Studies on the Development of Enzyme Linked Immuno-sorbent Assay(ELISA) for Hepatitis B Surface Antigen(HBsAg) by Monoclonal Antibodies of Different Affinity Constants

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Abstract ☐ Mouse monoclonal antibodies to Hepatitis B surface antigen(HBsAg) were prepared and their functional capabilities tested by the method of solid phase enzyme linked immuno sorbent assay(ELISA). HBsAg binding studies indicated that one monoclonal antibody 6E-1-1 bound more HBsAg at a faster rate than the other monoclonal antibodies. Also, for the binding inhibition studies with the selected monoclonal antibody 6E-1-1, one monoclonal antibody 8D-3-6 didn't exhibit binding inhibition for HBsAg. Then, a simultaneous ELISA method was developed for the immunodiagnosis of HBsAg. Different combinations of two monoclonal antibodies as solid phase and horseradish peroxidase(HRPO) labeled phase were studied. The combination of monoclonal antibody of higher affinity constant(6E-1-1) immobilized in a solid phase and monoclonal antibody of lower aff'nity constant(8D-3-6) as a HRPO labeled phase was more sensitive when two monoclonal antibodies of different affinity constants for HBsAg were prepared.

Keywords ☐ Sensitivity, enzyme linked immuno sorbent assay(ELISA), hepatitis B surface antigen(HBsAg), monoclonal antibody, affinity constant.

Acute hepatitis B causes significant mortality and morbidity, and chronic infection with the virus is associated with hepatocellular carcinoma, chronic active hepatitis and cirrhosis. The specific viral marker for acute and chronic hepatitis B infection is the presence of hepatitis B surface antigen(HBsAg) in the blood[1]. This high molecular weight complex viral protein is a surface component of the intact 42 nm hapatitis virus, but it is also present on a circulating 22 nm particle[2]. HBsAg is distinct from other hepatitis B-associated antigens such as hepatitis B core antigen and hepatitis B e antigen[3,4]. Thus, we attempted this study to detect the HBsAg as a first marker of Hepatitis B virus.

In the present investigation, we have immunized BALB/c mice with a highly purified preparation of HBsAg. Subsequently, splenocytes from these mice were fused with the SP2/0 myeloma cell line. Somatic cell hybrids(hybridomas) were produced, cloned, and have been maintained as continuously proliferating, stable cell lines in tissue cultur Clones of these hybrids were found to produc antibodies with exceptional binding avidity to th antigenic determinants unique to HBsAg.

Affinity constant of the monoclonal anti-HE was obtained by enzyme linked immuno sorbe assay(ELISA) method. Bertrand *et al.*[5] hav recently adapted the ELISA technique to determi the affinity constants of monoclonal antibodie The experimental values of affinity constant fou by them for two monoclonal antibodies are show to be very close to those obtained by convention radioimmunoassay(RIA). Thus we have recent applied this ELISA method to the determination affinity constant of monoclonal Anti-HBs.

Also, we have explored the use of monoclon antibodies directed towards epitopes on HBsAg the immunodiagnosis of hepatitis B. A simultaneo ELISA using monoclonal anti-HBs was construc ed and the correlations of the sensitivity of ass systems were compared on the basis of affini constant of monoclonal Anti-HBs used.

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EXPERIMENTAL METHODS

Materials

Human plasma determined by reverse passive hemagglutination assay(RPHA) to contain high titers of HBsAg and normal human plasma were obtained from Red Cross Blood Bank, Seoul. Protein A immobilized Sepharose CL-4B was purchased from Pharmacia Fine Chemicals Co. EIA TEST for HBsAg and anti-HBs were obtained from Dong-A phamaceutical Co., Ltd. One fourth inch polystyrene beads were purchased from Clifton Plastics Inc., Baltimore, PA. Microtiter plate were purchased from Nunc, Denmark. The remainder of the reagents were obtained from various, local sources and were of the highest grade available.

Purification of hepatitis B surface antigen(HBsAg)

HBsAg was purified by density gradient ultracentrifugation [6,7] from pooled human plasma determined by RPHA to contain high titers of HBsAg. Gradient fractions with the highest HBsAg activity were pooled and dialyzed against 0.01 M phosphate buffered saline(PBS, pH 7.2). The protein content was determined by the method of Lowry *et al.*[8]

Establishment of somatic cell hybrids producing monoclonal antibodies to HBsAg

The preparation of immunizing antigen, immunization schedule, cell fusion techniques and culture of somatic cell hybrids have been described at Chang's paper[9]. Hybridomas were cloned at limiting dilutions. Six cell lines(4D-1-1, 6E-1-1, 7C-6-4, 7F-2-1, 8C-4-1, 8D-3-6) to the common "a" epitopes on HBsAg were selected for intraperitoneal injection(2×10^7 cells) into pristane-primed BALB/c mice. Subsequently, the ascitic fluids were collected.

Purification of monoclonal antibodies from ascitic fluids

First, 6 ml of ascitic fluid was precipitated by two sequential 50% ammonium sulfate solutions. 80 mg of the pooled fraction was dialyzed against 0.01 M PBS(pH 7.2) followed by affinity chromatography on Protein A immobilized Sepharose CL-4B[10]. Pooled fraction was loaded to column(1.5×5.0cm) and was washed with 0.01 M PBS(pH 7.2). Finally, the column was eluted with 0.1 M glycine-HCl buffer(pH 2.8) at a flow rate of 10 ml/hr. The aliquots were collected with a fraction collector(Hellirack, LKB), and effluent absorbance was recorded by continuous flow through an UV absorption monitor(Uvicord II, LKB) at 280 nm. Purity of purified monoclonal antibodies used in these determinations were estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE). The protein content was determined by the method of Lowry *et al.* The anti-HBs activity was assayed with commercial EIA TEST.

Analysis of anti-HBs activity

Two separate assays were used for measurement of anti-HBs activities of the purified monoclonal antibodies. One was HBsAg binding studies, and the other anti-HBs binding studies. For the HBsAg binding studies, monoclonal anti-HBs(10 μg/ml of 0.1 M sodium carbonate buffer, pH 9.5) were coated on polystyrene beads. The beads were washed three times with distilled water. Subsequently, the beads were incubated with 200 μl of HRPO labeled HBsAg[11] at 40℃ for various times and washed three times with distilled water. Finally, 300 μl of 14.5 mM o-phenylenediamine(OPD) in 0.1 M sodium citrate buffer(pH 5.3) containing 5 mM H_2O_2 as a substrate was added and incubated at room temperature for 30 min. Reaction was stopped by 1 ml of 1 N sulfuric acid and anti-HBs activity was detected by measuring the optical density at the wavelength of 492 nm[12].

For the anti-HBs binding studies monoclonal antibodies were labeled with HRPO[13]. 200 μl HRPO labeled monoclonal antibodies were incubated with commerical HBsAg coated polystyrene beads(EIA TEST for anti-HBs) at 40℃ for various times and washed three times with distilled water. Finally, 300 μl of 14.5 mM OPD in 0.1 M sodium citrate buffer(pH 5.3) containing 5 mM H_2O_2 as a substrate was added and incubated at room temperature for 30 min. Reaction was stopped by adding 1 ml of 1 N sulfuric acid and anti-HBs activity was detected by measuring the optical density at the wavelength of 492 nm

Affinity constants of the monoclonal antibodies were obtained by ELISA method[5].

Epitope identification

Two inhibition assays of antigenic determinant specificities were carried out as described below. Pooled human plasma determined by RPHA to contain high titers of HBsAg was diluted with recalcified normal human serum to give a value of 1.0 at optical density at 492 nm in the ELISA system for HBsAg. The first assay involved 6E-1-1 monoclonal antibody as a HRPO labeled phase. 500 μl of diluted HBsAg positive plasma was incubated in solution with the 500 μl of monoclonal antibody(10 μg/ml of 0.01 M PBS, pH 7.2) for 20 hr at room temperature. Then, 200 μl of the reaction mixture was reacted with commercial

Table I. Purification of 6E-1-1 monoclonal antibody.

	Volume (ml)	Protein concentration (mg/ml)	Anti-HBs activity (EIA titer)	Specific anti-HBs activity (EIA titer/mg·protein)
Ascitic fluid	6	54.00	1260000	23300
$(NH_4)_2SO_4$ fractionation	2	90.00	3500000	38900
Affinity chromatography on Protein-A	15	2.57	350000	136200

solid phase(guinea pig anti-HBs coated polystyrene beads, EIA TEST) at 40℃ for 2 hr followed by washing three times with distilled water. Subsequently, 200 ml of HRPO labeled 6E-1-1 monoclonal antibody was incubated with the bound HBsAg at 40℃ for 1 hr followed by washing three times with distilled water. Finally, 300 ml of 14.5 mM OPD in 0.1 M sodium citrate buffer(pH 5.3) containing 5 mM H_2O_2 as a substrate was added and incubated at room temperature for 30 min. Reaction was stopped by adding 1 ml of 1 N sulfuric acid and bound HBsAg was detected by measuring the optical density at the wavelength of 492 nm. For the second inhibition assay we used 6E-1-1 monoclonal antibody as a solid phase(6E-1-1 monoclonal antibody coated polystyrene beads). 500 μl HBsAg positive serum diluted with recalcified normal human serum was incubated in solution with 500 μl of monoclonal antibody(100 μg/ml of 0.01 M PBS, pH 7.2) at room temperature for 20 hr.

Then 200 μl of the reaction mixture was reacted with 6E-1-1 monoclonal antibody coated polystyrene beads at 40℃ for 2 hr followed by extensive washing with distilled water. Subsequently, 200 μl of commercial HRPO labeled phase(HRPO labeled goat anti-HBs, EIA TEST) was incubated with the bound HBsAg at 40℃ for 1 hr followed by washing three times with distilled water. Finally, 300 μl of 14.5 mM OPD in 0.1 M sodium citrate buffer(pH 5.3) containing 5 mM H_2O_2 as a substrate was added and incubated at room temperature for 30 min. Reaction was stopped by adding 1 ml of 1 N sulfuric acid and bound HBsAg was detected by measuring the optical density at the wavelength of 492 nm.

Construction of a simultaneous ELISA for HBsAg

HBsAg sample used as pooled fraction from several units of human plasma determined by RPHA to contain HBsAg was two fold diluted with recalcified normal human serum. 50 μl of HBsAg blood sample and HRPO labeled monoclonal antibody were incubated at 40℃ for 1.5 hr in the monoclonal antibody (10 μg/ml of 0.1 M sodium carbonate buffer, pH 9.5) coated microtiter plate. After washing three times with distilled water, 100 μl of 14.5 mM OPD in 0.1 M sodium citrate buffer(pH 5.3) containing 5 mM H_2O_2 as a substrate was added and incubated for 30 min at room temperature. Reaction was stopped by adding 100 μl of 1 N sulfuric acid and HBsAg content was determined by measuring the optical density at the wavelength of 492 nm.

In the present investigation, we took different combinations of two monoclonal antibodies as solic phase and HRPO labeled phase, in order to deter mine which combination had the higher sensitivit; and the lower background.

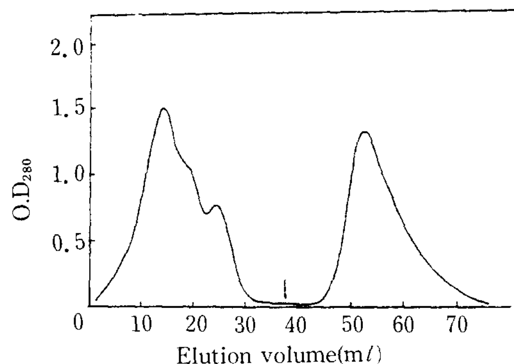


Fig.1. Elution patterns of 6E-1-1 monoclon: antibody.

6E-1-1 monoclonal antibody was chromat graphed on a 1.5×5.0cm column of Prote A-Sepharose CL-4B. Flow rate, 10 ml/l Washing buffer, 0.01 M PBS(pH 7.2 Eluent, 0.1 M Glycine-HCl(pH 2.8). T arrow indicates the initiation of elution.

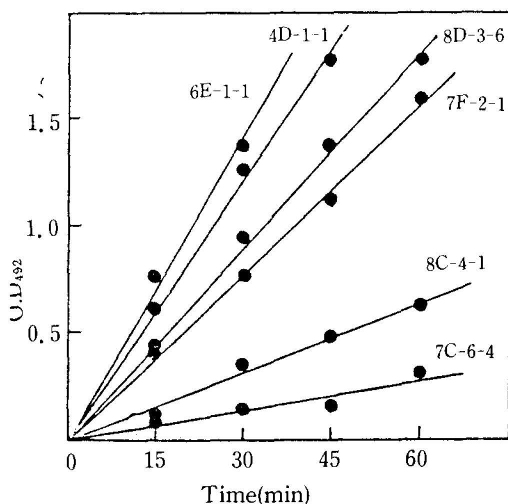
RESULTS

Purification of mouse monoclonal antibodies

Fourty nine mg of the monoclonal antibody was)btained by the purification procedure from 6 ml)f ascitic fluid of 6E-1-1 monoclonal ıntibody(Table I). When the monoclonal antibody)reparation purified by the affinity chromatogra-)hy on Protein A immobilized Sepharose CL-4 3(Fig.1) was analyzed on SDS polyacrylamide ;el electrophoresis, only two bands containing light :hain and heavy chain of the γ-immunoglobulin vere detected. The results obtained by SDS-PAGE lemonstrated that 6E-1-1 monoclonal antibody ıas been effectively purified.

HBsAg binding studies and anti-HBs binding studies

The capability of monoclonal antibodies to bind HBsAg was investigated by coupling to a solid)hase support. Fig.2 illustrates that 6E-1-1 nonoclonal antibody bound the most HRPO labeled HBsAg and at the highest rate compared to the)ther monoclonal antibodies tested. Equal concen- ration of monoclonal antibodies(10 μg/ml) was :oated on the polystyrene beads. Therefore, the lifferences in HBsAg binding are a result of the lifferent affinities of the monoclonal antibodies ather than of the concentration effects alone. Also, t was found that 6E-1-1 monoclonal antibody till demonstrated the greatest binding capacity for HBsAg when the reverse binding experiments were)erformed by incubating HRPO labeled mono-



ig.2. Binding of HRPO labeled HBsAg to monoclonal antibodies on the solid phase support.

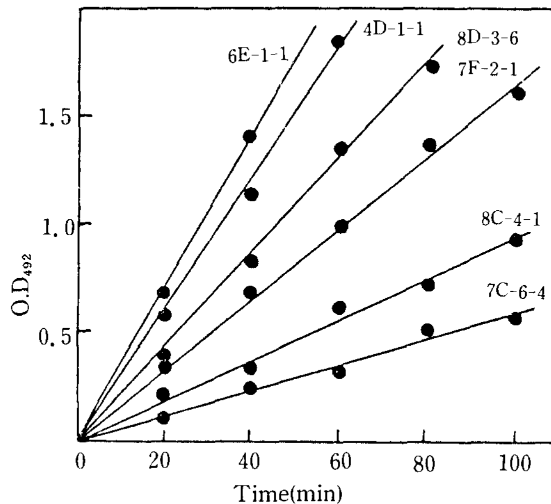


Fig.3. Binding of HRPO labeled monoclonal antibodies to HBsAg on the solid phase support.

clonal antibody with HBsAg coupled to polystyrene bead(Fig.3).

Epitope analysis

Through the binding studies of both HBsAg and anti-HBs, it was confirmed that 6E-1-1 monoclonal antibody demonstrated the greatest binding capacity for HBsAg. Thus, the binding inhibition for HBsAg between 6E-1-1 monoclonal antibody and the other 5 monoclonal antibodies to "a" specific antigenic determinants was investigated. Table II illustrates that only 8D-3-6 monoclonal antibody didn't exhibit binding inhibition with 6E-1-1 monoclonal antibody. Subsequently, it was showed that 4D-1-1, 7F-2-1, 8C-4-1 and 7C-6-4 monoclonal antibodies were sterically adjacent to the 6E-1-1 monoclonal antibody determinant.

Affinity constant

6E-1-1 monoclonal antibody and 8D-3-6 monoclonal antibody by the epitope analysis were selected. A solid phase ELISA system was used to determine the affinity constants of the monoclonal antibodies by Bertrand *et al.*[5] Table III shows the affinity constants of the 6E-1-1 and 8D-3-6 monoclonal antibody.

Construction of a simultaneous ELISA for HBsAg

In a given assay system, such as an ELISA, using one monoclonal antibody on the solid phase and another monoclonal antibody as HRPO labeled phase, different results may be obtained, according to the monoclonal antibody combination used. Thus the optimal combination should be empirically

Table II. Competitive inhibition of 5 mouse monoclonal antibodies with 6E-1-1 monoclonal antibody.

solid phase / HRPO labeled phase / monoclonal antibodies	OD_{492}	
	6E-1-1 monoclonal antibody / EIA TEST (goat anti-HBs)	EIA TEST (guinea pig anti-HBs) / 6E-1-1 monoclonal antibody
Negative control (Normal Human Serum)	0.035	0.021
Positive	1.035	1.265
4D-1-1	0.082	0.048
8D-3-6	0.995	1.171
7F-2-1	0.036	0.029
8C-4-1	0.054	0.026
7C-6-4	0.043	0.032

Table III. Affinity constants of the mouse monoclonal antibodies.

Monoclonal antibodies	Affinity constant
6E-1-1	$4.8\times10^{9}M^{-1}$
8D-3-6	$3.3\times10^{8}M^{-1}$

found among the available monoclonal antibodies.

So, we took different combinations of 2 monoclonal antibodies as solid phase and HRPO labeled phase, in order to determine which combination gave more sensitive results and lower background. Monoclonal antibody combinations were compared on the basis of affinity constants of monoclonal antibodies used. Correlations of the sensitivity of monoclonal antibody combinations were described below(Table IV). In conclusion, the combination of monoclonal antibody of higher affinity constant immobilized in a solid phase and monoclonal antibody of lower affinity constant as a HRPO labeled phase was more sensitive than reverse combination.

DISCUSSION

The considerable interest generated recently in the application of monoclonal antibody technology to immunodiagnostics has raised much concern over the precise specificity of a monoclonal antibody. Several authors have reported the successful production of anti-HBs monoclonal antibodies[14-16] and several firms are actually proposing in their catalogue such the antibodies.

In this studies, six cloned cell lines have been established which are stable and retain their capability to produce specific antibodies to HBsAg. Monoclonal antibodies derived from the six cell lines contained antibody that bound to "a" determinants of HBsAg. The binding avidities of the monoclonal antibodies, especially the 6E-1-1 antibody were particulary striking.

In an attempt to develop enzymeimmunoassay using monoclonal antibody, it is important to establish that the antibodies can be linked to a solid phase support and maintained their HBsAg binding characteristics. Fig.2 indicates that 6E-1-1 monoc

Table IV. Sensitivity obtained by different combinations of mouse monoclonal antibodies in simultaneous ELISA for HBsAg.

Solid phase	HRPO labeled phase	P/N ratio: Dilution of HBsAg positive sera pooled 1/2^{7}	1/2^{8}	1/2^{9}	1/2^{10}	1/2^{11}	1/2^{12}	Mean of Negative serum ($O.D_{492}$)
6E-1-1	8D-3-6	>57.1	>57.1	51.4	28.1	12.1	4.8	0.035
8D-3-6	6E-1-1	>41.7	21.5	10.5	4.4	2.0	1.1	0.048

clonal antibody was superior to the other monoclonal antibodies. When the monoclonal antibodies were labeled with HRPO and the binding to HBsAg on a solid phase support was assayed, 6E-1-1 monoclonal antibody performed better than the other antibodies at all time points(Fig.3).

Subsequently, we confirmed that 6E-1-1 monoclonal antibody demonstrated the greatest binding capacity for HBsAg. Thus we investigated the binding inhibition for HBsAg between 6E-1-1 monoclonal antibody and the other 5 monoclonal antibodies. Table II illustrates that 8D-3-6 monoclonal antibody only didn't exhibit binding inhibition with 6E-1-1 monoclonal antibody.

Affinity constants of the selected 6E-1-1 and 8 D-3-6 monoclonal antibodies were determined by using ELISA method[5]. Bertrand *et al.* have recently adapted the ELISA technique to determine the affinity constants of monoclonal antibodies. The experimental values of affinity constants found by them for 2 monoclonal antibodies are shown to be very close to those obtained by conventional radioimmunoassay(RIA). Thus we have applied this ELISA method to the determination of affinity constants of selected monoclonal antibodies.

We tried different combinations of 2 monoclonal antibodies as solid phase and as HRPO labeled phase, in order to determine what combination gave the more sensitive results and lower background. There are, as expected, correlations between avidity for HBsAg and affinity constant in ELISA allowing to predict the efficiency of a given monoclonal antibody as reagent. Initially, we used the 6E-1-1 monoclonal antibody immobilized in a solid phase and 8D-3-6 monoclonal antibody as a HRPO labeled phase. After, we used the 8D-3-6 monoclonal antibody immobilized in a solid phase, 6E-1-1 monoclonal antibody as a HRPO labeled phase. More sensitive assay design for measurement of HBsAg was the use of 6E-1-1 monoclonal antibody immobilized in a solid phase and 8D-3-6monoclonal antibody as a HRPO labeled phase(Table IV). In conclusion, these experiments suggest that the combination of monoclonal antibody of higher affinity constant immobilized in a solid phase and monoclonal antibody of lower affinity constant as a HRPO labeled phase was more sensitive when two monoclonal antibodies different in affinity constant for HBsAg were prepared. Though it would appear that one can take advantage of the special characteristics(affinity constant) of these monoclonal antibodies in the construction of a highly sensitive simultaneous enzymeimmunoassay for HBsAg, the efficacy of these monoclonal antibodies assay reported here must now be demonstrated by more extensive trials with more monoclonal antibodies for HBsAg.

LITERATURE CITED

1. Levene, C. and Blumberg, B.S.: Additional specificities of Australia antigen and the possible identification of hepatitis carriers. *Nature* **221**, 195(1969).
2. David, A.S. and Harvey, M.L.: The molecular biology of hepatitis B virus. *Ann. Rev. Med.* **35**, 219(1984).
3. Ling, C.M., Mushahwar, I.K., Overby, L.R., Berquist, K.R. and Maynard, J.E.: Hepatitis B e-antigen and its correlation with other serological markers in chimpanzees. *Infection and Immunity* **24**, 352(1979).
4. Mushahwar, I.K., Dienstag, J.L., Polesky, H. F., Mcgrath, L.C., Decker, R.H. and Overby, L.R.: Interpretation of various serological profiles of hepatitis B virus infection *A.J.C.P.* **76**, 773(1981).
5. Bertrand, F., Alain, F.C., Lisa, D.O. and Michel, E.C.: Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. *J. Immunol. Methods* **77**, 305(1985).
6. Stannard, L.M., Moodie, J., keen, G.A. and Kipps, A.: Electron microscopic study of the distribution of the Australia antigen in individual sera of 50 serologically positive blood donors and two patients with serum hepatitis. *J. Clin . Pathol.* **26**, 209(1973).
7. Gerin, J.L., Ford, E.C. and Puricell, R.H.: Biochemical characterization of Australia antigen. *Am. J. Pathol.* **81**, 651(1974).
8. Lowry, O.H., Resebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265(1951).
9. Chang, J.A.: Studies on the development of hepatitis B surface antigen detection system by monoclonal antibodies. Thesis for the Degree of M.S., Graduate School, Seoul National University (1987).
10. Affinity Chromatography mannual, Pharmacia Fine Chemicals, 48.
11. Guesdon, J.L., Ternynck, T. and Avrameas, S.: The use of avidin-biotin interaction in immunoenzymatic techniques. *J. Histochem. Cytochem* **27**, 1131(1979).
12. Gallati, V.H. und Brodbeck, H.: Peroxidase aus Meerrettich. *J. Clin. Chem. Biochem.* **20**, 221(1982).
13. Nakane, P.K. and Kawaoi, A.: Peroxidase

-labeled antibody. *J. Histochem. Cytochem.* **22**, 1081(1974).

14. Wands, J.R. and Zurawski, V.R.: High affinity monoclonal antibodies to hepatitis B surface antigen(HBsAg) produced by somatic cell hybrids. *Gastroenterology* **80**, 225(1981).
15. Soulier, J.P., Lee, C.H., Drouet, J., Muller, A. and Cana vaggio, M.: Monoclonal antibodies against HBs antigen. *Biotest Bulletin* **4**, 353(1983).
16. David, G.S., Present, W., Martinis, J., Wang, R., Bartholomew, R., Desmond, W. and Sevier, E.D.: Monoclonal antibodies in the detection of hapatitis infection. *Med. Lab. Sci.* **38**, 341(1981).