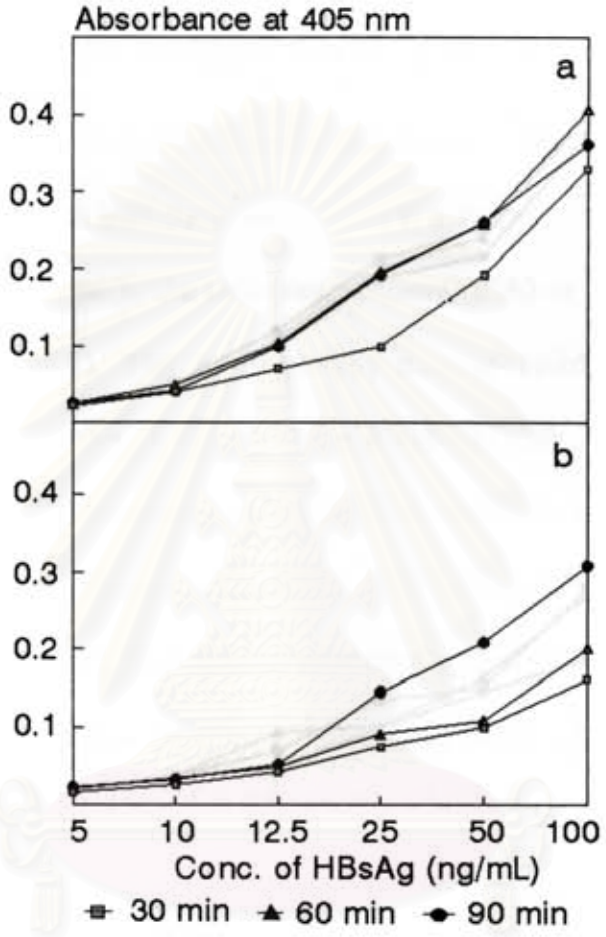

Figure 17 Determination of optimal temperature and reaction time of HBsAg and AP-1C5
a) 37 °C; b) room temperature

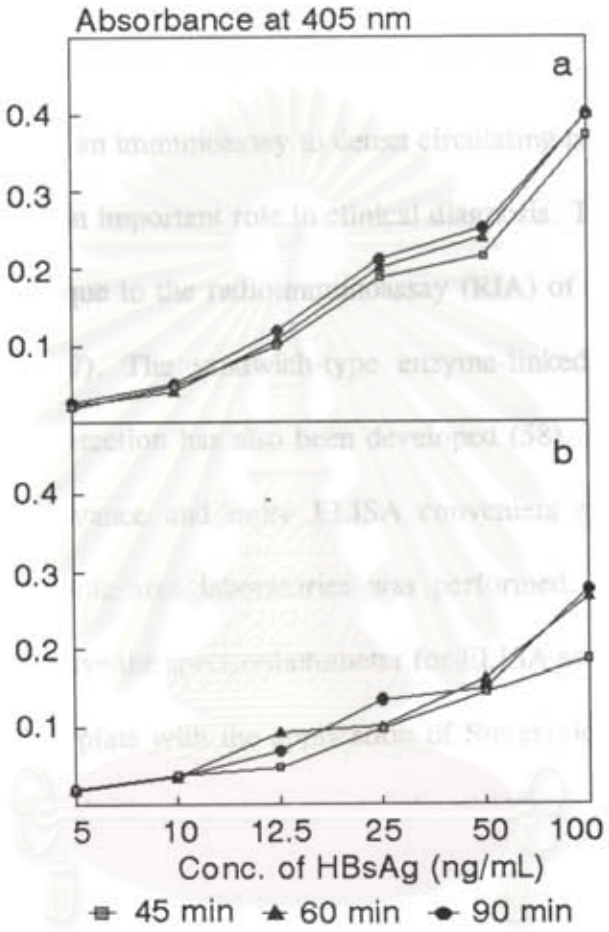

Figure 18 Determination of optimal temperature and reaction time of colour development
a) 37 °C; b) room temperature

CHAPTER V

DISCUSSION

The ability of an immunoassay to detect circulating hepatitis B surface antigen (HBsAg) may play an important role in clinical diagnosis. The application of monoclonal antibody technique to the radioimmunoassay (RIA) of HBsAg has been previously described (56,57). The sandwich-type enzyme-linked immunosorbent assay(ELISA) for HBsAg detection has also been developed (58). In this study, the attempt to establish the advance and more ELISA convenient system to use in Thailand, especially in remote area laboratories was performed. Since almost all laboratories in the country have the spectrophotometer for ELISA as a microtiter plate reader, thus, the microtiter plate with the application of Streptavidin-Biotin ELISA is selected for this study. Preliminary, two monoclonal anti-HBs were prepared from mouse ascites and purified by protein A-sepharose CL-4B column. Protein A from *S. aureus* was combined with the Fc part of γ-globulin (IgG) molecule (59). Monoclonal anti-HBs 129 (MAb 129) and monoclonal anti-HBs 1C5 (MAb 1C5) were purified through protein A-sepharose CL-4B column with different pHs of the elution buffer as shown in Figure 4 and then were taken to label with biotin and enzyme. As presented in Table 6 and Figure 13, it was indicated that MAb 1C5 could be used to label with enzyme alkaline phosphatase or with biotin better than MAb 129. So the determination of suitable labeled monoclonal anti-HBs pair of biotinylated monoclonal anti-HBs and enzyme alkaline phosphatase-monoclonal anti-HBs conjugate for detection of HBsAg was investigated. As illustrated in Figure 14, it was indicated

that the pair of B-129 and AP-1C5 conjugate gave higher dose response than the pair of B-1C5 and AP-129 conjugate. So the pair of B-129 and AP-1C5 conjugate was chosen in this Streptavidin-Biotin ELISA system. For coating the polystyrene microtiter plate with streptavidin, EDAC was used as a coupling reagent. Although the conditions during carbodiimide mediated coupling were not well-characterized (60). A representative assay for detection of HBsAg using various concentration of streptavidin coated onto the microtiter plate was shown in Table 7. The results indicated that the appropriated concentration was 1 μg/mL since the response was steady from 1 μg/mL to 25 μg/mL. In order to gain the best result, many kinds of reagent used in Streptavidin-Biotin ELISA had been optimized, the optimal condition of each reagent was determined by checkerboard titration (61,62). In conclusion, the suitable conditions selected were as the following: the concentration of streptavidin was 1 μg/mL, the dilution of B-129 and dilution of AP-1C5 conjugate were 1:2,000 and 1:250 respectively and the optimal incubation temperature and time for each reagent was shown in Table 9.

Whenever any immunological assay was developed, it must be standardized and the precision of the test can be determined by mean of a coefficient of variation (CV). In our ELISA test, it was found that the CV was ranging from 10.87 % to 18.24 %. The Streptavidin-Biotin ELISA still showed high sensitivity and specificity when compared with the commercial ELISA kit as presented in Table 12. The sensitivity of the assay comparing to the commercial reagent kit was 100% because there was no false negative serum. The specificity was 98.29% because two

sera from one hundred and seventeen were false positive. This false positive might occur in washing process because in the first trial, the distilled water was used to wash out the plate in each step and the false positive detected in the number of serum were twenty. After changing the distilled water to the washing buffer system, i.e. PBS-tween buffer, the false positive serum was reduced to two. Unfortunately, further study can not be done due to these two sera were run out. However the efficiency of the assay was 99.09% when compairing to the commercial ELISA kit.

The detectable concentrations of HBsAg by the Streptavidin-Biotin ELISA, Conventional ELISA in Table 12 were 12 and 25 ng/mL, respectively. This indicated that the streptavidin-biotin complex could increase the sensitivity of the conventional ELISA comparing to the local-made reagent. Although it could not detect 1 ng/mL of HBsAg as same as the commercial kit, but the sensitivity of the assay was better than the conventional ELISA.

The stability of the Streptavidin-Biotin ELISA reagent was tested in various conditions from 1 to 3 months period. Table 13 and 14 indicated that the reagent should not be left at 37°C or room temperature because the reagent was considered invalid. Although in the case of keeping the reagent at room temperature, the assay was still valid for 1 day, the mean of TNR decreased drastically from 42.85 to 13.75. For the 4°C storage, Table 16 represented the valid criteria of the assay of at least 3 months. All reagents were kept in liquid form especially the biotinylated monoclonal anti-HBs and the conjugate.

The Streptavidin-Biotin ELISA for HBsAg has been successfully developed with the cost effective concept by using local resources as much as possible. It is obviously seen that by implementation of this developed kit with the available instrument in rural areas, many essential laboratory diagnosis can be developed effectively. This research is an example model of the local developed ELISA kit which can be applied to detect antigens in both infectious and non infectious such as hormones, carcinoembryonic antigen(CEA), toxin and drugs.

CHAPTER VI

CONCLUSION

The Streptavidin-Biotin ELISA has been established for the detection of Hepatitis B surface antigen by means of the sandwich enzyme-linked immunosorbent assay. This system used two monoclonal anti-HBs, clone 129 and 1C5, which produced from BALB C mice. Monoclonal anti-HBs 129 (MAb 129) and monoclonal anti-HBs 1C5 (MAb 1C5) were mouse subclass IgG2a and IgG1, respectively. MAb 129 was labeled with biotin as a primary antibody binding with hepatitis B surface antigen and this biotinylated MAb 129 was used to bind with streptavidin onto a microtiter plate. MAb 1C5 was conjugated with enzyme alkaline phosphatase that could amplify the signal for the detection of HBsAg.

It was also found that Streptavidin-Biotin ELISA system yielded higher sensitivity than the local-made conventional ELISA but its sensitivity was lower than the commercial ELISA. When comparing the Streptavidin-Biotin ELISA to the commercial ELISA, it was fould that the sensitivity, specificity and efficiency of the assay were 100%, 98.29% and 99.09%, respectively. Thus, the Streptavidin-Biotin ELISA can be utilized for detection of HBsAg and in addition, this ELISA system is convenience to be used in Thailand especially, remote area laboratories due to the fact that almost all laboratories in the country have the spectrophotometer for ELISA as the microtiter plate reader. It can be foreseen that the cost-effective diagnosis system can be established in rural area laboratories without difficulties in the very near future by using this ELISA system.

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Appendix

Reagents and Preparation

1. Reagent for cell culture

1.1 RPMI 1640 medium

RPMI 1640	10.4 g
Sodium bicarbonate	2.02 g
Distilled water	1 L

The medium was sterilized by milipore membrane filtration of pore size 0.45 micron.

1.2 Complete RPMI medium

RPMI 1640	10.4 g
HEPES	1.19 g
$NaHCO_3$	2.016 g
D-glucose	3.6 g
Sodium pyruvate	1.1 g
L-glutamine	0.292 g
Distilled water	1 L

The solution of penicillin G, streptomycin and Kanamycin were added to the final concentrations of 10,000 units, 100 mg and 100 mg per litre of medium, respectively. The medium was sterilized by milipore membrane filtration.

Before using this medium, heat-inactivated fetal bovine serum was added to the final concentration of 10 %.

2. Reagent for purification

2.1 0.1 M Sodium phosphate buffer pH 8

1 M NaH_2PO_4	6.2 mL
1 M Na_2HPO_4	94 mL
Distilled water up to	1 L

2.2 2 M Tris

Tris	24.228 g
Distilled water	100 mL

2.3 0.1 M Citric acid

Citric acid	1.921 g
Distilled water	100 mL

2.4 0.1 M Sodium citrate pH 6

0.1 M Citric acid	100 mL

1M NaOH was added to adjust pH until pH changed to 6.

2.5 0.1 M Sodium citrate pH 4.5

0.1 M citric acid	100 mL

1 M NaOH was added to adjust pH untill pH changed to 4.5

2.6 0.1 M Sodium citrate pH 3

0.1 M Citric acid	100 mL

1 M NaOH was added to adjust pH untill pH changed to 3

2.7 Phosphate buffer saline (PBS)

NaCl	8.0	g
KCl	0.2	g
KH_2PO_4	0.2	g
Na_2HPO_4	1.15	g
Distilled water	1	L

3. Reagent for conjugation of enzyme alkaline phosphatase to monoclonal anti-HBs

3.1 0.05 M Sodium phosphate buffer pH 7.2

$Na_2 HPO_4.12 H_2O$	1.289	g
$NaH_2PO.2H_2O$	0.218	g
NaCl	0.877	g
Distilled water	100	mL

3.2 25 % glutaraldehyde

3.3 0.05 M Tris-HCl buffer pH 8 containing 0.001 M, $MgCl_2$, 0.04 % Sodium azide , 5 % BSA

Tris	0.6	g
Distilled water	80	mL

Adjust pH to 8 with concentrated HCl and added distilled water up to 100 mL. Then, sodium azide, BSA and 1 M $MgCl_2$ were added 0.004 g , 5 g and 0.1 mL , respectively.

4. Reagent for labeling biotin to monoclonal anti- HBs

4.1 0.1 M Sodium bicarbonate pH 8.3

$NaHCO_3$	8.4	g
Distilled water	1	L

4.2 PBS

similar to 2.7

5. Reagent for coupling streptavidin to polystyrene microtiter plate

5.1 3 mM Phosphate buffer pH 6.8

1 M Na_2HPO_4	1.46	mL
1 M NaH_2PO_4	1.54	mL
Distilled water	1	L

5.2 0.01 M Phosphate buffer saline pH 7.2

1 M Na_2HPO_4	7	mL
1 M NaH_2PO_4	3	mL
NaCl	8.75	mg
Distilled water	1	L

5.3 High salt buffer

1 M Na_2HPO_4	7	mL
1 M NaH_2PO_4	3	mL
NaCl	81.8	g
Distilled water	1	L

5.4 PBG (0.15 M Phosphate buffer pH 7.4, 0.001 M EDTA, 0.1 % gelatin)

1 M Na_2HPO_4	120	mL
1 M NaH_2PO_4	30	mL
Distilled water	1	L
EDTA	0.372	g
gelatin	1	g

5.5 0.2 M EDAC

EDAC	38	mg
3 mM Phosphate buffer pH 6.8	1	mL

6. Reagent for ELISA system

6.1 Diluent I

(0.04 M Phosphate buffer pH 7 containing 1 % BSA and 0.01% sodium azide)

1 M NaH_2PO_4	16	mL
1 M Na_2HPO_4	24	mL
BSA	1	g
Distilled water	100	mL

6.2 Diluent II

(0.01 M Tris-HCl buffer pH 8 containing 0.001 M $MgCl_2$,0.02 % sodium azide, 1 % BSA)

Trisma base	12.1	mL
Distilled water	800	mL

adjusted pH up to 8 with conc. HCl and added distilled water up to 1 L

1 M $MgCl_2$	1	mL
Sodium azide	0.02	g
BSA	10	g

6.3 PBS-Tween

PBS	1	L
tween 20	5	mL

6.4 Substrate buffer

1 M Diethanolamine buffer pH 9.8 containing 50 mM $MgCl_2$

Diethanolamine	96.46	mL
1 M $MgCl_2$	25	mL
Distilled water	1	mL

6.5 15 mM p-nitrophenyl phosphate

p-nitrophenyl phosphate	40	mg
Substrate buffer	10	mL

Vitae

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