Acta virol. 37: 417–428, 1993

ANALYSIS OF COMPUTER-PREDICTED ANTIBODY INDUCING EPITOPE ON JAPANESE ENCEPHALITIS VIRUS

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Received October 20, 1992; revised April 26, 1993

Summary. – Theoretical methods to delineate antibody inducing epitopes have been employed to predict antigenic determinants on envelope glycoprotein (gpE) of Japanese encephalitis (JE), West Nile (WN) and Dengue (DEN) I-IV viruses. A predicted region on JE virus gpE ^{74}CPTTGEAHNEKRAD^{87} was synthesized, conjugated to KLH (KLH-peptide) and used in immunization of mice. A mouse monoclonal antibody (MoAb IVB4) reactive to the peptide was also found to react with native JE virus gpE. Characterization of the idiotypic (ID) determinants with the help of polyclonal domain-specific anti-ID antibodies revealed that polyclonal anti-KLH-peptide antibodies and MoAb IVB4 are flavivirus-cross-reactive to Hx and NHx domains, respectively. The region 74–87 in JE virus gpE has been mapped as a linking area between Hx and NHx domains. Reactivity of the peptide with sera from JE patients and vaccinees also indicated the feasibility of using predicted peptides for diagnostic and prophylastic purposes.

Key words: Japanese encephalitis virus; synthetic peptide; B-cell epitope

Introduction

The gpE of flaviviruses is an antigen which is capable of inducing neutralizing antibody. Based on studies on homology of gpE and other structures of various flaviviruses, and reactivities of synthetic peptides with polyclonal immune sera, it appears that R1 domain contains type-specific and subgroup-specific epitopes. R2 domain comprises mainly flavivirus cross-reactive epitopes. Joining of R1 and R2, and R1 and R3 appears to be most important in eliciting type-specific and neutralizing antibodies (Roehrig *et al.*, 1989; 1990). Nowak and Wengler (1987) have postulated domain structure WN gpE. A similar model has been postulated by Mandl *et al.* (1989) for tick-borne encephalitis (TBE) virus gpE.

Domain structures of DEN and Murray Valley encephalitis (MVE) virus gpE were postulated by Roehrig *et al.* (1989, 1990) using data on beta turns and reactivities of the predicted B-cell peptides with various polyclonal sera. However, regarding JE virus no such information is available.

Domain structure prediction accompanied with reactivities of predicted synthetic peptides with immune sera can also be used to evaluate the accuracy of the B-cell antigenic determinant prediction methods. Based upon the knowledge of domain structure, the sequence of certain B-cell peptides can be predicted. The validity of the prediction is tested by the reaction of polyclonal immune sera raised against the whole virus with the predicted peptide. In these studies polyclonal antibodies raised against gpE or peptide were used to determine the antigenicity of the peptide (Innis *et al.*, 1989; Roehrig *et al.*, 1989). In very few studies MoAbs raised against the gpE have been used in binding assay with the oligopeptide (Aaskov *et al.*, 1989; Mandl *et al.*, 1989). In none of the studies, MoAb has been raised against the peptide to check conformation of the oligopeptide in free state and as a part of a protein. The results will be of practical use e. g. in diagnostic tool development or vaccine design, only if the conformations of the free peptide and the peptide as a part of the protein would be identical. Therefore we have carried out studies on an oligopeptide of gpE of JE virus.

Materials and Methods

Viruses. JE (strain P20778), WN (strain E101) and DEN II (strain TR 1751) viruses were grown in porcine stable kidney (PS) or Vero cells in Earle's based minimum essential medium (MEM) containing 10 % goat serum. Virus was pelleted at 37 000 rpm for 2 hrs from infected tissue culture fluids. Purified virus was used as antigen in ELISA and Western blot (WB) analysis (Cecilia *et al.*, 1988).

MoAbs. A panel of 16 MoAbs against gpE of JE virus (733913 strain) has been reported earlier from this laboratory (Kedarnath *et al.*, 1986). Epitope mapping by use of these MoAbs has revealed five domains on gpE of JE virus, namely (a) haemagglutination-inhibition (HI)-positive JE virus-specific (Hs), (b) HI-positive flavivirus-cross-reactive (Hx), (c) HI-negative JE virus-specific (NHs), (d) HI-negative flavivirus-cross-reactive (NHx), and (e) HI-negative autoreactive (NHA) (Cecilia *et al.*, 1988). Domain-specific inhibition in binding was observed with MoAbs directed against the homologous domains only.

Polyclonal anti-ID antibodies to domain-specific MoAbs were raised in rabbits (Ghanekar *et al.*, 1991). Biotin conjugates of purified anti-ID antibodies (using normal mouse IgG and homologous idiotype) have been prepared. These anti-ID antibodies show domain-specific reactivities i. e. anti-ID against Hs-1 MoAb (JE-specific) shows reaction with all antibodies of Hs type. Similarly anti-ID antibodies were prepared against MoAbs Hx-1 and NHx-1. Anti-ID against MoAb Hx-1 reacted with MoAbs Hx-1, 2, 3 and 4, but not with NHx-1 or NHx-2. Anti-ID against MoAb NHx-1 reacted with MoAb NHx-1 only, and not with any of the MoAbs of Hx group (Ghanekar *et al.*, 1991).

Human sera. Eighteen human sera were collected from JE patients during an epidemic at Gorakhpur (U. P., India). The diagnosis of JE was carried out for all cases with JE virus-specific IgM capture ELISA. Prevaccination and postvaccination sera from 8 children vaccinated with mouse brain purified vaccine were also collected. Known flavivirus-positive and -negative human sera served as controls.

Prediction of antigenic sites. gpE of JE, WN and DEN viruses have nearly 80 % homology (Roehrig *et al.*, 1989). To predict antigenic determinants we have used the strategy of Parker *et al.* (1986).

Hydrophilicity of overlapping heptapeptides of gpE from N-terminus to C-terminus were determined and in each heptapeptide the average value was assigned to the middle (4th) residue. The mean of these average hydrophilicity was termed as surface hydrophilicity (SH). If SH > 1.25 then residues are assumed to be on the surface of the protein. The accessible residues were determined using Jannin parameters (Jannin, 1979). The flexibility of any residue in the given sequence was calculated according to Parker *et al.* (1986). Using hydrophilicity, accessibility and flexibility values a composite plot was made. An oligopeptide was said to be an antigenic determinant if at least 6 amino acid residues were predicted as antigenic in the composite profile. Computer program used to predict antigenic determinant is available on request for PC compatible systems as well as for Micro VAX II.

Conjugation of synthetic peptide to KLH. A synthetic peptide of sequence ^{74}CPTTGEANHE-KRAD^{87} was obtained from Multiple Peptide Systems, San Diego, California. The peptide was purified on C-18 column to a 80 % purity and coupled to Keyhole limpet haemocyanin (KLH) through free sulphydryl group of cysteine by the MBS method of conjugation by the supplier. The conjugation of the peptide to KLH (KLH-peptide) was in a molar ratio 1:1.

Immunization of mice. KLH-peptide (100 μg) was used in 50 % complete Freund's adjuvant (CFA) to immunize five adult BALB/c mice by subcutaneous route at multiple sites. Two booster doses of KLH-peptide were administered with incomplete Freund's adjuvant (IFA) at weekly intervals by intraperitoneal route. Control BALB/c mice were immunized with KLH (100 μg) in CFA. Blood was collected at weekly intervals through orbital bleeding. Immune serum was separated and stored at -20 °C till tested.

Generation of MoAb reactive to synthetic peptide. The protocols described by Kedarnath *et al.* (1986) were used for the generation of hybridoma. Splenocytes of BALB/C mice immunized with JE virus were fused with Sp2/0 cells with the help of polyethyleneglycol (Sigma). Out of 75 hybrid wells tested only one reacted with the peptide in ELISA. The hybrid cells were cloned and their monoclonality was ascertained using IgG subtyping kit (Hyclone). Ascitic fluids (AF) from the hybridoma were obtained by injecting cells in pristane primed BALB/c mice.

ELISA. Protocols for ELISA described by Cecilia *et al.* (1988) were employed. Microplate (Nunc) wells were coated with the peptide or purified JE virus (1 μg per well) overnight. Goat anti-mouse IgG conjugated to horse raddish peroxidase (HRP) was used for probing. Goat anti-human whole immunoglobulin conjugated to HRP was used while testing human sera. As substrate served o-phenylenediamine. Positive/negative (P/N) ratio higher than 2.0 was taken as positive reaction.

In the case of *antigen capture ELISA* 1:50 dilution of AF was coated and blocked as above. The captured virus from infected tissue culture fluids was probed with biotin-conjugated MoAbs followed by avidin-HRP. Idiotypic reactivity of bound antibody was checked with biotin-conjugated purified rabbit anti-ID antibodies (Ghanekar *et al.*, 1991).

Binding inhibition of MoAb-conjugates to JE virus by anti-KLH-peptide sera (diluted 1:10) was also studied. Percentage inhibition was calculated from the decrease of absorbancy readings (taking the absorbancy for unblocked MoAb for 100 %).

Immunofluorescence (IF) was carried out on acetone-fixed virus-infected PS cell cultures using FITC-conjugated rabbit antimouse serum (Gore *et al.*, 1990). Virus-infected cells stained with polyclonal immune AF were used as positive control. Uninfected cells stained with immune AF and virus-infected cells stained with non-immune AF served as negative controls.

HI tests. Acetone-extracted sera and AF were used in the HI tests. HI tests were carried out with standard protocols using purified mouse brain antigen and goose red blood cells (Clarke and Casals, 1958; Kedarnath *et al.*, 1986). Eight units of antigen were employed for HI tests.

WB analysis was carried out using purified JE and WN viruses (Towbin *et al.*, 1979). Proteins were transferred to nitrocellulose membrane using semidry blot apparatus (Sartorius). Reactivities of immune serum and MoAbs were visualized using goat anti-mouse IgG conjugated to alkaline phosphatase (Biorad) with TMB/BCIP as substrate.

Radioimmunoprecipitation (RIP) was carried out using ^{35}S-methionine labelled JE virus (strain 733913) grown in Vero cells. Antibody-antigen complexes were pulled out with the help of formalinized *Staphylococcus aureus* (FSTA) (Richert *et al.*, 1979). FSTA was prepared by the method described earlier (Gore *et al.*, 1983). The method of Kale *et al.* (1991) was used for growth and ^{35}S-methionine labelling of JE virus. The labelled cell lysates in 500 μl of RIP buffer (1 % Trition X-100,

0.5 % sodium deoxycholate, 0.1 % SDS, 0.1 mol/l NaCl, 1 mmol/l EDTA and 1 mmol/l PMSF in 0.01 mol/l sodium phosphate buffer pH 7.5) and 25 μl of MoAb-containing AF were mixed and incubated overnight at 4 °C. 50 μl of 10 % FSTA in RIP buffer was then added and incubation was continued for another 4 hrs at room temperature. Immune complexes were pelleted and washed extensively, and polyacrylamide gel electrophoresis (PAGE) was carried out.

Table 1. Predicted antigenic determinants on gpE of JE, WN and DEN viruses

Predicted antigenic determinant		Predicted antigenic determinant	
Residues No. in gpE	Sequence	Residues No. in gpE	Sequence
JE		WN	
75–92	PTTGEAHNEKRADSSYVC	35–42	SKDKPTID
147–157	TTSENHGNYSA	75–87	PTMGEAHNEKRAD
226–237	TSPSSTAWRNRE	180–195	GEVTVDCEPRSGIDTS
234–251	EEAHATKQS	222–247	SSAGSTTWRNRETLMEFEEP-HATKQS
309–319	SFAKNPADTGH	252–260	GSQEGALHQ
328–335	YSGSDGPC	309–316	RTPADTGH
363–369	TSSANSK	384–399	RGEQQINHHWHKSGSS
DEN I		DEN II	
35–42	AKDKPTLD	48–55	TEAKQPAT
66–79	SNTTTDSRCPTQGE	65–89	LTNTTTESRCPTQGEPSL-NEEQDKR
153–160	NETTEHGT	144–171	HSGEEHAVGNDTGKHGKEI-KITPQSSIT
223–235	GASTSQETWNRQD	223–230	GADTQGSN
271–276	QTSGTT	241–249	KNPHAKKQD
339–350	SQDEKGVTQNGR	254–260	GSQEGAM
393–398	KKGSSI	326–335	YEGDGSPCKI
		393–398	KKGSSI
DEN III		DEN IV	
72–78	SRCPTQG	68–89	ITTATRCPTQGEPYLKEEQDQQ
84–89	EEQDQN	144–159	HNGDTHAVGNDTSNHG
147–156	DQHQVGNETQ	223–236	GADTSEVHWNYKER
222–230	ATTKTPTWN	254–260	ESQEGAM
269–274	QTSGGT	265–275	AGATEVDSGDG
338–346	EDGQGKAHN	392–397	RKGSSI
391–396	RKGSSI	467–473	TNSRNTS

Results

Prediction of B-cell epitopes

Amino acid sequences of gpE of JE, WN and DEN I-IV viruses obtained from Database NBRF-PIR were aligned with the help of ALIGN program (Dayhoff *et al.*, 1983). Predicted antigenic determinants on gpE of these viruses are shown in Table 1. There are 5–8 B-cell antigen determinants on each of these viruses. As can be seen from these data the regions 75–92 and 220–250 were common to all the viruses. The region 392–397 is common to DEN I-IV but not to JE and WN viruses. The region 309–320 is common to JE and WN but not to DEN viruses. A JE virus-specific region was found between 147 and 157. Sequences common to DEN I-II and WN viruses but not to JE, DEN III and IV viruses were present in the region 35–50.

In order to test experimentally the correctness of the prediction approach we have chosen the predicted region of ^{74}CPTTGEAHNEKRAD^{87} (peptide 74–87). This peptide region had high score in the composite plot. Thus from theoretical consideration the region 75–92 was most likely to be antigenic. Within this region sequence homology was higher for JE and WN than for DEN I-IV viruses. Thus antibodies raised against the peptide 74–87 would crossreact only with WN and not with DEN I-IV viruses, eventhough the region is antigenic. To test this hypothesis the peptide 74–87 was synthesized and conjugated with KLH (KLH-peptide) and used for immunization in mice.

Induction of antibody by KLH-peptide

As can be seen (Fig. 1) the seroconversion to peptide 74–87 was observed in ELISA in sera from mice collected 7 days after the first dose of immunization. Besides, there was an increase in the immune response to peptide after second and third doses. The control sera from mice immunized with KLH alone did not bind to the peptide 74–87. This shows that the peptide was capable of inducing an antibody response.

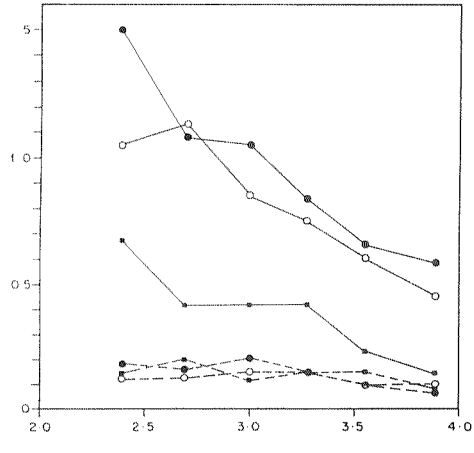

Fig. 1
Reactivity of anti-KLH-peptide serum with the peptide 74–87 in ELISA
Sera collected 7 (■), 15 (○) and 21 days (●) post immunization. (___) anti-KLH-peptide serum, (--) anti-KLH serum. Abscissa: log of reciprocal of serum dilution; ordinate: absorbancy.

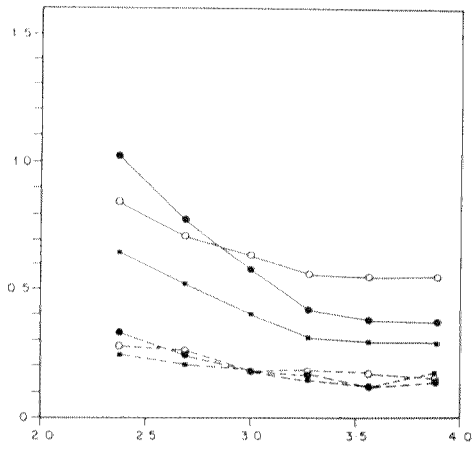

Fig. 2
Reactivity of anti-KLH-peptide serum with JE virus in ELISA
For legend see Fig. 1

Reactivity of antipeptide sera with gpE of JE, WN and DEN II viruses

The reactivity of anti-KLH-peptide sera with JE virus and the peptide 74–87 was similar in ELISA (Fig. 2). The antisera to KLH-peptide reacted only with JE virus-infected PS cells in IF (Fig. 3), while they did not with WN and DEN II virus-infected cells. The anti-KLH immune sera did not react with any of the JE, WN and DEN II virus-infected cells in IF (data not shown). The reactivity of the anti-KLH-peptide immune sera with JE and WN viruses was further tested by WB analysis (Fig. 4) and HI tests. HI titers of anti-KLH-peptide sera were 20 and 10 HI units with JE and WN virus antigen, respectively, and below 10 units with DEN II virus antigen after three doses of antigen. The control KLH immune sera did not show any HI activity. In WB analysis KLH-peptide immune sera could react with gpE of JE virus. Identity of the gpE was confirmed by staining with MoAb reactive to JE virus. However, KLH-peptide immune sera did not react with WN virus proteins. It is possible that denaturation of WN gpE by SDS and reducing agents might have affected its structure. The neutralization tests *in vivo* and *in vitro* demonstrated that these antipeptide antibodies were of non-neutralizing type.

Characterization of MoAb reactive to the peptide

The MoAb reactive to the peptide (IVB4, IgG2a subclass) reacted with JE and WN viruses as well as with the peptide in ELISA (P/N ratios 6.72, 4.41 and 7.03, respectively). The reactivity of the MoAb IVB4 was tested also with another peptide (SIGKAVHQVF, peptide 436–445), which was a T-helper epitope of JE virus gpE (Kutubuddin *et al.*, 1991). It was observed that MoAb IVB4 reacted with these peptides if the NaCl concentration was low (0.15 mol/l). P/N ratio was 1.93 for the peptide 74–87 and 2.13 for the peptide 436–445. However, when salt concentration was raised 0.5 mol/l NaCl, the reactivity with the second

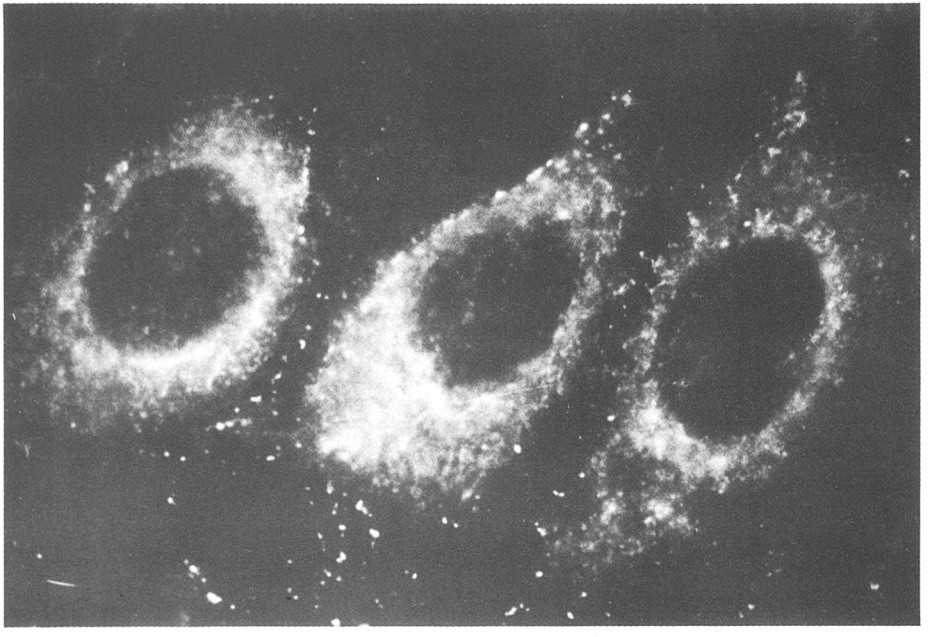

Fig. 3
Reactivity of anti-KLH-peptide serum with JE virus-infected PS cells in IF

peptide was lost (P/N ratio was 1.9 for the peptide 74–87 and 1.59 for the peptide 436–445).

Reactivity of the MoAb IVB4 with JE virus gpE was confirmed by RIP (Fig. 5). MoAb IVB4 could precipitate JE virus gpE similarly to other JE virus-specific MoAbs. These results clearly point out that conformation of the free peptide and that of the peptide in the gpE are similar. MoAb IVB4 did not react with JE and WN viruses in HI and neutralization tests, but it reacted in IF with JE virus-infected PS cells.

Analysis of the domain contributed by the peptide

Reactivity of domain-specific anti-JE virus gpE MoAbs with the peptide was tested. It was found that although the MoAbs could react with JE virus antigen, none of them reacted with the peptide. These studies pointed out that the 74–87 epitope was not mapped earlier (Cecilia *et al.*, 1988). To analyze further the domain constituting the peptide, binding inhibition assays were performed. It was observed that anti-KLH-peptide antibodies did not inhibit binding of Hs-1, Hs-2 or Hs-3 MoAbs (inhibition 0–8 %) to JE virus antigen. However, binding of Hx-1, Hx-2 and Hx-3 MoAb could be inhibited to 39.6 %, 49.3 % and 62.5 %, respectively by the anti-KLH-peptide serum. Thus antibodies raised against the

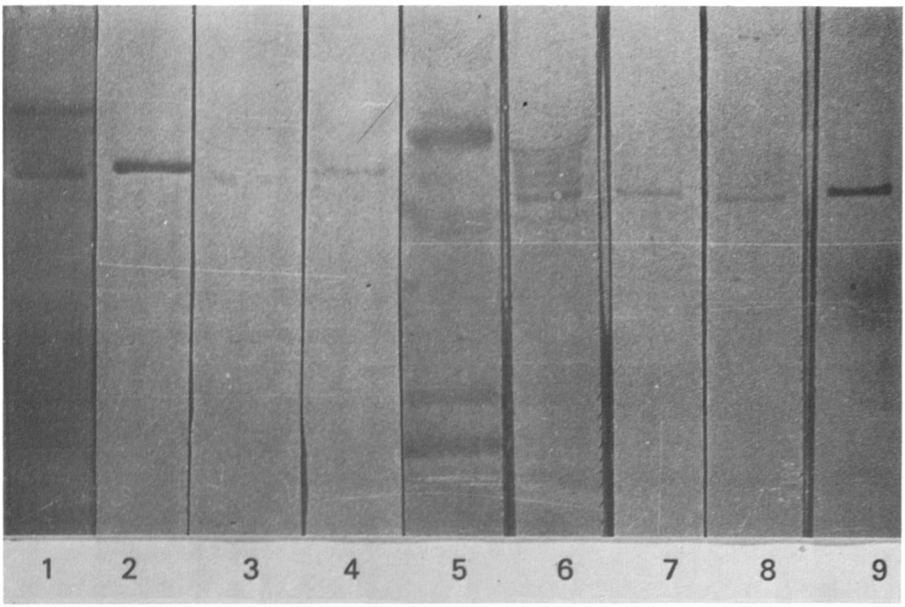

Fig. 4
WB analysis of reactivity of anti-KLH-peptide serum
Viruses: JE (lanes 1–4) and WN (lanes 6–9). Antisera: anti-KLH-peptide serum (lanes 2, 7), anti-KLH serum (lanes 3, 8), and MoAb Hx-2 (lanes 4, 9).
Molecular weight markers 67 K, 45 K, 22 K, and 14 K (lane MW). Amidoblack-stained blots (lanes 1, 6).

peptide 74–87 could be mapped into Hx domain.

Polyclonal anti-KLH-peptide serum was found to react with anti-ID antibodies raised against MoAb Hx-1 (P/N ratio was 7.17 for Hx-1 and 1.19 for Hs-1). MoAb IVB4 reacted with anti-ID antibodies raised against MoAb NHx-1 and inhibited the binding of anti-ID antibodies to NHx-1 with homologous MoAb by more than 85 %. This indicated that polyclonal and monoclonal antiboddies reactive to the peptide could be mapped within domains Hx and NHx.

Reactivity of human sera with the predicted antigenic peptide

It is important to test the reactivity of human sera with the antigenic peptides. Sera collected from JE patients and JE vaccinees were tested for the reactivity with the peptide. 1:250 dilution of the serum sample was tested in ELISA against the JE virus antigen and the peptide. As controls served flavivirus-positive and -negative human sera. JE virus antigen and the peptide were found to react with JE patient and vaccinee sera. The reactivity was weak though sufficient in the case of peptide (data not shown). Correlation coefficients (r) of P/N ratios of reactivities with JE virus antigen and the peptide in the cases of JE patient sera

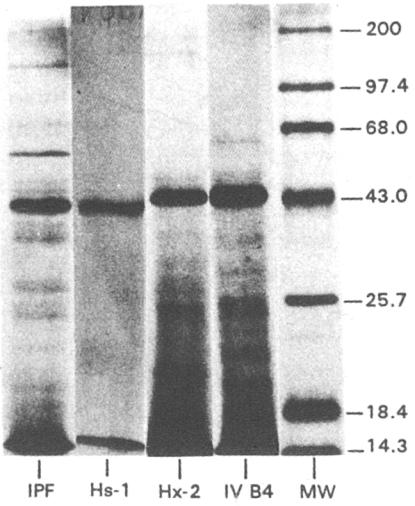

Fig. 5
RIP of JE virus-infected cell lysate with MoAbs
IPF: polyclonal serum; Hs-1: MoAb Hs-1; Hx-2: MoAb Hx-2; IVB4: MoAb IVB4; MW: molecular weight markers with M_r values (K) indicated on the right side of the gel.

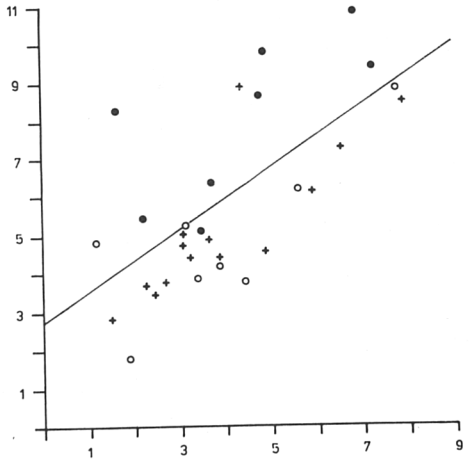

Fig. 6
Correlation of reactivity of human sera with peptide 74–87 and JE virus antigen
Sera of JE patients (x), sera of vaccinees (●), and control sera (○). Abscissa: reactivity with peptide; ordinate: reactivity with JE virus antigen.

and sera of vaccinees were similar (0.629 and 0.644, respectively). The calculated mean correlation coefficient was thus 0.643 and the Y axis intercept value 2.88 (Fig. 6).

Discussion

The antigenic structure of Murray valley encephalitis (MVE) virus gpE (Roehrig *et al.*, 1989) was found to be similar to that of JE virus gpE in this study. Roehrig *et al.* (1989) predicted many continuous regions in MVE virus gpE to be antigenic. Our method has delineated seven B-cell antigenic determinants in JE virus gpE, five of which are found to be common to MVE virus gpE. Predicted antigenic determinants of DEN II virus gpE, given in this study are very similar to those obtained earlier by Roehrig *et al.* (1990). The determinant 180–195 on WN virus gpE is unique for this virus. The 75–92 region in antigenic in gpE of all flaviviruses even though the sequence of this region is different in each protein.

Induction of the antibody reactive to JE virus by the KLH-peptide indicates that this region is accessible and occurs on the surface of the native gpE confirming the correctness of the prediction method. The antiserum to KLH-peptide could react with the native JE virus in IF, HI tests and WB analysis as well as in ELISA. However, these antibodies were negative in neutralization tests.

As pointed out earlier most of the studies with the prediction and analysis of antigenic determinants use either polyclonal antiviral or antipeptide sera. We have selected a MoAb (IVB4) reactive with the peptide. This MoAb also reacts with whole gpE in RIP. This further confirms the antigenic ability of the peptide. As mentioned earlier, a reactivity of the MoAb IVB4 with the control peptide at the low salt concentration, but not at the high salt concentration was observed. Peptides tested for reactivity are those which are predicted to be antigenic determinants and thus have high hydrophilicity index and concomittant charged residues. While testing reactivities in ELISA, a low salt concentrations will not neutralize the charges on the peptide and thus peptide will have high flexibility and will take various conformations. One of the conformations is recognized by the MoAb IVB4. Similarly, while testing predicted sequences with polyclonal sera in ELISA, some of the conformations of peptides may give a positive signal. This can be considered as a general phenomenon that suggests a need for more careful analysis of results obtained by studying reactions of peptides with polyclonal immune sera. Thus there is a possibility that some of the peptides reactive with polyclonal immune sera may not have identical conformation of native protein and may thus be artifacts.

The binding inhibition ELISA and the ELISA with the anti-ID sera was carried out to analyze the domain in which the predicted peptide occurs. Anti KLH-peptide sera showed low HI activity. Roehrig *et al.* (1989) have shown that the sera raised against the MVE virus peptide 77–97 (conjugated at ^{97}cys to

carrier) are weakly positive in HI reaction. The anti-KLH-peptide sera could inhibit binding of MoAbs Hx-1, 2 and 3 to JE virus in ELISA. Furthermore, anti-KLH-peptide sera could bind anti-ID antibody to MoAb Hx1. These data indicated that polyclonal anti-KLH-peptide immune response is of Hx-type. Anti-ID binding assay results and HI-non-reactivity data point out that the MoAb IVB4 can be mapped within the NHx domain. The difference in reactivities of KLH-conjugated and free peptide in inducing two different types of antibodies can be explained on the basis of conformation of the peptide attained in free state and in KLH-bound form. Taking into consideration domain-specific inhibition by anti-ID antibodies (Ghanekar *et al.*, 1991), one can conclude that the peptide could be mapped within different domains. This peptide thus links domains Hx and NHx.

The reactivity of human sera to peptide in ELISA also shows a promising result. However, JE virus-specific dominant sequences are to be worked out for realization of their diagnostic value.

It is thus concluded that the approach described above can be used in mapping the antigenic structure of the proteins. However, care should be taken to avoid artifacts. Such studies can provide data which can be used in understanding the three-dimensional strucure of proteins. Studies on human sera have pointed out that the above mentioned approach can reduce efforts in diagnostic tools and/or vaccine development considerably.

Acknowledgements. Supply of antiidiotypic conjugates by Mrs. S. A. Ghanekar is gratefully acknowledged. Selection and characterization of MoAbs was carried out by Ms. S. S. Chandra. Authors are also thankful to Mrs. V. J. Lad, Mr. V. M. Ayachit, and Mr. A. A. Koshy for technical assistance. Secretarial assistance of Mrs. A. V. Shendrikar is also acknowledged. We acknowledge support of DIC (DBT) at University of Poona, Pune.

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