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Elisas for Rotavirus Diagnosis, Typing, and Analysis of Antibody Response

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1. Introduction

Enzyme-linked immunosorbent assays (ELISAs) have been widely used to detect antigens and antibodies of a vast number of infectious agents, including viruses. While general approaches to develop and use ELISAs for analysis of antigens and antibodies are the same, different infectious agents differ widely in their complexity, antigenic structure, stability, diversity, and availability of antigens, making it necessary to consider their special characteristics to develop and use these assays. In this review I describe the development and use of ELISAs to detect and analyze rotavirus antigens and antibodies.

Rotaviruses belonging to group/species A (RVA) are the most important cause of severe gastroenteritis in infants throughout the world, and they also cause diarrhea in most mammalian species that have been studied. The main symptoms in infants are vomiting and diarrhea, which are also common to other gastroenteritis agents. Infections can be asymptomatic, mild, or severe leading to dehydration and in some instances death, while in adults asymptomatic infections occur throughout life, with raising levels of circulating anti-rotavirus IgG with increasing age. In the clinical practice diagnosis is facilitated due to the age range of infants with severe symptoms, higher incidence of infections in the cold season, and watery diarrhea. Current vaccines are contributing to relieve the burden of RVA gastroenteritis; however more vaccines are needed due to their high cost and suboptimal efficacy in developing countries (Estes & Kapikian, 2007).

Rotavirus (RV) is a genus of the family *Reoviridae*, classified in 5 species (A-E), formerly known as groups, and 2 tentative species (F and G) (Matthijnssens *et al.*). RV species are identical morphologically, with no cross-reaction antigenically. RVA have a genome consisting of 11 segments of dsRNA that code for 6 structural (VP1-VP4, VP6 and VP7) and 6 non-structural (NSP1-NSP6) proteins. The viral genome in the core of the virion is closely associated with the viral RNA polymerase VP1 and the guanilyl- and methyl-transferase VP3 (RNA-capping enzyme). The virion has icosahedral symmetry and consists of 4 structural proteins in 3 concentric layers: the inner layer formed by 60 dimers of VP2, the intermediate layer formed by 260 trimers of VP6, and the outer layer formed by 260 trimers of VP7 and 60 spikes of VP4 (Jiang *et al.*, 2008). RVA antigens obtained from infected cells contain mature triple-layered particles (TLPs), single-layered particles (SLPs), double-layered particles (DLPs), empty particles of all 3 classes which lack the viral genome, and multimeric complexes of viral proteins that have not been fully characterized (Gallegos &

Patton, 1989). The mature TLPs are unstable in low calcium, in contrast to DLPs which are remarkably stable in environmental settings such as seawater (Loisy *et al.*, 2004). When calcium is chelated the outer layer of the virion disassembles due to the effect of low calcium on the stability of trimeric VP7 capsomers (Dormitzer *et al.*, 2000).

The surface proteins of TLPs consist of a thin layer of the glycoprotein VP7 with a diameter of 90 Å, and VP4 spikes that traverse the VP7 layer, extending outward to a length of 120 Å, and interacting deeply with the intermediate VP6 layer. VP4 needs to be cleaved by trypsin into VP5* and VP8* to enhance viral infectivity, with VP5* subunits occupying the foot, body and stalk regions of the spikes and VP8* the distal heads. Cryo-electron microscopy of TLPs grown in the absence of trypsin show that VP4 is structurally disordered, *i. e.* the spikes cannot be seen, whereas upon trypsination the spikes become conformationally stable and structurally ordered (Jiang *et al.*, 2008). By cryo-electron microscopy the spikes of trypsin-treated TLPs appear to have trimeric foots (Li *et al.*, 2009), with dimeric bodies and two VP8* heads at the tips (Settembre *et al.*). In spite of this apparently dimeric structure, the long bilobed spikes undergo an irreversible conformational change at pH 11 and become trilobed and stunted. The alkali-treated TLPs are non-infectious and bind 3 anti-VP5* Mabs per spike (Pesavento *et al.*, 2005). From these data, it seems that VP4 spikes are trimeric with flexible linked subunits prior to cleavage. Upon treatment with trypsin the spikes rigidify, with one floppy and 2 structured subunits, while treatment with alkali leads to a conformational rearrangement whereby the stalks fold backand rigidify the floppy subunit, thus revealing their trimeric structure (Kim *et al.*).

The outer layer of the TLPs is the machinery for virus attachment and entry into the target cells. In the entry process, VP8* initially interacts with sialic acid (SA)-containing receptors, followed by sequential interactions of VP5* with hsc70 and integrins (Lopez & Arias, 2006). In Madin-Darby canine kidney (MDCK) cells infected with rhesus rotavirus (RRV) entry seems to occur through an endocytosis pathway, since the input RRV proteins colocalize with the early endocytic pathway proteins Rab4 and Rab5. In addition, the entry process is dependent on endocytic calcium concentration, since bafilomycin A1 and elevated extracellular calcium result in accumulation of intact TLPs in the actin network, suggesting a delay in RV decapsidation (Wolf *et al.*). A recombinant VP5* fragment has a trimeric, folded-back structure, that seems to mimic a conformational rearrangement that occurs when the virion uncoats. Such conformational change has been detected with conformational-specific Mabs against the recombinant VP5* fragment. The folding-back of VP5* resembles the conformational changes in enveloped virus fusogenic proteins. It has been proposed that an intermediary in the process of folding back of VP5*, that is able to bind liposomes, leads to membrane disruption of endosomes and virus entry (Trask *et al.*).

RVA replication and early morphogenesis occur in cytoplasmic inclusion bodies known as viroplasms. In MA104 cells the incoming DLPs in the cytoplasm transcribe the viral genome to produce the 11 viral mRNAs. Two of the viral proteins produced contain leader sequences that are targeted to the endoplasmic reticulum (ER), VP7 and NSP4. On the other hand, several viral proteins involved in viral replication and in the initial assembly steps accumulate in viroplasms: NSP2, NSP5, NSP6, VP1 to VP3, and VP6. Initially, viral mRNAs are used as templates to produce the genomic dsRNAs, in a process that occurs simultaneously with assembly of the inner layer of the virion. Further assembly of the intermediate VP6 layer depends on NSP4, and presumably occurs at the interphase between

viroplasms and the ER. The DLPs bud into the lumen of the ER, using NSP4 as a receptor. NSP4 presumably also functions as a VP4 receptor, such that budding serves to incorporate both surface proteins of the virion into the transiently enveloped DLPs. Finally, the envelope is lost as the virus matures within the ER to produce TLPs (Patton *et al.*, 2006).

NSP4 primary translation product has 20 kDa that increases to 28 kDa after glycosilation in the ER. The N-terminus contains 3 hydrophobic domains, H1 (aa 7-21) that resides mostly in the ER lumen, H2 (aa 29-47) that traverses the ER lipid bilayer, and H3 (aa 67-85) that is embedded in the surface side of the ER membrane. The protein emerges from the ER approximately at amino acid 44, hence the remaining 131 amino acids reside in the cytoplasm (Bergmann et al., 1989; Chan et al., 1988). The carboxy-terminus of NSP4 has several domains: a heptad repeat (aa 95-137), VP4-bindig (aa 112-148), calcium-binding (aa 114-135), and DLP-binding (aa 161-175) (Estes, 2001). Silencing of NSP4 expression by RNA interference leads to a severe defect in the assembly of both DLP and TLP RVA particles. Upon NSP4 silencing, several viral proteins show altered subcelular distribution, most notably VP6 accumulate as long fibers throughout the cytoplasm instead of being assembled in the viroplasms to form the intermediate layer of the virions. In addition, when NSP4 expression is silenced several proteins that accumulate in the viroplasms show reduced expression levels and viroplasms are small, suggesting that the failure to incorporate VP6 affects the assembly of other proteins that reside in the viroplasms. On the other hand, silencing of VP7 by RNA interference leads to the accumulation of enveloped DLPs in the lumen of the ER and no infectious virions are formed, indicating that VP7 has a role in envelope removal and TLP maturation in the ER (Lopez et al., 2005). Since interference of VP7 expression does not block DLP budding into the ER, the second viral ER-resident protein, NSP4, is most likely responsible of DLP budding into the ER.

The group specificity of RVs depends on VP6, the major antigen of the virion. Within species A, RVs have been classified in 4 subgroups (SG) of VP6, I, II, I/ II, and non-I/II, based on the reactivity with 2 Mabs (Kapikian & Chanock, 1996). In addition, RVAs have been classified in dual serotypes, since both of the surface proteins of the virion induce neutralizing antibodies. By neutralization assays with hyperimmune sera to purified TLPs 15 VP7 serotypes have been identified, known as G serotypes since VP7 is a glycoprotein. The predominance of anti-VP7 neutralizing antibodies in neutralization assays with sera hyperimmune to the whole viral particles preclude typing of VP4 with this assay. In order to determine the serotype-specificity of VP4, hyperimmune sera to recombinant VP4 were developed and used in neutralization assays to identify 14 VP4 serotypes, known as P since VP4 is protease-sensitive. Serotype P1 was further classified as P1A and P1B, based on one-way cross neutralization with hyperimmune sera to the prototype P1A and P1B strains (Hoshino & Kapikian, 1996). RVA G and P serotyping performed by neutralization assays are time consuming, and have been superseded by G and P genotyping based on identities among cognate genes. So far, 25 G genotypes (G[1] to G[25], with square brackets) have been identified with G[1] to G[14] corresponding to the serotypes G1 to G14 (without square brackets). For VP4, 35 P genotypes have been identified, P[1] to P[35], however the numbering of the P genotypes do not coincide with the numbering of P serotypes, since more than one P genotype may correspond to a single P serotype (Matthijnssens *et al.*).

Recently a complete genome classification system was developed for RVA strains based on nucleotide-identity cutoff values for the open reading frames of each of the 11 gene segments. This system assigns a one-letter code to each gene and successive Arabic numbers

for the different genotypes, such that the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 are described using the abbreviations Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ix-Hx. Based on this whole-genome system, human RVA strains were classified in 3 gene constellations that are characteristic of the Wa-like family and porcine RVA strains, the DS-1-like family and bovine RVA strains, and the AU-1-like family that has a mixed canine and feline RVA gene constellation. As an example of the diversity of human and animal RVA strains, 3 genotypes have been identified among human RVA strains based on VP6 (I1, I2, and I3), NSP2 (N1, N2, and N3), or NSP4 (E1, E2, and E3). The diversity considering both human and animal RVA strains is much higher, since 16 I, 9 N, and 14 E genotypes have been identified so far. Among human RVA strains the genotypes I1, N1, and E1 cluster together in the Wa-like family, I2, N2, and E2 in the DS-1-like family; and I3; N3, and E3 in the AU-1-like family (Matthijnssens *et al.*, 2008; Matthijnssens *et al.*).

2. Group/species A rotavirus ELISAs

A commonly used format for ELISAs to diagnose RVAs is based on 2 hyperimmune anti-RVA sera raised in different animal species, one as capture antibody, and the second as detector, followed by an enzyme-conjugate to the second species. Ideally, the capture and conjugate antibodies should be produced in the same species to prevent reactivity of the conjugate to the capture antibody (Yolken & Leister, 1982).

RVA ELISAs depend mostly on VP6, presumably due to the predominance of antibodies to VP6 in the polyclonal sera used for capture and detection, and the predominance of VP6 as an antigen in the assays. The yield of DLPs is usually higher than TLPs in MA104 cells infected with RVAs. In addition, VP6 is the major antigen in TLPs, since it accounts for 51% of its mass (Liu et al., 1988). Using plates coated with rotavirus TLPs or DLPs to screen monoclonal antibodies (Mabs) by solid-phase radioimmunoassay yielded predominantly anti-VP6 Mabs, 70% (Greenberg et al., 1983), as compared to Mabs specific to other viral proteins like VP7, VP4 or VP2. VP6 shows extensive cross-reactivity among RVA strains, such that hyperimmune sera produced to any mammalian RVA can be used in ELISAs to detect a heterotypic response. On the other hand, avian RVA strains are distantly related to mammalian strains (Matthijnssens et al., 2008), hence the performance of hyperimmune sera to mammalian RVA strains may be lower than homologous antisera to detect avian RVA strains by ELISA.

RVA ELISAs have been used in numerous studies to diagnose RVA infections from different animal species. The performance of the assay to detect RVAs from different animal species was similar since 33 human RVAs obtained from stools of naturally infected infants could not be differentiated by an RVA ELISA from strains derived from calves, piglets, foals, monkeys, and mice (Yolken *et al.*, 1978). In one study 1,163 children were analyzed for the presence of RVA particles using two ELISA kits. The kits were evaluated in laboratories of 7 different countries. One of the kits, the DAKO-ELISA, had a sensitivity of 97% and a specificity of 97% as compared to the WHO-ELISA kit. On the other hand, in individual laboratories the range of sensitivity of the DAKO-ELISA was 90-100%, and the range of specificity was 85-100% (Flewett *et al.*, 1989).

3. Subgroup rotavirus ELISAs

RVAs have been classified in four SGs according to the reactivity determined by ELISA of Mabs that specifically reacts with SG I (266/60) or SG II (631/9) (Greenberg *et al.*, 1983).

Other Mabs have been described that also react with SG I or II (Gerna *et al.*, 1989; Liprandi *et al.*, 1990; Taniguchi *et al.*, 1984), however the Mabs developed by Greenberg *et al.* are the most widely used in epidemiological studies.

The antigens used to produce the Mabs 255/60 and 631/30 were the simian genotype I2 strain RRV, and the human genotype I1 strain Wa, respectively. Some murine and avian RVA strains do not react with SG-specific Mabs (Svensson *et al.*, 1990; Theil & McCloskey, 1989), and an equine RV strain reacts with both SG I and II Mabs (Hoshino *et al.*, 1987). No clear correlation has been observed between the subgroup specificity and the VP6 genotyping system, since several of the VP6 genotypes contain strains with different SG specificities (Matthijnssens *et al.*, 2008).

The SG Mabs recognize conformational epitopes that are present in the trimeric but not the monomeric forms of VP6 (Gorziglia *et al.*, 1988). By using *in vitro* translated VP6 from the SG II human Wa strain, single amino acid mutations at position 172 were sufficient for recognition by the SG I Mab 255/60, while retaining the reactivity with the SG II Mab 631/9. On the other hand, the SG I porcine rotavirus YM VP6 with a double mutation at positions 305 and 306 acquires reactivity with the SG II Mab 631/9 and simultaneously loses its capacity to interact with the Mab 255/60 (Lopez *et al.*, 1994). In addition, a single mutation at position 315 was sufficient to change the SG specificity of the murine strain EW from non I/II to II (Tang *et al.*, 1997). All the residues that determine SG specificity are located closely together on the top surface of VP6, which interacts with VP7. Since the SG epitopes also form the VP7 binding structure, the constraints of such interaction have been proposed to be the limiting factor that restricts their antigenic variability (Mathieu *et al.*, 2001).

Epidemiologic studies of the prevalence of SG I and II in infants with gastroenteritis have been done by ELISA. Subgrouping differentiates the SG II Wa-like family from the SGI DS-1-like and AU-1-like families. Among the SG I specimens, the serotype G2 strains are preliminarily assigned to the DS-1-like family. On the other hand, the SG II specimens belonging to the serotypes G1, G3, G4, and G9 are preliminarily assigned to the Wa-like family. Sugroup I strains with serotypes other than G2 are likely to be AU-1-like, animal strains, or genomic reassortants. Apart from the highly prevalent Wa-like and DS-1-like strains, and their characteristic genomic constellations, other SG/serotype combinations are rarely detected (Beards *et al.*, 1989).

4. Serotype-specific rotavirus ELISAs

The development of neutralizing anti-VP7 and anti-VP4 monoclonal antibodies, and their use in epitope-specific ELISAs served to analyze the complexity of RVA serotype-specificity. ELISAs to serotype VP7 have been widely used and are highly sensitive, however the sensitivity has been variable in different studies, prompting for the use of more than one serotype-specific Mab to increase the sensitivity of the method (Ward *et al.*, 1991). On the other hand, ELISAs for VP4 serotyping have been of limited use, presumably due to the antigenic complexity of this protein (Coulson, 1993; Padilla-Noriega *et al.*, 1998). VP4 and VP7 differ in their antigenic structure, exposure on the surface of the virion, conformational flexibility, immunogenicity, role in virus entry to the target cells, and the potency and mechanism of neutralization by antibodies.

Studies of neutralization escape mutants of the G3 simian rotavirus SA11, selected with anti-VP7 Mabs, identified single amino acid substitutions in 3 regions, A, B, and C, localized at amino acids 87-96, 145-150, and 211-223, respectively (Dyall-Smith *et al.*, 1986). Further studies with RVA strains of different G serotypes identified the variable regions designated A and C as major targets recognized by anti-VP7 neutralizing Mabs that are serotype-specific, or cross-reactive (Hoshino et al., 1994; Kobayashi et al., 1991). The breadth of serotype recognition was affected by valency, since the IgM Mab 57-8 has far broader neutralizing range that IgG Mabs (Mackow *et al.*, 1988a). The epitopes recognized by neutralizing Mabs are considered to be operationally related if the escape mutant selected with one Mab acquires resistance to a second Mab. By analysis of the cross-neutralization patterns of several anti-VP7 Mabs with their neutralization escape mutants, the antigenic regions A and C have been shown to be operationally related, and collectively constitute a single large neutralization domain (Morita *et al.*, 1988; Taniguchi *et al.*, 1988a).

Recent analysis of the high resolution structure of VP7 allowed grouping various epitopes identified by Mab escape mutations at intersubunit boundaries designated 7-1a and 7-1b, of the calcium dependent VP7 trimers, corresponding to antigenic regions A and C, respectively. Alternatively, epitopes recognized by neutralizing anti-VP7 Mabs were identified at intrasubunit boundaries designated 7-2, corresponding to antigenic region B. Mabs that bind the 7-1 region are partially dependent on bivalency for neutralization, and Mabs that bind epitopes in the 7-2 region are completely dependent on bivalency for neutralization. In general anti-VP7 Mabs neutralize by cross-linking VP7 trimers (Aoki *et al.*).

The mechanisms of neutralization by Mabs that bind at intersubunit or intrasubunit boundaries of VP7 trimers seem to be different. Mabs that recognize intersubunit boundaries inhibit uncoating of the virion outer layer (Aoki *et al.*; Ruggeri & Greenberg, 1991). Mabs that recognize intrasubunit might interfere with VP4 conformational changes needed for viral entry (Aoki *et al.*).

Anti-VP7 Mabs have been produced that neutralize specific G serotypes by neutralization assays (Coulson *et al.*, 1986; Heath *et al.*, 1986; Shaw *et al.*, 1985; Taniguchi *et al.*, 1987). The structure of the G3-specific neutralizing Mab 4F8 bound to the surface of the G3 strain RRV has been determined (Aoki *et al.*). 4F8 Fabs bind to the intersubunit region 7-1 of VP7 trimers on the surface of the virion. Other serotype-specific Mabs bind the same region on VP7, as determined by the location of mutations that escape neutralization, hence suggesting that binding to the interface of VP7 trimers by divalent Mabs is needed for neutralization by serotype-specific Mabs.

Several sets of serotype-specific anti-VP7 Mabs have been used in ELISAs to detect RVA serotypes G1 to G4 (Coulson *et al.*, 1986; Heath *et al.*, 1986; Shaw *et al.*, 1985; Taniguchi *et al.*, 1988a). The specificity of some of these Mabs has been confirmed by comparison with conventional neutralization tests (Urasawa *et al.*, 1988; Ward *et al.*, 1991). Further development of Mabs that specifically neutralize other RVA serotypes, like G6, G8, G9, and G10, have been useful to increase the coverage of serotyping ELISAs to detect emerging or zoonotic RVA infections (Coulson et al., 1999; Keklar & Ayachit, 2000). Serotype-specific ELISAs have been used in numerous studies to determine the prevalence of different G serotypes in different parts of the world (Beards et al., 1989; Keklar & Ayachit, 2000;

Urasawa et al., 1989). Serotype G1 is the most important serotype in humans throughout the world, accounting for more than 50% of all infections, and other serotypes of the Wa-like family, *i. e.* G3, G4, and G9, are also important, together with the single G2 serotype of the DS1-like family (Santos & Hoshino, 2005). The prevalence of different G serotypes differ by geographic region in the same epidemic season, and it also changes in the same area in consecutive epidemic seasons (Urasawa *et al.*, 1989).

The sensitivity of serotype-specific Mabs to detect all RVA strains in different epidemiologic studies has been variable. Because of epitope variation between RVA strains, the use of several Mabs directed at different epitopes may increase the sensitivity of the method (Ward *et al.*, 1991).

A study of the repertoire of neutralization epitopes on VP4 was done by sequencing the VP4 gene of RVA strain SA11 selected after 39 passages in the presence of hyperimmune anti-SA11 serum. The antiserum selected many mutations in the largest variable region of VP4, localized at amino acids 92-192 in VP8*, and in four positions in VP5*, one in amino acid 393, close to the variable region localized at amino acids 384 to 388, and three in amino acids 453, 588, and 736 (Gorziglia *et al.*, 1990). Studies of neutralization escape mutants selected with neutralizing anti-VP4 Mabs have identified mutations in VP8*, within the variable region of VP8* or very close (Mackow *et al.*, 1988b; Padilla-Noriega *et al.*, 1995; Zhou *et al.*, 1994). Fewer neutralization escape mutants are localized in the second cleavage product of VP4, VP5*, some in the segment 385-392, and also in positions 305, 428, 433, and 494 (Kobayashi *et al.*, 1990; Padilla-Noriega *et al.*, 1995; Taniguchi *et al.*, 1988b). By analyzing the cross-neutralization patterns of several anti-VP4 Mabs with their neutralization escape mutants, 3 independent neutralization domains were identified in human RVA KU, one in VP8* and two in VP5* (Kobayashi *et al.*, 1990). One of the domains in VP5* of KU has been identified only in this strain, using a single Mab, YO-2C2, that selects a neutralization escape mutant in position 305. The same approach was used to study the antigenic structure of the human rotavirus ST3, with a panel of neutralizing Mabs that included 2 anti-KU Mabs that served to define neutralization domains previously established in this strain. By using this approach, 2 neutralization domains were identified in ST3, one in VP8*, and one in VP5* that is operationally related to the large neutralization domain in KU, SA11, and RRV.

From the above studies, VP4 serotypes depend on 3 operationally defined neutralization domains, 2 major domains, one in VP5* and the second in VP8*, and one minor domain on VP5*. The 2 large neutralization domains consist of several operationally related epitopes, and the minor domain consists of a single epitope defined by the Mab YO-2C2.

In one study a VP4 serotyping ELISA was used to characterize the antigenic diversity of VP4 in 569 stool specimens of individuals naturally infected with RVAs. Five neutralizing Mabs were used that had been preliminarily characterized as specific for VP4 serotypes P1A, P1B, and P2. In order to validate the VP4 serotyping ELISA, the genotype of a subset of samples was determined by PCR. Three different patterns of reactivity were found among genotype P[4] strains, corresponding to serotype P1B, and 5 different patterns of reactivity were found among P[8] strains, corresponding to serotype P1A. No genotype P[6] strain was detected in this study, corresponding to serotype P2. The conclusion was that P serotypes can be identified by the patterns of reactivity obtained with multiple Mabs used in serotype specific ELISA (Padilla-Noriega *et al.*, 1998).

5. Anti-rotavirus ELISAs

ELISAs to detect the presence of anti-RVA IgG in individuals or seroprevalence in populations have been successfully developed, using particulate rotavirus antigen. As with ELISAs to detect rotavirus antigen, the dominant antibodies in these assays are anti-VP6. Anti-rotavirus IgM and IgA ELISAs have also been developed, however the sensitivity of these assays is limited (Menchaca *et al.*, 1998; Midthun *et al.*, 1989).

In one study, purified RRV DLPs were used as antigen in ELISA to detect anti-RVA IgG in 125 serum samples from asymptomatic Galapagos sea lion (GSL) pups. Due to the lack of anti-GSL IgG, protein A-peroxidase was used to detect IgG binding to the coat antigen. The sensitivity of the assay was determined by testing 6 serum samples from adult California sea lions (CSL) and one anti-VP6 Mab that had been produced using the human Wa strain as antigen. Adult sera of species susceptible to rotavirus usually contain serum anti-RVA IgG, hence the 6 control sera from adults were expected to contain anti-RVA antibodies. The signal produced in the anti-RVA ELISA by adult sera could be competed to background levels by purified RRV antigen, thus demonstrating the specificity of the assay. Antibodies to rotavirus were detected in 22% of the GSL pups, demonstrating that rotavirus infections are prevalent at an early age in this species (Coria-Galindo *et al.*, 2009).

6. Recombinant rotavirus protein IgG ELISAs

An RVA ELISA to measure the humoral immune response to RRV VP4 and its cleavage products VP5* and VP8* was developed by coating plates with the recombinant baculovirus-expressed proteins, followed by incubation with paired serum samples from infants vaccinated with 2 RVA vaccines, RRV and RRV-derived human RVA reassortant vaccine. In experimental vaccination with live attenuated virus the serotype specificity of the infecting virus is known, thereby allowing the detection of homologous immune responses to the recombinant proteins used in the assay. The presence of anti-VP4, anti-VP5*, and anti-VP8* antibodies was then detected with an anti-IgG conjugate. Of the 44 vaccinated infants studied a high percentage seroresponded to VP4 and VP8*, but fewer infants seroresponded to VP5*, indicating that VP5* is less immunogenic than VP8* (Padilla-Noriega *et al.*, 1992). VP5* induces mostly cross-reactive neutralizing antibodies while VP8* induces mostly strain-specific neutralizing antibodies, hence the low immunogenicity of VP5* might adversely affect the efficacy of rotavirus vaccines. In addition, the failure of natural rotavirus infections to induce a strong cross-reactive neutralizing antibody response might contribute to the short duration of protective immunity, allowing repeated RVA infections throughout life.

An RVA ELISA to measure the humoral immune response to RRV VP7 was developed by coating plates with the recombinant baculovirus-expressed VP7, followed by incubation with paired serum samples from mice immunized with recombinant VP7 (Fiore *et al.*, 1995). The recombinant VP7 was not immunoprecipitated by Mabs that recognize antigenic region 7-1, which are the most potent RVA neutralizing antibodies (Ruggeri & Greenberg, 1991). This finding suggest that unlike TLPs, the recombinant VP7 would not be able to induce neutralizing Mabs to the antigenic region 7-1, due to a conformational difference between the recombinant protein alone or assembled in TLPs.

NSP2 is highly immunogenic in natural RVA infections. In one study, recombinant NSP2 from the N5 strain SA11 was used as antigen in ELISA to detect the IgG and IgA serum

antibody responses from 27 children hospitalized for primary RVA gastroenteritis. Heterotypic anti-NSP2 IgG and IgA responses were detected in 100% and 75% of children, respectively. It is of interest that the strong antibody response to NSP2 in natural RVA infections has been useful to detect IgA antibody responses by ELISA to this non-structural protein with high sensitivity (Colomina *et al.*, 1998; Kirkwood *et al.*, 2008).

7. DLP-binding ELISA, a functional assay

A DLP-binding assay was developed that captures purified DLPs from the simian RVA SA11, using recombinant SA11 NSP4 bound to ELISA plates, coupled to detection of the captured DLPs with and anti-VP6 Mab (Jagannath et al., 2006). Upon fusion to the carboxy terminus of glutathione-S-transferase (GST), the C-terminal 20 amino acids of NSP4 were identified as sufficient for DLP-binding, however a C-terminal peptide failed to inhibit the receptor activity of the full-length protein, indicating that DLP binding may somehow depend also on other parts of the protein (Au et al., 1993; O'Brien et al., 2000; Taylor et al., 1993). The N-terminus of NSP4 is relevant for DLP binding, since a deletion mutant lacking the N-terminal 85 amino acids is severely affected in this function. In contrast, ΔN72, a deletion mutant lacking the N-terminal 72 amino acids is fully competent for DLP binding. Site-specific mutations Y166, P168, and M175 in the C-terminus of ΔN72 are totally unable to bind DLPs, supporting the notion that the C-terminus is the primary DLP-binding site, while site-specific mutations in other parts of this protein, F76, Y85, and Y131, exhibit loss of binding which can be regained using higher concentrations of recombinant NSP4 bound to the plates. The dependence of binding upon NSP4 concentration may result from enhanced affinity and cooperativity, suggesting that upon binding of the C-terminus to DLPs, a conformational rearrangement generates a secondary site of interaction in the N-terminus of NSP4, resulting in enhanced affinity (Jagannath et al., 2006).

8. Rotavirus epitope-specific competition ELISAs

RVA epitope-specific competition ELISAs were developed with neutralizing anti-VP4 and anti-VP7 Mabs. These assays were used to analyze the serotype-specificity of the immune response in individuals vaccinated or naturally infected with RVA. Two basic formats have been used for RVA competitive binding ELISA, one to detect binding of Mabs to virus particles bound to the plate and another that allows virus particles to bind Mabs in solution.

To perform competitive biotinilated-Mab ELISA (CBME), the virus has to be purified and bound directly to a plate with a buffer at high pH (*i. e.* bicarbonate buffer, pH 9.6) in the presence of calcium to prevent outer layer disassembly. In order to allow competition to occur, the coated plates are incubated with serial dilutions of the competing antibody and subsequently with a biotinilated Mab. Finally, to detect the amount of biotinilated antibody bound to the viral particles in the solid phase the plates are incubated with avidin-horseradish peroxidase conjugate (Shaw *et al.*, 1987). In a variant of CBME, the epitope-blocking assay (EBA) the virus is bound indirectly to the plate by using a hyperimmune anti-rotavirus serum to the homologous virus strain. At the competition phase, the plates are incubated with serial dilutions of the competing antibody, and subsequently with the Mab of interest. Finally, the amount of Mab bound to the viral particles is determined with anti-mouse IgG conjugated to horseradish peroxidase (Matson *et al.*, 1992).

One format that allows virus particles to bind Mabs in solution, the competitive Mab capture ELISA (CMCE), is performed in plates coated with a Mab. In a separate plate, serial dilutions of the competing antibody are incubated with the viral antigen, and the antigen-antibody complexes are added to the plate containing the capture Mab. Finally, the amount of virus bound to the plates is detected in two steps, by incubation with hyperimmune anti-rotavirus serum and then with horseradish peroxidase-conjugated anti-IgG to the species used to produce the hyperimmune serum (Burns *et al.*, 1988).

The antigenic structure of VP7 from human and simian RVA strains has been analyzed by competition ELISAs. One study compared 3 different sets of neutralizing anti-VP7 Mabs for their ability to bind virus particles in CCME. The antigen used in the competition assays was matched to the antigen used to produce the Mabs that included serotypes G1 to G4. Mabs that recognize epitopes in antigenic regions A and C competed completely, indicating that regions A and C are adjacent to each other on the virus particle (Raj *et al.*, 1992). In contrast, a CBME study with the G3 strain RRV as antigen showed nonreciprocal competition between Mabs that recognize regions A and C of VP7 (Shaw *et al.*, 1987). It is possible that viral particle binding to the plates in CBME rigidifies the outer layer therefore restricting interactions that can occur in solution. From these results, a single large neutralization domain was identified in VP7 with several operationally related epitopes in antigenic regions A, B, and C.

A study of neutralizing and non-neutralizing anti-VP4 Mabs for their ability to bind SA11 particles was performed by CCME. The study included 13 Mabs produced using the P5B P[2] simian strain SA11 as antigen, and two cross-reactive anti-VP4 Mabs made to the P5B P[3] simian strain RRV and the P9 P[7] strain OSU. Of the 6 neutralizing Mabs used in competition assays, 4 selected neutralization escape mutations in VP8* (Zhou *et al.*, 1994), the single Mab 2G4 selected neutralization escape mutants in VP5* and reacted by immunoprecipitation with VP5* (Mackow *et al.*, 1988b; Mackow *et al.*, 1990), and one Mab has not been characterized. As determined by CCME, VP4 has one neutralization domain with 3 antigenic sites, 2 in VP8* and one in VP5*. The different patterns of competition among antigenic sites seem to reflect functional differences between VP5* and VP8* and overall conformational flexibility of VP4, since: i) neutralizing antibodies directed to the antigenic site 1 in VP8* enhanced binding of all non-neutralizing antibodies; ii) the single neutralizing Mab directed to site 2 in VP5* competed with all Mabs, either neutralizing or non-neutralizing; and iii) the single Mab directed to site 3 in VP8* partially competed with all other Mabs, either neutralizing or non-neutralizing. It is of interest that CCME allows conformational alterations of VP4 to occur in solution, thereby revealing that all anti-VP4 neutralizing Mabs seemed to alter the conformational structure of VP4, leading to enhanced binding by other Mabs (Burns *et al.*, 1988). By considering the specificity of the Mabs used in CCME to the cleavage products of VP4, this assay revealed that neutralizing anti-VP8* Mabs are able to allosterically alter the conformation of VP5*.

Studies of the antigenic structure of RRV VP4 by CBME were performed with 11 neutralizing and one non-neutralizing Mab. Of the neutralizing Mabs, 4 selected escape mutations in VP8*, and a single Mab (2G4) on VP5*. As determined by CBME VP4 has one neutralization domain with 2 antigenic sites, one in VP8* and one in VP5*, that is operationally related to the epitope recognized by the single non-neutralizing Mab. The different patterns of competition among antigenic sites determined by CBME seem to reflect more clearly the functional differences between VP5* and VP8* than CCME, since: i) there was no binding enhancement between any pair of Mabs; ii) the single neutralizing Mab

directed to the site denominated B in VP5* competed with only one of 10 Mabs directed to the antigenic site A. From these results, CBME, based on the interaction of RVA particles with antibodies in the solid phase, restricts the allosteric changes induced in solution by neutralizing anti-VP8 Mabs on VP5*.

9. Conclusion

The antigenic structure of RVA and the diversity of the antibody response in RVA infections have been studied using a number of ELISAs. Group/species A rotavirus ELISAs and typing ELISAs have been widely used to diagnose RVA infections and to determine the SG and serotype specificities of the field specimens. Anti-rotavirus IgG ELISAs have been used to determine the prevalence of RVA infections in human and animal populations. Recombinant RVA protein IgG ELISAs have been relevant to dissect the antibody response to individual RVA proteins, and to describe the magnitude of the antibody response to the surface proteins of the virion. Epitope-specific ELISAs have been used to analyze the repertoire of neutralizing antibodies induced by natural RVA infections or vaccination, and the antigenicity of cross-reactive and serotype-specific epitopes on the surface proteins of the virion. The knowledge gained on the serotypic diversity and the antigenic structure of RVA particles by using diverse ELISAs are relevant in the design of new vaccines to protect broadly against all relevant RVA serotypes.

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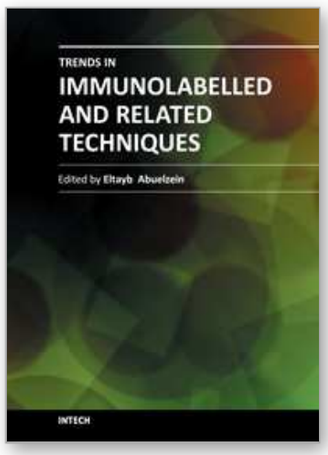

Trends in Immunolabelled and Related Techniques

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The book is coined to provide a professional insight into the different trends of immunoassay and related techniques. It encompasses 22 chapters which are grouped into two sections. The first section consists of articles dealing with emerging uni-and-multiplex immunolabelled methods employed in the various areas of research. The second section includes review articles which introduce the researchers to some immunolabelled techniques which are of vital significance such as the use of the conjugates of the Staphylococcus aureus protein "A" and the Streptococcus Spps. protein "G" in immunolabelled assay systems, the use of bead-based assays and an overview on the laboratory assay systems. The book provides technological innovations that are expected to provide an efficient channel for developments in immunolabelled and related techniques. It is also most useful for researchers and post-graduate students, in all fields, where immunolabelled techniques are applicable.

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