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Immunochemical Detection of PreS2 Epitope of Hepatitis B Surface Antigen

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Abstract.- This study was undertaken to detect immunochemically the PreS2 epitope of HBsAg of HBV present in serum samples. Polyclonal antibodies against synthetic peptide of PreS2 epitope of HbsAg were raised in rabbit, purified by affinity chromatography and characterized by ELISA and immunodotblot assay. Affinity resin was prepared by coupling the purified HbsAg with commercially available yanogens bromide activated sepharose 4-B. Fifty clinically HBV positive human blood samples with medical history were collected from different hospitals of Lahore whereas five normal serum samples were collected as a negative control. The HBV in the sera samples was inactivated and used for immunochemical tests. An ELISA of all the serum samples was performed which revealed the presence of PreS2 epitope of HBV in positive sera. Immunodotblot and western blot analysis also showed the reactivity of PreS2 epitope towards the antibodies by giving purple blue color reaction product. So, in all these immunoassay techniques, PreS2 region of HBV was used as a marker to identify Hepatitis B infection in our local population. The results indicated that the immunoassay techniques used in this study can be exploited in routine diagnostic purposes and for the identification of antigen at the early stage of disease or during the treatment of the disease.

Key words: PreS2 epitope, hepatitis B surface antigen (HbsAg), hepatitis B virus (HBV).

INTRODUCTION

The Hepatitis B virus (HBV) is a double-stranded DNA virus about 42nm in diameter of *Hepadnaviridae* family which also infects ducks, ground squirrels and woodchucks (Tiollais and Buendia, 1991; Scmuness, 1978). In acute infection, hepatitis B virus takes about 1 to 6 months from the time of infection for the disease to manifest itself. (Chen *et al*., 2005). Those who are unable to produce an effective immune response allow the virus to replicate for long periods in their livers causing chronic hepatitis HBV infection, cirrhosis of the liver, and hepatocellular carcinoma (Gilbert *et al*., 2005). The WHO report (1994) documented that more than 300 million people are infected with HBV worldwide and Central South Asian population show intermediate endemicity (2-7%) and South East Asian has high endemicity (8-10%). Clinical results shows that an HBV infection in Pakistan is increasing day by day due to bad hygienic conditions and the published results showed that 10-14% are the carriers of HBV virus (Malik and Tariq, 1995).

The various viral components are produced by hepatitis B virus during reproduction (Xuanyong and Timothy, 2004). Some enter the blood stream and cause detectable changes, some may only be determined via liver biopsy and others require sophisticated reliable tests. It includes Hepatitis B DNA, Hepatitis B DNA polymerase (DNAp), Hepatitis B Core Antigen (HbcAg), Hepatitis B e Antigen (HbeAg), HBx Protein and the most important Hepatitis B Surface antigen (HBsAg) (Stibbe and Gerlich, 1983). There are three different types of hepatitis B surface antigens located in the lipid bilayer of viral envelop; small hepatitis B surface antigen (SHBsAg), middle hepatitis B surface antigen (MHBsAg), and large hepatitis B surface Antigen (LHBsAg). The PreS2 domain of MHBSAg and LHBsAg is a proteolysis sensitive site and it has a 5-16 amino acid sequence which can block a human serum albumin receptor-binding site (Ueda *et al*., 1991). After natural infection, PreS1 and PreS2 antibodies appear first. PreS2 epitope domain has been identified using a panel of monocolonal antibodies (Mimms and Floreni, 1990). PreS region constitutes the most variable part of the HBV genome and this region is important for virus attachment and cell entry. On the basis of limited availability of HBV serology in our country,

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it needs to produce the immunological tests specific for HBV detection in our local population at initial stage of disease.

MATERIALS AND METHODS

All chemicals used in this study were purchased from Sigma. The abbreviation used are, pNPP (para-Nitrophenyl Phosphate), NBT (Nitroblue tetrazolium), BCIP (5-Bromo-4-Chloro-3- Indolyl Phosphate), TBS (Tris buffered saline), EDTA (Ethylene diamine tetra acetic acid), BSA (Bovine serum albumin).

Ammonium sulfate precipitation of anti-PreS2 antibodies

Two ml of immunized rabbit serum (against synthetic PreS2 peptide) was mixed with equal volume of saturated ammonium sulfate and incubated overnight at 4°C. Next day the tubes were centrifuged at 4°C at 5000 rpm for 10 minutes, supernatant was removed and the pellets were resuspended in 1ml of TBS pH 7:4. The resuspended pellets were dialysed against TBS as described by Harlow and Lane (1988).

Affinity purification on cyanogen bromide activated sepharose 4-B

Coupling procedure

One gm of cyanogen bromide activated sepharose 4-B gel was suspended in 200 ml of 1mM HCl for one hour and centrifuged at 600 rpm at 25°C for 15 minutes. Supernatant was discarded and the gel was mixed with ligand (100 µl of purified HBsAg, 5 µg/ml) in the coupling buffer (0.01M sodium carbonate pH:9). The antibodies were purified on the gel using affinity chromatography columns as described by Harlow and Lane (1988).

Concentration of antibodies

To concentrate the affinity purified antibodies, the solution was added in an eppendorf spin column (Sigma) with the filter pore size of 130 kDa and centrifuged at 10,000 rpm for 10 minutes at 4°C. The filtrate was discarded and filtration tube was refilled with more purified antibodies each time. At the end, antibodies were resuspended in TBS buffer and stored at -20°C for further use (Harlow and Lane, 1988).

Characterization of antibodies

Enzyme linked immunosorbent assay (ELISA)

The modified ELISA procedure was used to characterize the purified antibodies as described by Harlow and Lane (1988). Briefly, 100µl of the purified antibody (1:20 dilution in TBS) was coated on the 96 well microtiter plate and kept at 37°C for 45 minutes. Unbound antibodies were removed by emptying the plate and 200µl of 5% skim milk in 1 x TBS (blocking buffer) was added in the well and incubated for 45 minutes at 37°C. Blocking buffer was removed and washed 5-6 times with 1 x TBS. 100µl of alkaline phosphatase conjugated secondary antibody (goat anti-rabbit IgG, 1:5000 dilution in 1 x TBS) was added in each well and incubated for 45 minutes at 37°C. Again wells were washed for 5-6 times in 1 x TBS then 100µl of freshly prepared pNPP substrate was added. Yellow color was developed after 15 minutes.

Immunodotblot analysis

The modified immunodot blot procedure was used to characterize the purified antibodies as described by Harlow and Lane (1988). Briefly, nitrocellulose membrane was cut in 3x5 cm and 10µl of purified antibody was spotted on the membrane and was allowed for 5 minutes at 4°C. The nitrocellulose membrane was stained with acidic Ponceau S stain for 5 minutes in order to check the presence of protein. The membrane was destained with several changes of 1xTBS and dipped in 5% skim milk in 1xTBS (blocking buffer) for 45 minutes at 37°C to block non specific binding sites. After 5-6 times washing with 1xTBS, membrane was dipped in alkaline phosphatase conjugated secondary antibody (Goat anti-rabbit IgG 1:5000 dilution). After 45 minutes membrane was washed for 5-6 times with TBS (10ml/wash) and color reaction was developed by dipping nitrocellulose strips in NBT/BCIP solution.

Collection of blood samples

Clinically investigated HBV positive blood samples of 50 individuals with brief medical history were collected from different hospitals of Lahore. Five normal blood samples for negative control were also collected (Table I).

Table I.- Detail of blood donors and protein concentration of HBV positive and normal healthy samples.

Sample No.	Sex	Age (Years)	Sample Collected	Medical History	Concentration (µg/ml)
1	F	35	Shaikh Zayed Hospital	Chronic Hepatitis B	0.745
2	F	43	Shaikh Zayed Hospital	Chronic Hepatitis B	0.765
3	F	26	Shaikh Zayed Hospital	Acute Hepatitis B	0.749
4	M	35	Shaikh Zayed Hospital	Chronic Hepatitis B	0.752
5	M	80	Shaikh Zayed Hospital	Liver carcinoma	0.756
6	M	35	Shaikh Zayed Hospital	Chronic Hepatitis B	0.761
7	M	30	Shaikh Zayed Hospital	Acute Hepatitis B	0.799
8	F	28	Shaikh Zayed Hospital	Liver Cirrhosis	0.761
9	M	55	Shaikh Zayed Hospital	Chronic Hepatitis B	0.749
10	F	37	Shaikh Zayed Hospital	Acute Hepatitis B	0.758
11	M	24	Shaikh Zayed Hospital	Acute Hepatitis B	0.769
12	F	30	Shaikh Zayed Hospital	Chronic Hepatitis B	0.791
13	F	58	Shaikh Zayed Hospital	Liver carcinoma	0.801
14	M	27	Shaikh Zayed Hospital	Acute Hepatitis B	0.777
15	M	20	Shaikh Zayed Hospital	Acute Hepatitis B	0.757
16	F	68	Shaikh Zayed Hospital	Chronic Hepatitis B	0.789
17	F	23	Shaikh Zayed Hospital	Acute Hepatitis B	0.668
18	F	46	Shaikh Zayed Hospital	Chronic Hepatitis B	0.753
19	M	32	Shaikh Zayed Hospital	Acute Hepatitis B	0.699
20	M	59	Shaikh Zayed Hospital	Chronic Hepatitis B	0.682
21	M	56	Shaikh Zayed Hospital	Acute Hepatitis B	0.985
22	F	60	Zenat Laboratory	Liver Carcinoma	0.781
23	M	26	Zenat Laboratory	Chronic Hepatitis B	0.746
24	M	25	Zenat Laboratory	Chronic Hepatitis B	0.876
25	M	30	Zenat Laboratory	Chronic Hepatitis B	0.765
26	F	46	Zenat Laboratory	Acute Hepatitis B	0.599
27	F	65	Zenat Laboratory	Chronic Hepatitis B	0.628
28	F	40	Zenat Laboratory	Acute Hepatitis B	0.741
29	M	40	Zenat Laboratory	Acute Hepatitis B	0.682
30	M	52	Zenat Laboratory	Chronic Hepatitis B	0.755
31	F	29	Zenat Laboratory	Acute Hepatitis B	0.952
32	F	50	Zenat Laboratory	Acute Hepatitis B	0.764
33	F	34	Zenat Laboratory	Acute Hepatitis B	0.759
34	M	45	Zenat Laboratory	Chronic Hepatitis B	0.902
35	M	39	Zenat Laboratory	Acute Hepatitis B	0.755
36	M	23	Zenat Laboratory	Chronic Hepatitis B	0.796
37	M	53	Zenat Laboratory	Chronic Hepatitis B	0.658
38	F	36	Zenat Laboratory	Chronic Hepatitis B	0.729
39	F	28	Zenat Laboratory	Acute Hepatitis B	0.701
40	M	37	Zenat Laboratory	Liver cirrhosis	0.568
41	M	56	Mayo Hospital	Liver cirrhosis	0.765
42	M	72	Mayo Hospital	Liver carcinoma	0.742
43	F	34	Mayo Hospital	Acute Hepatitis B	0.756
44	M	63	Mayo Hospital	Acute Hepatitis B	0.791
45	M	44	Mayo Hospital	Chronic Hepatitis B	0.754
46	M	26	Mayo Hospital	Chronic Hepatitis B	0.771
47	M	19	Mayo Hospital	Acute Hepatitis B	0.91
48	F	23	Mayo Hospital	Acute Hepatitis B	0.672
49	F	54	Mayo Hospital	Acute Hepatitis B	0.738
50	M	37	Mayo Hospital	Liver cirrhosis	0.747
Control					
1	F	22	Institute of Biochemistry and Biotechnology	Normal	0.946
2	F	21	Institute of Biochemistry and Biotechnology	Normal	0.942
3	F	20	Institute of Biochemistry and Biotechnology	Normal	0.893
4	F	23	Institute of Biochemistry and Biotechnology	Normal	0.945
5	M	35	Institute of Biochemistry and Biotechnology	Normal	0.976

Inactivation of HBV in positive serum samples

Collected sera samples were processed for inactivation of Hepatitis B virus. 100 µl of each sample was mixed with 100µl of 0.05M Tris-Cl and 100µl of 1% SDS. Then the samples were incubated at 60°C for 1.5 hr. After incubation, the samples were centrifuged at 10000 rpm for 10 minutes to remove the pellet. Supernatants were stored at -20°C.

Bradford assay

Bradford Assay was used for estimation of total protein concentration of purified antibody and total serum protein by using BSA as standard (Bradford, 1976).

ELISA of serum samples

100µl of (1:100 dilution in TBS) each inactivated serum sample and control serum sample was coated on microtitre plate and incubated at37°C for 45 minutes and preceded for ELISA as mentioned above using purified anti-preS2 antibodies as primary Ab and Alkaline phospatase conjugated goat anti rabbit IgG as secondary antibody.

Immunodotblot analysis of serum samples

3µl of 10 inactivated sera and 3µl of positive control and negative control were spotted on the nitrocellulose membrane and preceded for Immunodotblot as mentioned above using purified anti-preS2 antibodies as primary antibody and alkaline phosphatase conjugated goat Anti rabbit IgG as secondary antibody.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE 12% was carried out as described (Laemmli, 1970). Different dilutions (1:8, 1:10, and 1:12) of HBV positive serum sample with total volume of 10µl each were made and mixed with 2.5µl of loading dye (4x) and kept in boiled water bath for 7minutes to prepare sample for loading onto the gel. The samples and protein marker were electrophoresed at 120V. It was stained with coomassie brilliant blue R-250 and then destained. "Gene Snap" photographed gel system was used to prepare the images whereas analyzed by "Gene Tool" using the computer software programme "Gene Genius Gel Documentation System analysis". The best dilution was considered for the next step of western blotting.

Western blotting

The best Dilution *i.e.* 1:10 of HBV positive serum was considered. This dilution was run on SDS-PAGE with a positive and a negative control. After electrophoresis the gel was soaked in transfer buffer for 15 minutes and then placed in Invitrogen XCell™ Blot Module semidry transblot cell and processed according to the procedure given in the equipment manual. Electrophoresed proteins were transferred onto nitrocellulose membrane using constant voltage (15V) for one and half hour. The nitrocellulose membrane was taken out from transbolt apparatus and was stained with acidic Ponceau S, for approximately 5 minutes in order to check the transfer of proteins. The membrane was destained with several changes of 1xTBS and water. The membrane was dipped in 5% skim milk 1X TBS (blocking buffer) for 45 minutes at 37°C to block non specific binding sites. After 5-6 times washing with 1xTBS, membrane was dipped in purified diluted (1:1000 dilution) anti-PreS2 antibodies. After 45minutes of incubation at 37°C with constant shaking at 100-150 rpm, membrane was washed in TBS for 5-6 times. Then the membrane was dipped in alkaline phosphatase conjugated secondary antibody (goat anti-rabbit IgG alkaline phosphatase conjugated, 1:5000 dilution). After 45minutes of incubation at 37°C with constant shaking at 100-150 rpm, again membrane was washed in TBS for 5-6 times. Then, color reaction was developed by dipping nitrocellulose paper in NBT/BCIP solution (Towbin *et al.*, 1979).

RESULTS AND DISCUSSION

The research study was focused to detect immunochemically the PreS2 epitope of Hepatitis B surface antigen (HBsAg) derived from local HBV isolates using anti-PreS2 anibodies. The study will be helpful in the diagnostic study of HBV infection in our local environment. Rabbit anti-PreS2 antibodies were partially purified by ammonium sulphate precipitation method and were further

purified by immunochromatography using CN-Br activated sepharose 4-B resin. The purified antibodies were characterized by ELISA and immunodot blot analysis which confirmed the presence of anti-PreS2 antibodies (Fig. 1).

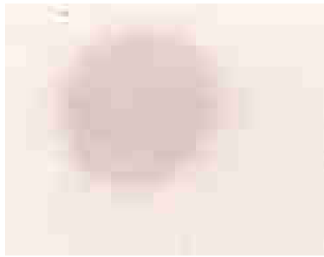

Fig. 1. The spot after immunodotblot is confirming the presence of purified antibody after immunoaffinity chromatography.

Fifty clinically investigated HBV positive serum samples were collected from different hospitals of Lahore with their medical history. Normal samples were also collected as a negative control. The purified anti-PreS2 antibodies were used to detect the PreS2 epitope of HBsAg in the local blood samples. Serum samples analyzed by ELISA confirmed the HBV infection whereas normal serum samples were devoid of HBV infection. This indicated the specificity of anti-PreS2 antibodies for PreS2 epitope of HBsAg and the results were neither due to non-specificity of antibodies nor due to non-specific reaction of secondary antibodies. Immunodot blot analysis of the sera also confirmed the presence of PreS2 epitope (Fig. 2). In the SDS-PAGE analysis, the best dilution of the serum sample *i.e.* 1:10 was considered for western blotting. Western blotting (Fig. 3) showed a number of bands depicting the presence of degraded HBsAg (Tiollais and Buendia, 1991; Ueda *et al.*, 1991). These purified anti-PreS2 antibodies also confirmed the purified 56 KDa protein of HBsAg.

The HBsAg is an envelope protein of the virus, and comprises three co-terminal proteins (large, middle and small) due to the presence of multiple transcripts and alternative translocation initiation sites in the gene. The large HBsAg peptide also composed of PreS2 epitope is the most abundantly expressed and is used in diagnosis of Hepatitis B infection.

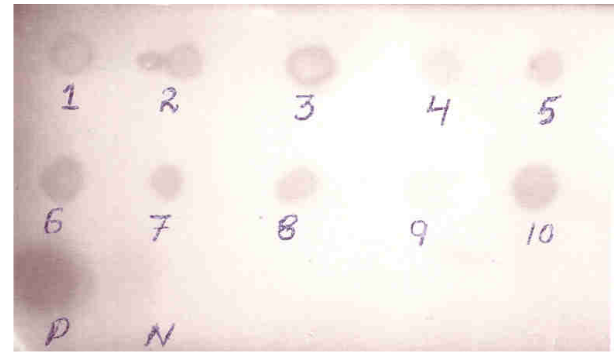

Fig. 2. Immunodotblot Analysis. A purple blue formazen reaction product of Alkaline Phosphatase was deposited on 1-10 (HBV Positive), P (Positive control) normal serum was devoid of reaction product.

Fig. 3. The strip (1) is the negative Control, (2) is the highly infected serum with a number of bands (3) showing the purified HBsAg.

Immunological techniques have been developed for the past several years to search a unique marker to identify the hepatitis B infection. In Pakistan precise and specific diagnostic studies of human infectious diseases still need attention. So there is need to develop such diagnostic immunoassays for detection and screening of the local population. Development of such

immunochemical based test against HBV found in our local environment will not only improve the ability to identify and treat the disease at its early stage but will also provide leading way to biological approach for the development of new drugs and tests for diagnosis.

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