Journal of Virological Methods 236 (2016) 1–4

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Detection of subtle differences in analogous viral capsid proteins by allowing unrestricted specific interaction in solution competition ELISA

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Article history:
Received 26 February 2016
Received in revised form 14 May 2016
Accepted 15 June 2016
Available online 16 June 2016

Keywords:
Antigen-antibody interaction
Hepatitis E virus
Viral capsid protein
Solution interaction
Binding analysis
Monoclonal antibody

A B S T R A C T

Assay artifacts were reported in plate-based immuoassays during the assessment of specific molecular interactions owing to the surface induced aggregation/conformational changes. To circumvent surface adsorption and associated artifacts, we used a solution competition ELISA by allowing unrestricted interaction between binding partners to occur in solution for better discrimination between epitopes with subtle differences. A difference of two orders of magnitude in binding to neutralizing antibodies for two truncated versions of the hepatitis E virus capsid protein was observed, while other assays showed comparable antigenicity with the same monoclonal antibodies. Discrimination of epitopes with high degree resemblance in analogous viral capsid proteins was demonstrated quantitatively based on their specific interactions. Therefore, the solution competition ELISA is a method of choice when the detection of subtle differences of two highly analogous proteins is desired.

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Biomolecular interactions are generally studied in plate- or bead-based formats to ensure specificity with different wash cycles incorporated. This is true for most of the antibody (Ab)-antigen (Ag) interactions where enzyme-linked immunosorbent assay (ELISA) is a most widely used technique in clinical, industrial or research settings. By adsorbing a binding partner on the solid surface, it enabled the determination of the specific interaction in the following steps by quantitative assessment of the immune complexes formed on the surface (or bead). Restrictions on the protein conformation or certain artifacts such as aggregation of the surface adsorbed protein molecules could preclude the capacity of the method to reflect the true solution conformation for induced fit for binding partner or to discriminate the subtle differences. For instance, certain bacterial capsular polysaccharides could adopt different conformation in solution as compared to surface adsorbed form (Sun et al., 2001).

Similarly, in protein molecules, artifacts have been observed when physically adsorbed or covalently attached to a solid surface in traditional ELISA methods. A principal problem was due to the fact that the conformation of the analyte could have been altered upon passive adsorption, leading to certain degree of denaturation or aggregation, a phenomenon noted by several research groups (Butler et al., 1997; Schwab and Bosshard, 1992). In a recent report, adsorbing hen egg white lysozyme on synthetic material induced tertiary unfolding of proteins, thus changing its activity or binding behaviors of this enzyme (Thyparambil et al., 2014).

Since the activity or function of a protein is dependent on its higher order of structures, alteration on protein conformation would impact the surface features or the accessibility of Ag epitopes or Ab binding sites (Butler et al., 1997). If the function of a protein were to bind and catalytically convert a substrate at its active site, the enzymatic activity would be impacted upon surface adsorption due to alteration in conformation or protein dynamics/flexibility. For example, the adsorption of Ribonuclease A using a wide range of surface chemistries caused changes in protein structure and was shown to lose approximately 60% of its native-state enzymatic activity, reflecting some conformational changes (Wei et al., 2014).

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http://dx.doi.org/10.1016/j.jviromet.2016.06.007
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To circumvent the surface adsorption, solution interaction for the binding partners of interests could be allowed during the assay. For example, rabbit antisera had an equal interaction with cognate *apo*-cytochrome *c* and native cytochrome *c* in a solid-phase ELISA, but in the solution-phase radiolabeled immunoassay, anti-*apo*-cytochrome *c* antibodies could identify the difference between *apo*-cytochrome *c* and native cytochrome *c*, which highlighting the advantage of methods of enabling solution interaction for binding partners in interests (Schwab and Bosshard, 1992). Another example of striking difference observed between solution form versus surface-adsorbed Ag is for Serotype 6A pneumococcal capsular polysaccharide. One monoclonal antibody (mAb) (Hyp6BM6) was found to bind specifically 6A pneumococcal capsular polysaccharide only in solution. This specific interaction was abolished likely due to the adoption of a different conformation when the same polysaccharide Ag was passively adsorbed onto the plastic plate, while the primary structures remain the same (Sun et al., 2001).

These reports outlined the phenomena of surface-induced alterations on the higher order structure of biomolecules and identified the altered biological functions due to such a change in the macromolecule conformation. In the solution, the interaction between Ag and Ab was unrestricted with both binding partners remain in solution, the solution competition ELISA would preserve the integrity conformation of proteins mimicking the native binding interaction in biological systems to a greater extent, reflecting the true affinity and specificity for the binding interactions (Friguet et al., 1985). With the solution competitive ELISA, we showed that up to 2-orders of magnitude of difference in binding activity to neutralizing mAbs against viral capsid protein were observed, whereas comparable antigenicity between recombinant hepatitis E virus (HEV) capsid protein p239 was observed in two other commonly used surface-based assay formats using the same reagents.

The recombinant HEV viral antigens used in the study were the dimeric E2 (aa 394–606), the capsid protein of HEV (Zhang et al., 2001) and p239 (aa 368–606), a 26 amino acids extension from N-terminal of E2, self-assembles into particle form (with MW: 3158 kDa). Both viral antigens are truncated version of the sole HEV capsid protein (Li et al., 2005). The latter is a vaccine antigen in a prophylactic HEV vaccine- Hecolin®. The structure/model of HEV recombinant capsid proteins E2 and p239 were shown in Fig. 1A and B. Both antigens showed similar reactivity with a panel of different murine anti-HEV mAbs (Li et al., 2005). For comparison, the mAbs (3A11, 8C11 and 8G12) were used in three different assay formats: the sandwich ELISA (Assay A), the direct binding ELISA (Assay B) and the solution competitive ELISA (Assay C).

As tabulated in Table 1, Assay A was performed by coating microtiter plates with mAb 3A11 as capture Ab, and 8C11 (with HRP label) as detection Ab in a commonly used sandwich format. The difference of antigenicity between p239 and E2 was analyzed by comparing the EC_{50} values derived from difference binding curves through curve fitting (Table 1; Fig. 1C). In a more straightforward Assay B, Ag was coated on the surface, followed by serially diluted mAb solutions and polyclonal HRP-conjugated goat-anti-mouse antibody (GAM-HRP) was used to detect the immuno complexes formed on the solid surface (Table 1 & Fig. 1D). No more than 2-fold difference in binding activity was observed for these two antigens (particulate p239 vs. dimeric E2) in these two assays.

Unlike in the above mentioned methods where quantitation comes from the detection of the immuno complexes formed on the solid surface, Assay C was used to assess the solution interaction of

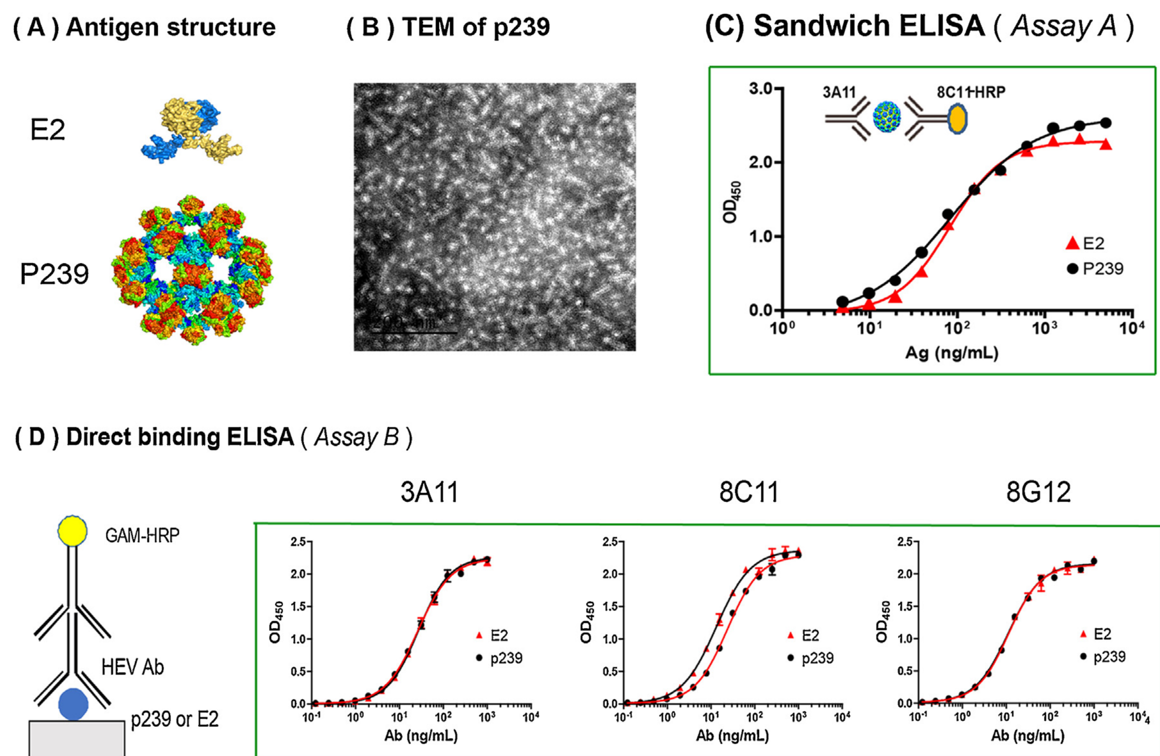

Fig. 1. The sandwich ELISA and the direct binding ELISA methods used in the antigenicity analysis of recombinant protein particulate p239 and dimeric E2 of hepatitis E virus (HEV). (A) Structure/model of the HEV capsid proteins. *Upper panel:* Surface diagram of E2 dimer (MW-46 kDa) was rendered using the known crystal structure of HEV-like particles (PBD: 2ZTN). Two monomers were shown in light blue and yellow colors, respectively. *Lower panel:* A model of particulate p239 (MW ~3158 kDa), the vaccine antigen in Hecolin®, was derived based on its sequence and crystal structures of relevant proteins. (B) Transmission electron microscopy (TEM) of p239 particulate antigen. The scale bar is 200 nm. (C) Binding profiles of E2 and p239 in a sandwich ELISA- Assay A (3A11: Ag: 8C11-HRP). (D) *Left panel*: Illustration of the direct Ag binding ELISA (Assay B) generally used for affinity ranking and specificity tests, *Right panels*: Three representative murine mAbs (3A11, 8C11 and 8G12) were used in the direct binding ELISA to compare their different binding affinity when E2 or p239 was used as coating antigen. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

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