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IMMUNOELECTRONMICROSCOPIC TECHNIQUE TO
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WITH END STAGE RENAL DISEASE

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Charlotte A. Currie

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M. J. Patterson
Major professor

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IMMUNOELECTROMICROSCOPIC TECHNIQUE TO DETECT DANE PARTICLES IN PATIENTS WITH END STAGE RENAL DISEASE

By

Charlotte A. Currie

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ABSTRACT

IMMUNOELECTRONMICROSCOPIC TECHNIQUE TO DETECT DANE PARTICLES IN PATIENTS WITH END STAGE RENAL DISEASE

By

Charlotte A. Currie

Urine and serum samples from patients with end stage renal disease were examined by immunoelectronmicroscopy (IEM) for the presence of Dane particles. These patients had previously been included in a statewide survey to determine prevalence, among chronic maintenance hemodialysis and renal transplant patients, of hepatitis B virus (HBV) markers (HBsAg: hepatitis B surface antigen, anti-HBs: antibody to HBsAg, anti-HBc: antibody to hepatitis B core antigen, HBeAg: hepatitis B e antigen, anti-HBe: antibody to HBeAg) detectable by radioimmunoassay.

Thirty one of thirty two urines from HBsAg seropositive patients were negative for whole HBV virions by IEM. Subsequent urinalysis data indicated that the positive specimen contained occult blood, the probable source of virions detected by the procedure.

Thirty three serum samples demonstrating anti-HBc in the absence of HBsAg and anti-HBs, were all negative for Dane particles by IEM. Refinement of the pseudoreplica technique for IEM provided a highly sensitive and practical system to detect Dane particles.

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REVIEW OF THE LITERATURE

Historical Introduction of IEM

Techniques which detect, by electron microscopy, the specific binding of antibody to antigen are generally refered to as immune electron microscopy or immuno-electron-microscopy (IEM) (5,63). Currently, many procedures have been established and depending upon the nature of materials under investigation, variations of these can be employed (87). This review focuses on the development of methods of IEM for detection of viral antigens, particularly those found in clinical specimens.

Anderson and Stanley (8) in 1941, were the first to use IEM to visualize the interaction between tobacco mosaic virus (TMV) and its specific antibody. In an attempt to observe immunological reactions directly, viral particles were examined following treatment with specific rabbit antiserum, normal serum and no serum added. Representative micrographs demonstrated morphological differences between the viruses incubated with specific rabbit antiserum, and those treated with normal or no serum. Viruses which were coated with specific antibody appeared wider and more dense with a fuzzy, indistinct outer boundary. In contrast, mixtures of TMV with antisera to tomato bushy stunt, potato latent mosaic or tobacco ring spot viruses resulted in little or no adsorption of molecules onto the virus particles.

Furthermore, prolonged reaction time resulted in immune complex formation readily observed in the electron microscope.

In a review of IEM, Almeida and Waterson (5) pointed out two significant improvements to the original IEM scheme, shadow-casting and negative staining, as well as some limitations. In early transmission electron microscopy, image contrasting was a result of density differences between the supporting film and the virus particles, and definitive studies were difficult. With the advent and refinement of the shadow-casting technique (40, 52, 66, 116), three dimensional studies became possible. In this technique, the specimen was dried onto the grid, which was then placed under vacuum and metal evaporated onto the surface. Major shortcomings associated with shadow-casting included poor resolution, due to limitations imposed by granular size of the metal used, and structural damage to the specimen resulting from the trauma of pressure changes.

The introduction of negative contrasting by Brenner and Horne (21) in 1959, allowed much greater resolution and visualization of fine structure. Viral particles were embedded in an electron dense heavy metal, usually phosphotungstic acid (PTA), and contrast was thus provided by negative staining in a fashion similar to bacterial negative staining. In initial studies involving tobacco mosaic virus and turnip yellow mosaic virus, equal volumes of virus suspensions and 2.0% solutions of PTA, pH 7.4, were poured into an atomizer and directly sprayed onto carbon coated grids. This approach yielded high contrast and excellent preservation of external ultrastructure. In addition, any cavities accessible to the phosphotungstate were also stained, thus revealing some internal structure.

As part of an interpretive study on structural detail of polyoma viruses, Almeida and co-workers (6), demonstrated that variations in particle diameter were more likely to occur in shadowed preparations than in those prepared in PTA. This phenomenon was a manifestation of flattening of the virus. It appeared that in negative staining, the particles were enveloped and supported by a protein-phosphotungstate complex that helped to retain the original size and shape of the viral structure or complex under investigation. The implications, therefore, as to the usefulness of such a technique in transmission electron microscopy were substantial.

The early work with negative contrast staining provided a means to reconfirm information about morphological arrangement and orientation of TMV and turnip yellow mosaic virus. Subsequently, Lafferty and Oertelis (70) and Anderson and associates (9), in 1961, adopted this method of negative staining for the sole purpose of visualizing and studying the structure of immune complexes. Suspensions of viruses and antisera were mixed with phosphotugstate and drops were mounted on carbon-formvar-coated grids. Both groups reported that at high magnification, aggregated particles with thin fibers forming connective virion-virion bridges were observed. Individual particles, not within complexes, appeared to have fibers forming loops upon their surfaces. Lafferty and Oertelis, in an investigation into the mechanism of the stabilization reaction during the combining of virus and antibody, observed two-site attachment between rabbit antibody molecules and influenza virus. Anderson and collegues, demonstrated the specificity of agglutination of bacteriophages P22 and P221, and polio virus, with their respective homologous antisera.

Immunoelectromicroscopy has undergone considerable evolution since the establishment and incorporation of negative staining. Of particular medical importance, was the use of IEM in the elucidation of fine structure of viruses of clinical significance. Some of the viruses so visualized include: polyoma, human papilloma, and other papovaviruses (2,96), influenza virus (71), poliovirus type 1 (57), rubella virus (15), hepatitis A (43,44) and B (30) viruses, herpes simplex (114), varicella and herpes zoster (41) viruses, rhinoviruses (58), coronaviruses (59), Norwalk agent (60), rotavirus (16,46), adenovirus type 3 (91), and smallpox virus (111). For some of these viruses, laboratory isolation has not been achieved and demonstration by IEM remains an integral part of the definitive identification.

Current Methods

For routine clinical use, virus antibody reactions are not generally visualized with embedded, thin sectioned materials. Rather, virus remains suspended in its original fluid or is resuspended for processing. As few as 10^6 virus particles are sufficient to yield suitable specimens, making IEM one of the most sensitive methods of detecting antigen-antibody complexes (5). Many methods reported in the literature have the advantages of being rapid, easy and adaptable to a variety of specimen types. Among these are the classical or direct technique, agar techniques, the Derrick technique and its modifications, and decoration.

The Classical Technique

The development of the classical or direct method has been primarily attributed to Anderson and co-workers (9) and Lafferty and Oertelis (70), as a result of some of the preliminary work of Anderson and Stanley (8) and Williams and Wyckoff (116).

Basically, antiserum or purified antibody is incubated with the virus, the suspended immune complexes centrifuged, and the pellet resuspended in distilled water and mixed with PTA (neutral). The preparation is then applied to a grid that has previously been coated with a support film (usually Parlodion, Formvar, and/or vacuum evaporated carbon), excess fluid is removed by filter paper, and the grid is air dried.

For a particular virus or specimen type, it may be necessary to modify one or more steps in the technique. Almeida and Waterson (5) found that 0.1 ml of antiserum, undiluted and at dilutions of 10^{-1} and 10^{-2}, provided suitable proportions to react with 0.2 ml of a suspension of wart virus containing 10^{8} particles/ml, when mixed in 0.7 ml of physiological saline. Ratios are dependent upon the particular virus concentration and the antiserum titer. These determinations are usually made by conventional methods (e.g. RIA, CF, HI, etc.), prior to preparation for immune electron microscopy.

Incubation times vary from 30 minutes to overnight, but one hour at 37 C followed by 4 C overnight (5), has received routine acceptance. Feinstone and coworkers (43), using a one hour incubation at room temperature in studies on stool filtrates, found this quite satisfactory in the detection of hepatitis A virus.

The centrifugation step is another source of considerable variation in the classical technique. Different centrifugal forces, dependent upon the size of the virus, are used to pellet the immune complex. Almeida and Goffe (3) used 480 X g for 30 minutes to sediment wart virus (nucleic acid molecular weight, 5×10^{6} daltons) immune complexes. For smaller viruses, for example picornaviruses (nucleic acid M.W., $2\text{-}3 \times 10^{6}$ daltons), centrifugation at 27,000 X g has been recommended. Best (15) pelleted rubella (nucleic acid M.W., 4 x 10^6 daltons) virus-antibody complexes twice at 12,000 X g, and used phosphate buffered saline for resuspension.

Some investigators have omitted pelleting entirely. Norrby and associates (91) used dialysis on membrane filters in an analysis of the specificity of, and interaction between adenovirus type 3 and IgG antibodies against different viral antigenic moieties. In preliminary experiments on serological crossreactivity among picornaviruses, Chaudhary and fellow workers (29) noted a tendency of centrifugation to cause clumping in control preparations. To avoid this, a prolonged incubation in the cold (4 C) for 24-48 hours, following the initial 37 C incubation, was substituted for the centrifugation step, and good complex formation was obtained.

Specimens are mounted onto the grid in the classical technique either directly in droplets or via a modification of the method of Doane (39). A commonly employed method follows: one drop of the immune reaction mixture is added to deionized water on parafilm; a coated grid is quickly touched to the surface, one drop of PTA, pH 7.0 is placed on top; excess is removed by blotting with filter paper; the grid is air dried. Alternative stains, other than 1-3 % PTA at a pH of 6-7, include 24% sodium silicotungstic acid, pH 7 (91,109) or 0.5-1% uranyl acetate in methanol or water, pH 4.6 (10,74).

Milne and Luisoni (87) have described the advantages and disadvantages of the classical technique in a recent review of IEM. The favorable aspects included: 1) good resolution of both virus and antibody, 2) virion trapping in recognizable clumps allowing specific identification, in circumstances where the concentration of virus is below levels of detectability by conventional transmission electron microscopy, and 3) increased sensitivity due to the concentration and partial purification provided in the pelleting step. Potentially unfavorable features included: 1) the additional time required for the incubation and centrifugation steps, 2) non-specific clumping associated with centrifugation, 3) unsuitability of the initial virus suspension for immune complex formation, and 4) degradation of the quality of the image due to co-pelleting of impurities in the virus-antibody preparation.

Agar Techniques

The development of agar techniques followed from attempts to eliminate two of the problems associated with the classical technique: contamination and lengthy preparation time. Agar filtration was first used by Kellenberger and Arber (64) for particle counting by conventional electron microscopy. The basic technique included the following general steps. Solutions of Parlodion were poured over the surface of dried agar plates. Agar, prepared in the same medium as that used to suspend the viral particles under investigation, was dried in an oven to remove added water. This increased absorption capacity, while maintaining the same medium concentration of the particle suspension. The Parlodion film served as a filter through which the liquids and salts diffused into the agar gel. After drops of the preparation were spread over the surface and filtration was completed, the film was fixed and floated off at the surface of $La(NO_3)_3$. Specimens were mounted onto grids by approaching the floating membrane from below and securing it. By addition of a step including the mixing of known quantities of latex spheres with particles of unknown concentration and calculating the resulting ratios from electron micrographs, this method was suitable for particle counting.

The Agar-diffusion Filtration Method- Steere (107) showed that an agar film acts as a molecular sieve absorbing fluid together with interferring salts, macromolecules, and tissue debris of $\leq$15 nm in diameter, while retaining the larger particles on its surface. Kelen and associates (62, 63) developed this basic observation, as well as the ideas of Kellenberger and Arber, for use in IEM. Agar surfaces were employed to trap immune complexes and absorb impurities. In 1971, the agar-diffusion filtration method (ADF) (62) was introduced for demonstrating the presence of the hepatitis B surface antigen and its corresponding antibody in serum specimens. Serial dilutions of specimens were prepared in a range to include the predetermined complement fixing surface antigen or antibody titer, depending upon the assay, and equal volumes of the appropriate concentrations of serum and "detector reagents" (either antigen or antibody) were incubated in microtiter plates with U-shaped wells. Thin drops were deposited on agar covered slides, and an inverted coated grid was left floating on top. As the fluid phase of the drop containing normal serum components diffused into the agar layer, the drop gradually flattened until the grid settled onto the surface of the agar, thus trapping the antigen-antibody complexes between the two surfaces. Grids were removed with forceps and negatively stained as described above. In addition to simplicity and speed of preparation (less than one hour), this method was reported to have both high specificity and sensitivity equal to or greater than that of complement-fixation (62).

The main advantage of the ADF technique over the classical method (63) was that it incorporated partial purification and concentration in one step. Additionally, with the elimination of the blotting step of direct methods, all of the virus particles contained in the original drop of fluid became attached to the grid.

<u>The Serum-Agar Method</u> - The ADF method was modified by Anderson and Doane (7) for rapid type-specific identification of crude cell culture isolates of enteroviruses (Echo 6, 7, 9 and 11, Coxsackie A9, B1, B4, and B5, and Polio 1, 2, and 3). In this technique, agar was used as a reservoir of antibody, and the formation of immune complexes was achieved by rapid diffusion of homologous antibodies into the viral specimen. The technique included the following steps. Dilutions of antisera were added to a solution of

cooled molten agar (1%). This was transferred into wells of microtiter plates, allowed to solidify at room temperature, and coated grids were placed on the surface. One to two small drops of viral specimens were added, and the grids were air dried for 30-60 minutes, and negatively stained.

In comparison to the classical technique, the serum-in-agar technique had several practical advantages (38). It was rapid and simple to perform, required only small amounts of virus and antiserum, and the antibody-coated wells could be stored at 4 C for several months prior to their use. Also, since specific virus identification was not dependent on optimal dilutions, this technique appeared especially suitable for detection of viruses directly from clinical specimens.

Methods for Agar Gel Precipitin Lines - Several methods have been described in the literature to study the nature of the components forming precipitin lines in the Ouchterlony gel diffusion technique. Watson and co-workers (113), in an attempt to examine polyoma virus-antibody precipitates by electron microscopy, used an extraction method developed by Weintraub, Rigetli, and Townsley (115). The entire length of the precipitin line was cut from the agar plate and suspended in a drop of distilled water in a test tube. The gel was mixed into the fluid by stirring, followed by immersion in an electrosonic bath for 30 seconds. Using a platinum loop, a droplet of the suspension was placed on the coated grid and the excess blotted. The grid was washed and negatively stained. Examination of the extracted material revealed aggregates of polyoma and antibody, that had a similar appearance to those complexes formed in the direct method. Beale and Mason (13) modified this technique by freezing the fragments of agar containing the precipitin lines corresponding to the D and C antigens of polio virus type 1. The agar fragments were cut into small pieces, thawed and negatively stained. By this method, it was confirmed that the D and C lines were associated with complete and empty virus particles, respectively. Other investigators fixed and embedded precipitin lines in studies on mouse mammary tumor virus (17), bovine papilloma virus (75), and the configuration of IgM antibodies attached to polio virus type 1 (109). However, certain disadvantages were recognized in this method also. Svehag and Bloth (109) reported that impurities in the gel, together with the thickness of the antibody layer, tended to obscure specimen detail.

<u>The Pseudoreplica Technique</u> - In a study on the ultrastructure of disrupted herpesvirus capsids, Palmer, Martin and Gary (93) reintroduced the drop pseudoreplica technique, which had been described earlier. Briefly, a drop of viral material was allowed to air dry on a small block of 2.0% agarose and then overlaid with 0.5%, liquid Formvar. After excess fluid was drained, the resulting membrane, with entrapped virus particles, was floated off at the surface of

a 2.0% solution of PTA, pH 5. Copper grids were placed on the film which was retrieved using a metal peg, and excess stain was removed with filter paper. Uranyl acetate, 0.5%, was substituted for PTA by Nahmias (10) and Lee and associates (74), in studies directly examining clinical specimens of body fluids for herpes viruses.

The major advantage of this method was its rapidity, since processing required less than 30 minutes. In addition, viruses were concentrated approximately tenfold. This technique was used for rapid detection of viruses easily demonstrated by routine clinical methods, and also facilitated detection and identification of virus groups not easily demonstrable in body fluids by other methods. Nahmias (10) visualized adenoviruses, rotaviruses, papovaviruses, cytomegaloviruses, herpes zoster and herpes simplex viruses. He also reported that specimen volumes of only 25 microliters were satisfactory when examining urine, stool and fecal suspensions, oral secretions, tears, vesicular fluids, serum, cerebrospinal, ventricular and other body fluids.

Lee, Nahmias and Stagno (74) detected cytomegalovirus (CMV) particles in urines and oral specimens, obtained from symptomatic and asymptomatic infants who had congenitally or postnatally acquired CMV infections. The virus were readily detectable in specimens that had been stored or shipped at 4 C for several days, again supporting the value of pseudoreplication as a rapid diagnostic tool.

The Derrick Technique

Derrick (35, 36, 37) described a method in which grids coated with specific antiserum were used for electron microscopy of plant viruses. Potato virus Y (PVY), tobacco mosaic virus (TMV), cucumber mosaic and tobacco etch viruses were specifically attached to, and concentrated on, specimen grids coated with a film of specific antiserum. One of the advantages of this technique (87) was that the grids could be washed at two points, after antibody was adsorbed to the grid and after virus was adsorbed to the antibody. These washes helped to remove salts and other impurities, without disturbing antibody or viral antigen.

Coated grids were floated for 30 minutes at 24 C on antiserum diluted in 0.05 M Tris buffer, pH 7.2. Immediately after a wash in the same buffer, the grids were floated on 0.1 ml drops of crude virus extracts for one hour at 24 C. Grids were washed in the same buffer used to prepare the viral extracts (Tris buffer containing 0.9% NaCl), followed by a wash in water, and were air dried and shadowed. Positive staining with 1.0% uranyl acetate could be substituted for shadow-casting. In a quantitive assay, Derrick (37) reported that approximately 50 times more TMV and 20 times more PVY were trapped on grids coated with their specific antisera, than on control grids which were coated with unrelated antibody. Other refinements in the same study showed that the number of entrapped virus particles could lengthening the incubation time of the virus step, or by raising the incubation temperature to 37 C.

The Modified Derrick System - Milne and Luisoni (87) simplified the Derrick procedure and shortened incubation times so that processing was completed in less than 25 minutes, rather than over 90 minutes. Phosphate buffer, 0.1 M, pH 7.0 was used instead of Tris buffer, and uranyl acetate was replaced by PTA. Diluted serum was incubated on a grid (held in forceps) in a humid box for 5 minutes at 24 C. The grid was washed with 20 consecutive drops of buffer followed by 30 drops of distilled water, and stained. Thirty to fifty times more plant viruses, from crude and purified preparations, were trapped on grids coated with homologous antiserum, than on those coated with unrelated antiserum.

The Derrick Technique Combined with Decoration - Although the terminology was introduced by Milne and Luisoni, the technique of decoration was first described by Yanagida and Ahmad-Zadeh (118) in a study to locate bacteriophage structural proteins. Virus was adsorbed onto coated grids, which were washed and floated (specimen side inverted) on diluted antiserum. Antigenic sites were thus coated or "haloed" by antibody molecules (decoration). Clumping was prevented since the virus particles were fixed in place and not free to move. This method has also been used to visualize specific antigenic sites on viral capsids (110, 117, 118).

To confirm a specific immune reaction between plant viruses and their respective homologous antisera, Milne and Luisoni decorated each particle first trapped by the Derrick system (87). The procedure required about 35 minutes and trapped 50 times more virus particles than the control (coated with normal serum). A grid was incubated with diluted antiserum for 5 minutes, washed with 20 drops of phosphate buffer, incubated with the virus for 15 minutes and washed again with 20 drops of phosphate buffer. Another drop of diluted antiserum was added and the grid was incubated 15 minutes longer. This was followed by the routine wash of buffer and distilled water, as previously described, and the grid was stained for examination.

This method had the shared advantages of both systems. Decoration was useful in high resolution work, in which antibody attachment sites must be clearly visible. It was also an attractive method in those situations in which samples contained components which interfered with immune reactions. Modified Derrick methods could be used on crude materials and were useful in trapping viruses present in low concentrations (87).

The use of many electron microscopic techniques as research and diagnostic tools, is becoming increasingly more practical. The transmission electron microscope has contributed to many areas of clinical medicine such as hematology, nephrology, gastroenterology, and the study of metabolic disease, as an aid in diagnostic procedures, a guide to treatment, and the characterization of pathogenic mechanisms. More specifically, viral diseases can be diagnosed in minutes, under ideal conditions, from the time the sample is taken until identification under the electron microscope (24). In those circumstances in which in vitro cultivation of some viruses has not been possible, IEM has proved to be invaluable. One such elucive virus is hepatitis B virus.

Hepatitis B

Despite intensive efforts to characterize the agents causing different forms of hepatitis, the understanding that hepatitis B was a separate entity did not become clear until the 1930's. At that time the disease was referred to as catarrhal or homologous serum jaundice, and was recognized as a complication of injections and serotherapy (45, 101). Early epidemiological studies suggested its transmission was associated with the inoculation of blood products from one individual to another, but little importance was attached to observations of transfer by physical contact from servicemen (88) and experimentally infected volunteers (20) to their spouses. Following the transmission experiments at the Willowbrook State School for the mentally retarded (69), the concept of transmission by modes other than serum took on new significance. Krugman, Giles and Hammond, in 1967, reported evidence that two clinically, epidemiologically and immunologically distinct types of infection (subsequently designated hepatitis A and B) were occurring in the institution. Plasma (designated MS-1) from a patient suffering from a first attack of hepatitis, caused disease after an incubation period of approximately 30 days in experimental groups. Other experimental groups received plasma (MS-2) from the same patient during a second attack of hepatitis, and developed hepatitis following a much longer incubation time of about 90 days. The investigators suggested that MS-1 was infectious or short-incubation hepatitis (hepatitis A) and MS-2 was serum or long-incubation hepatitis (hepatitis B). Subsequent studies at Willowbrook defined other properties of these two agents, one of the most significant being that hepatitis B could be transmitted by oral ingestion of serum (69). In 1968, Prince (99) recognized a relationship between the Australia antigen, a lipoprotein discovered by Blumberg, Alter and Visnich (18) in 1965, and the viremic phase of hepatitis B. The antigen, later renamed Hepatitis B surface antigen (HBsAg), received its first name following its detection in the sera of Australian aborigines, during a study of varients in serum proteins. Prior to Prince's studies, the association of Australia antigen with hepatitis B had been suggested by Blumberg and co-workers, but this was not fully established (19). Giles (51) in 1969, tested sera from patients in the Willowbrook studies for HBsAg and showed the complete correlation between the presence of the antigen and MS-2 infection.

HBsAg has been used as a marker of hepatitis B infection in many clinical and epidemiological studies. These studies have provided a more complete understanding of the nature of the virus, in spite of the fact that all efforts to cultivate it in vitro have been unsuccessful. In 1970, Dane, Cameron and Briggs (30) identified the hepatitis B virion (subsequently called the Dane particle) by immune electron microscopy of HBsAg positive serum. The following year Almeida, Rubenstein and Scott (4) described the nucleocapsid core or hepatitis B core antigen (HBcAg), also by using IEM. Experiments to develop animal models by Maynard and coworkers (84), showed that chimpanzees were susceptible to infection, although clinical effects are minimal.

Viral Characteristics - Three morphological forms are associated with the hepatitis B virus (HBV): 1) the Dane particle, 2) the spherical form of HBsAg, and 3) filamentous or tubular forms of HBsAg. The complete virion, or Dane particle, exists as a complex double layered structure measuring 42 nm in diameter (30). It has a 27 nm core containing circular, double stranded DNA with a molecular weight of 1.6×10^6 daltons, and DNA polymerase. Single stranded regions may be present on the DNA molecule (72). Studies suggest the core has icosahedral symmetry, subunit capsomeres of variable size (appx. 4 nm) and 8-10 nm long spikelike structures projecting from the surface (23). The existence of subpopulations of Dane particles has been demonstrated (61) in cesium chloride gradients. Full core particles containing DNA have buoyant densities of 1.36 g/ml, and empty cores, densities of 1.30 g/ml.

A 7 nm envelope surrounds the core, which is antigenically distinct from antigens on the 27 nm core (HBcAg). This material, the hepatitis B surface antigen

(HBsAg), is produced in excess by infected hepatocytes and is released into the blood as spherical particles and as tubular or tadpole-like filaments. Both forms have diameters between 17 and 25 nm (averaging 22 nm), but the tubular forms exhibit a variable length from 100-700 nm. It has been suggested that the tubular forms are stacked discs, which dissemble into the spherical particles under appropriate conditions (54).

The constitution of HBsAg has been studied extensively (67). Estimates of its molecular weight range between 2.4 to 4.6×10^6 daltons (27, 65). It has a density of 1.21 g/ml in CsCl (80), and has the chemical characteristics of lipoprotein. Approximately 20-30% is lipid (cholesterol, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine are predominant) (108), and 5% is carbohydrate (28). A protein content of 40-60% has been suggested (56), with 14% tryptophan (102) and large amounts of proline and leucine.

HBsAg is antigenically complex and its immunoreactivity is not fully established (67). It appears to carry a common or group-specific determinant designated "a" (76) on its surface, and at least two other subdeterminants "d" or "y" (73) and "w1", "w2", "w3", "w4", or "r" (12). Eight subdeterminant categories or subtypes are recognized, ayw1, ayw2, ayw3, ayw4, adr, ayr, adw2, adw4, as well as two mixed subtypes adyw and adyr (67). There appears to be no correlation between subtype and severity of illness (50) and the distribution is probably related to geographic factors (86), and thus is of epidemiological importance.

Antibodies against HBcAg and HBsAg, are designated anti-HBc and anti-HBs, respectively. A third antigen, immunologically and chemically distinct from HBcAg and HBsAg, was identified by Magnius and Espmark in 1972 (80, 81). Hepatitis B e antigen (HBeAg) appears to be of viral origin, and closely associated with HBV DNA polymerase and circulating Dane particles in serum (53). It exists in serum as a complex of three molecular species of soluble proteins varying in size. One component has been characterized and has a buoyant density of 1.29 g/ml in CsCl (79), and an S-value of 11.6 (89). In urine, HBeAg is a heat labile soluble protein with a buoyant density of 1.34 g/ml, S-value of 4 and molecular weight of approximately 40,000 daltons (89).

<u>Humoral Immune Response to HBV Infection</u> - The appearance and disappearance of HBc-, HBs-, and HBe-antigens and the appearance of their respective antibodies, generally follow a characteristic pattern, during the course of and convalescence from most HBV infections (77). The first seromarker to appear is HBsAg, followed by HBeAg, and both reach maximum titer at approximately two to three months postinfection. Anti-HBc is the first antibody produced by the host. It appears about three months after infection, usually during the early acute phase of illness. It may

persist for several years, and is considered to be one of the best markers of current or past infection. Disappearance of the IgM class of anti-HBc probably indicates HBV clearance, while persistence may signal continuing viral replication and chronic disease (33). Seroconversion to anti-HBe follows the appearance of HBeAg and precedes the disappearance of HBsAg. Eventually, anti-HBs, the protective antibody, is elicited and the host convalesces. Anti-HBs is also long lasting, persisting similarly to anti-HBc, and confers immunity to future infections.

There is an "immunologic window period" between the disappearance of detectable HBsAg and appearance of anti-HBs in which anti-HBc and anti-HBe, or anti-HBc only, may be detectable. Studies (55) have shown that the patient's blood may be infectious during this period.

The antigen-antibody system markers have been related to the status or severity of infection. HBsAg seropositivity indicates current infection and presence of HBeAg suggests severe liver damage, high infectivity and a relatively poor prognosis. The presence of Dane particles or HBcAg are also an indication of continuing viral replication and infectivity. Testing for these markers however, is not routine, due to limitation in the sensitivity of detection methods (33). Failure to seroconvert to anti-HBe during the carrier state implies on-going active infection and chronic liver disease is a probable outcome (90). The presence of anti-HBe may indicate a reduced level of infectious virions because of decreased viral replication. In the acute stage, the presence of anti-HBe indicates the beginning of convalescence, and in the chronic HBsAg carrier, its appearance is often associated with improvement in the patient's underlying liver disease (33).

Transmission - Human chronic carriers are the main reservoir of hepatitis B virus. Plasma, serum and their products serve as the principle source of infection; however, it is clear that transmission is accomplished by routes other than direct inoculation. As reviewed by Maynard (83), several mechanisms are currently recognized.

Direct percutaneous parenteral inoculation of contaminated serum, plasma or transfusion of infective blood or blood products remains the major mode of transfer. Included also are other procedures involving use of needles, such as tattooing, acupuncture, ear-piercing, indiscriminate drug use, improper decontamination of syringes and needles during mass immunization, and accidental needle punctures among health care employees. Second in importance as a route of transmission, is non-needle percutaneous transfer of infective serum or plasma through minute breaks in the skin. Larger skin lesions from impetigo, burns, or scratches serve as entry sites as well.

Mucosal surfaces, especially oral and conjunctival mucosa, appear to provide the third portal of entry for HBV from infective serum or plasma. Serum/blood-oral and serum/blood-eye mechanisms are implicated in certain occupational groups. At high risk are hospital personnel, laboratory technicians and dentists.

The fourth mechanism, the introduction of infectious body secretions onto mucosal surfaces, or through breaks in skin, is a more recently recognized route of infection. Secretions have been studied from patients with acute disease and chronic carriers. HBsAg has been detected in feces (112), urine (11, 112), saliva (112), nasopharyngeal secretions (92), menstrual blood (85), vaginal secretions (32), bile (97), breast milk (78), cerebrospinal fluid (34), sweat (22), and synovial fluid (98). Recently, HBsAg positive saliva and semen have been shown to cause infection by both of the parenteral transmission routes in experimental studies in gibbons (104). This substantiates epidemiological data implicating sexual activity with transmission and high rates of hepatitis B among prostitutes (94), homosexuals (48), and spouses of partners with the disease (103).

Indirect transfer of infective serum or plasma via inanimate objects or arthropods, has been suggested as the fifth specific mode of transmission. HBsAg has been detected in mosquitoes in Africa (100), however attempts experimentally to transfer the disease from mosquitoes to chimpanzees have been unsuccessful (14).

Airborne transmission may be a possible but comparatively insignificant mechanism of transfer. Similarly oral transmission via saliva is probably infrequent (104).

Hepatitis in renal dialysis units - Outbreaks of hepatitis B in hemodialysis units were recognized in the 1960's as a major hazard affecting both patients and staff (82). Because dialysis patients may receive a renal transplant, and move back and forth between the dialysis and transplant populations the disease has also been associated with transplantation units (47).

Studies (49, 82) have shown that the virus is usually introduced into dialysis units through blood transfusions. Efforts to prevent this entry have included restricted blood transfusion (26) and the substitution of reconstituted frozen red cells for whole blood, when transfusion of patients or priming of dialysis machines is necessary (25). The virus may also enter the hemodialysis unit via new patients or staff members who are infectious, or by the infection of patients or staff in other parts of the hospital followed by transmission into the unit, although these are less common mechanisms (68).

Evidence suggests that there are multiple routes of infection from infectious carriers and acutely ill viremic patients to fellow patients or staff, once the virus has been introduced into the unit (68). Strongly implicated are

malfunctioning dialysis machines (e.g. rupture or leaks in dialysis membranes) (105), accidental needle penetration among the staff, and transmission of blood through breaks in the skin (106). Other investigators have speculated that contaminated surfaces in the unit or airborne transmission may play some role (1, 31, 42). Household contacts, especially spouses, are also at risk of developing a hepatitis B infection (95).

As previously mentioned, HBsAg has been detected with some frequency, in urine of HBsAg seropositive patients. Recent development of a very sensitive solid phase radioimmunoassay (RIA) for HBeAg has made possible the detection of HBeAg in urine of HBsAg seropositive endstage renal disease (ESRD) patients (89). One purpose of this study, was to assess the infectivity of ESRD patient urines by detection of Dane particles by immunoelectronmicroscopy.

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ABSTRACT

HEPATITIS B e ANTIGEN AND MORPHOLOGIC FORMS OF HEPATITIS B VIRUS IN URINES FROM PATIENTS WITH END STAGE RENAL DISEASE

By

C.A. Currie, I.K. Mushahwar, T.J. Kelly, G.H. Mayor, and M.J. Patterson

Urines from hepatitis B surface antigen (HBsAg) persistently seropositive patients with end stage renal disease were studied for markers of hepatitis B e antigen (HBeAg) and antibody to HBeAg (anti-HBe) by radioimmunoassay (RIA) and for Dane particles by immunoelectron-microscopy (IEM). Fifty-one percent of the HBsAg positive urines were positive for HBeAg and 14 percent for anti-HBe. The over-all prevalence of HBeAg and anti-HBe was 59 percent. Some urines were positive for HBeAg by RIA while not having detectable HBsAg. When serial urines from these patients were inspected, HBe antigenuria was an intermittent and persistent marker. Despite the presence of HBsAg and HBeAg in the urines, careful examination by transmission electron microscopy in samples prepared by the pseudoreplica technique for IEM were negative for complete hepatitis B virions.

HBe ANTIGEN AND MORPHOLOGIC FORMS OF HEPATITIS B VIRUS IN URINES FROM PATIENTS WITH END STAGE RENAL DISEASE.

By

C.A. Currie, I.K. Mushahwar, T.J. Kelly, G.H. Mayor, and

M.J. Patterson

From the Departments of Microbiology and Public Health, and Medicine, Michigan State University, East Lansing, Michigan, and Abbott, Laboratories, North Chicago, IL.

This research was supported by a grant from the Michigan Department of Public Health.

Reprint requests should be sent to M.J. Patterson, Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824

INTRODUCTION

The finding of hepatitis B e antigen (HBeAg) in the presence of hepatitis B surface antigen (HBsAg) in serum, generally correlates with hepatitis B virus (HBV) infectivity (1,2,3). Patients on chronic maintenance hemodialysis or recipients of renal transplants have been shown to have HBsAg and HBeAg in serum (4,5). Additionally, almost all such patients persistently HBsAg seropositive have detectable HBsAg in their urines when two or more randomly timed urine samples are assayed (6). The link between surface antigenuria and infectivity in unclear but transmission of HBV by other fluids has been documented (7), and HBV remains a significant risk for patients with end stage renal disease. Thus, it is important to evaluate urine as a vehicle of HBV transmission.

Accordingly, in this study, urines from renal dialysis and transplant patients who are persistantly HBsAg seropositive were examined for HBeAg and Dane particles. A sensitive solid phase radioimmunoassay (RIA) was used for HBeAg detection and immunolectronmicroscopy (IEM) was employed for virion detection.

MATERIALS AND METHODS

Patients and Specimens - Fifty-one urine samples were obtained from end stage renal disease (ESRD) patients in Michigan. These patients were selected from a population previously surveyed to determine prevalence of various hepatitis B serum markers (HBsAg, anti-HBs, anti-HBc, HBeAg, and anti-HBe) detectable by radioimmunoassay (RIA) (5,8,9).

Twenty-four urine samples examined in this study were obtained from patients on chronic maintenance hemodialysis, while 27 samples were from renal transplant recipients. All samples were obtained from patients who were HBsAg sero-positive at the time of sampling. Multiple serial urine samples were obtained from ten patients, while 23 patients contributed single specimens. All urines were coded and stored at -20 C until processing.

Urine specimens were evaluated for HBsAg by RIA (AUSRIA II, Abbott Laboratories, North Chicago, Illinois) and for the presence of occult blood or proteinuria by colorometric tests (Multi Stix, Ames Company, Elkart, Indiana).

Forty-four (86 percent) of the 51 urines were evaluated for HBeAg and anti-HBe by RIA (HBe, Abbott Laboratories, North Chicago, Illinois) and 32 (63 percent) of the 51 were selected for IEM.

RIA - HBeAg and anti-HBe were monitored using sensitive solid phase radioimmunoassays. To detect HBeAg, unconcentrated urine (0.2 ml) was incubated overnight at room temperature with an anti-HBe pre-coated polystyrene bead. Bound antigen was quantitated using 0.2 ml ^{125}I-labeled antiHBe, incubated for 4 hours at 45 C with the washed bead. Urine samples with $\geq$ 2.1 times the net negative control counts per minute (cpm) of ^{125}I were presumptive positives for HBeAg. These specimens were then confirmed as positive for HBeAg by specific antibody inhibition.

Anti-HBe was detected using a two-step inhibition assay. Urine (0.1 ml) was incubated for 2 hours at room temperature with known HBeAg (0.1 ml), quantitated to yield 5-10 x 10^3 cpm at the end of the assay in the presence of urine negative for anti-HBe. Following this initial incubation step, an anti-HBe precoated polystyrene bead was added and the procedure continued as for HBeAg above. Urine samples were positive for anti-HBe when cpm were <50 percent of the net negative control cpm.

Clarification Procedures - Prior to treatment for immunoelectronmicroscopy (IEM), all urines were clarified by ultracentrifugation (Beckman L3-50) at 20,000 rpm and 4 C for 30 minutes, using a fixed angle rotor (Beckman 50 Ti). Alternative methods of clarification included centrifugation at 3000 rpm (Beckman L5-50) for two hours at 4 C or filtration through a 0.2 micron filter unit (Nalge Company, Rochester, New York) with applied vacuum.

Controls for IEM - Positive controls were obtained by adding serum, positive for HBsAg by RIA and known to contain Dane particles, to aliquots of urine. The positive serum was diluted 10^{-2}, 10^{-3}, and 10^{-4} in urines from both a HBsAg seronegative individual with normal renal function and an ESRD patient with proteinuria. Normal urine, without serum added, was employed as the negative control.

Agar - The agar blocks used in the pseudoreplica technique were prepared with Bacto-Agar (Difco Laboratories, Detroit, Michigan). Agar was dissolved in distilled water to a final concentration of 2 percent (w/v). This was autoclaved, delivered to standard size Petri plates, and refrigerated until needed. Blocks (approximately 12 x 12 mm) were cut with a scalpel and allowed to warm to room temperature before use.

Buffers - Sterile 0.01 M phosphate buffered saline (PBS), (0.01 M phosphate, 0.13 N NaCl) pH 6.9 was the diluent in all procedures in which serial dilutions are indicated. In the modified Derrick system, 0.1 M phosphate buffer, pH 7.0 was included as a routine wash.

Plastic Support Film - Parlodion (Ted Pella, Inc., Tustin, California) was used to manufacture specimen support films in all procedures. Film solutions (0.05 percent) were made by dissolving broken nitrocellulose strips (Parlodion) in amyl acetate. After being tightly covered with parafilm and wrapped in aluminum foil, the solutions were placed in a dessicator for 48 to 72 hours to allow complete dissolution of the plastic. The solution was then filtered through Whatman #1 filter paper and used immediately, either to coat grids directly or to be used in the pseudoreplica technique.

To coat grids, the Parlodion solution was cast upon clean microscope slides and floated off onto distilled water. Four hundred mesh copper grids (Ted Pella, Inc., Tustin, California) were placed upon the membrane. The entire film was retrieved by parafilm, placed in Petri plates and stored in a dessicator.

Negative Stain - A two percent solution of phosphotungstic acid was adjusted to pH 6.0-6.7 by titrating with 5 N KOH. Following filtration through a 0.2 micron Nalgene filter unit (Nalge Company, Rochester, New York), 0.05 percent powdered bacitracin (Sigma Chemical Company, Saint Louis, Missouri) was added to facilitate spreading.

Pseudoreplica Technique - Grids were prepared for IEM according to a modification of the pseudoreplica technique described by Palmer and associates (10).

Antiserum - Dilutions of hyperimmune hepatitis B immune globulin (HBIG)(John R. Kateley, Jr., Ph.D., Department of Laboratories, E. W. Sparrow Hospital) were prepared in 0.01 M PBS for urine reaction mixtures prior to concentration for electron microscopy. Measurements of antibody titers showed that undiluted HBIG contained approximately 5-6 x 10^3 RIA units of anti-HBs and small amounts of anti-HBc.

Immune Complex Reaction Mixure - Two drops of diluted HBIG were mixed with an equal volume of clarified urine in 12 x 75 mm test tubes and covered with parafilm. Tubes were incubated for one hour, 37 C. Alternative incubation methods evaluated, included refrigeration at 4 C overnight following incubation for one hour at 37 C, or incubation for one hour at room temperature (24 C) only.

Grid Preparation - One drop of each reaction mixture was deposited on an agar block, positioned at a corner of a microscope slide, and spread with the edge of a Pasteur pipette. Excess fluid was drained from the agar onto filter paper by tilting the slide, and the remaining thin fluid layer was allowed to air dry. One drop of liquid Parlodion was carefully delivered and spread by slowly rotating the slide. This was drained as above and the film was air dried resting at an angle of ninety degrees. The entire membrane was floated off onto the surface of the negative stain solution (PTA). Two four hundred mesh copper grids were placed on areas with an even appearance using forceps, and the film was removed with parafilm. To avoid pooling of PTA, excess moisture was blotted with filter paper. Grids were removed when completely dry and examined by transmission electron microscopy (TEM).

Modified Derrick System - The technique to form immune complexes directly on grids, described by Derrick (11) and modified by Milne and Luisoni (12), was further modified here to detect HBsAg and Dane particles in urine.

One drop of HBIG was delivered to a grid held by forceps and previously coated with Parlodion. For each specimen, dilutions of HBIG formed the initial layer. After a five minute incubation period in a 37C humid box, the grid was washed with 20 consecutive drops of 0.1 M phosphate buffer, pH 7.0, and drained by touching the grid edge with filter paper. One drop of urine was placed on the grid, incubated under the same conditions for fifteen minutes, washed and drained as above. One drop of HBIG (10^{-2} dilution) was layered on top, creating a sandwich effect, and the grid was incubated 5 minutes as above. The grid was again washed with 20 drops of buffer followed by 30 drops of filtered distilled water. The grid was then drained and negatively stained with one drop of PTA. Excess fluid was removed with filter paper, and the grid was air dried.

Transmission Electron Microscopy - Grids were examined in a Philips 300 Transmission Electron Microscope operated at 80 kV. To determine the presence of Dane particles, 5 to 6 squares per grid were scanned at magnifications of approximately 25,000x and 67,000x for a total of 20 to 30 minutes. Micrographs were taken using Kodak Electron Image Film 4463 or Kodak Electron Microscope Film 4489 (Eastman Kodak Co.).

Negatives were developed in Kodak D-19 Developer and fixed in Kodak Rapid Acid Fixer. After a 20 to 30 minute wash in running water, negatives were briefly immersed in Kodak Photo-Flo and dried in a hot air drier.

RESULTS

RIA - The results of HBeAg and anti-HBe testing on the 44 urines using solid phase radioimmunoassays are as follows. Nineteen of the 37 HBsAg positive urines had concomitant HBeAg. Five had anti-HBe, and 13 had neither marker. In no instance were HBeAg and anti-HBe detected simultaneously. Unlike the patterns observed in serum, where HBsAg always coexists with HBe or anti-HBe, two of seven urines negative for HBsAg showed HBeAg. The remaining five were negative for both e antigen and antibody.

When the total ESRD urines were separated into two subgroups, urines obtained from patients on dialysis and those who received transplants, the results shown in Appendix 1 were obtained. Of 18 urines from dialysis patients, 12 had HBsAg. Nine of these 12 had HBeAg and 2 anti-HBe. One of the 6 HBsAg-negative urines had HBeAg. Among urines obtained from transplant patients, 25 of 26 were surface antigen positive, with 10 of these having detectable HBeAg and 3 others anti-HBe. The one HBsAg-negative urine was HBeAg-positive.

The over-all prevalence of HBe markers in urines from ESRD patients (59 percent) was not significantly different when patient subgroups were examined; 67 percent of the dialysis urines and 54 percent of transplant urines demonstrated one or the other marker. No significant differences in urine frequency ratios (HBeAg to anti-HBe) were seen among dialysis versus transplant patient urines.

Ten ESRD patients were followed by collection of serial urine samples 5 to 16 months after initial urine sampling. The results are shown in Appendix 2. Tests for HBsAg in these urine samples were positive in all but one instance (designated by the open circle). Urines designated with the letter D were taken during a time when the patient was being maintained on chronic hemodialysis. Where not marked, the patient has received a kidney transplant. Some patients have moved from the dialysis to the transplant population during the course of the study. HBeAg and anti-HBe results are shown within the symbols. Appearance and disappearance of detectable HBeAg in urine is seen (top patient) as well as the disappearance of detectable levels of pre-existing anti-HBe (third patient, coincident with movement from the dialysis to the transplant population).

Development of IEM Technique - Results of the examination by IEM of known HBsAg-positive urines using modifications of the pseudoreplica and Derrick techniques are shown in Table 1. HBsAg was readily detected in patient urines using the pseudoreplica technique following incubations for one hour at 24C or 37C. In control preparations, Dane particles, as well as HBsAg, occurred singly and in complexes with or without the excess HBsAg spherical and filamentous morphological forms. Fewer complexes were formed during room temperature incubations, and single particles of HBsAg appeared to be surrounded by hazy material. Incubation at 37 C followed by overnight incubation at 4 C, was an unsatisfactory procedure, since preparations appeared cloudy and any complexes that may have been formed were totally obscured.

When incubated for one hour at 37 C, Dane particles and excess HBsAg were abundant and easily detected in 10^{-2} and 10^{-3} dilutions of positive control serum. Fewer morphological forms were visualized at the higher dilution (10^{-4}), although they were still present in moderate to abundant amounts. At all three dilutions, most virions were highly complexed with antibody, but the most dilute serum had more antibody coated single virions and HBsAg particles (Fig.1A). Representative micrographs demonstrating all three morphological forms (ie. Dane particles, spherical and tubular forms) in control urines are shown in Figures 1B, 1C, and 1D.

Fewer immune complexes were formed in patient urine using the Derrick system. For all specimens, two dilutions of HBIG (10^{-1} and 10^{-2}, representing 500-600 and 50-60 RIA units) were chosen to form the initial layer, and more virions and/or HBsAg were trapped on grids coated with the more concentrated antiserum. While control preparations were comparable, patient specimens did not appear as clean as those processed by pseudoreplication.

TABLE 1: COMPARISON OF IEM TECHNIQUES FOR EXAMINATION OF HBsAg POSITIVE URINE

PREPARATION METHODS	CONTROL URINES	PATIENT URINES
1) PSEUDOREPLICA 24C for 1 hour	5-10 aggregates containing 0-2 virions per aggregate and 10-30 single excess HBsAg particles, occasional single antibody coated virions per grid square.	5-10 HBsAg aggregates without virions, and 10 to 30 single excess HBsAg particles per grid square.
2) PSEUDOREPLICA 37C for 1 hour	50-100 HBsAg aggregates containing 0-15 virions each, occasional single excess HBsAg particles 2-10 single antibody coated virions per grid square.	20-70 HBsAg aggregates without virions, occasional single excess HBsAg particles per grid square.
3) PSEUDOREPLICA 37C for 1 hour, 4C for 12 hours	Obscured*	Obscured*
4) DERRICK 37C for 20 minutes	30-50 aggregates containing 0-15 virions, 5-10 single HBsAg particles; occasional single antibody coated virions per grid square.	Occasional HBsAg aggregates containing no virions, 5-40 single HBsAg particles per grid square.

* Preparation method 3 resulted in essentially opaque/cloudy grids in which any aggregates or virions present were completely obscured.

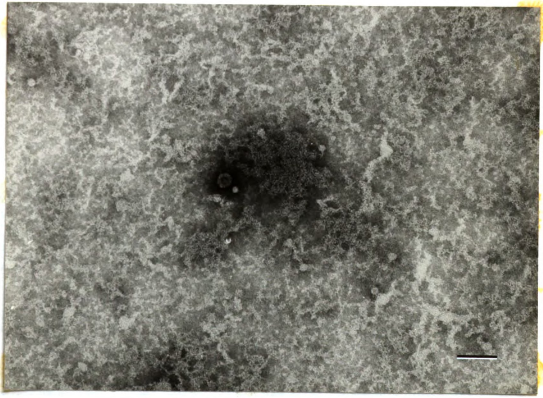

Figure 1A: Typical negatively stained single antibody-coated hepatitis B virion and HBsAg particles observed following incubation of highly diluted serum with HBIG. Bar represents approximately 112 nm.

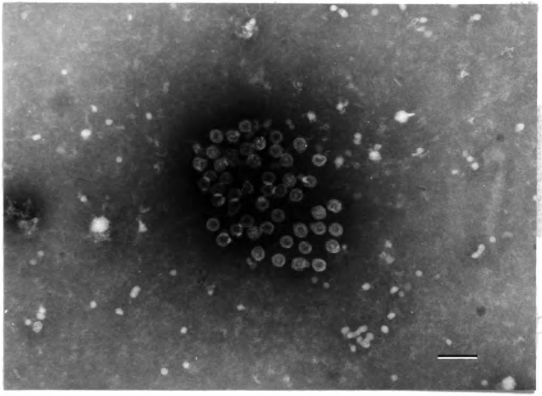

Figure 1B: Negatively stained Dane particles in aggregate. Bar represents approximately 100 nm.

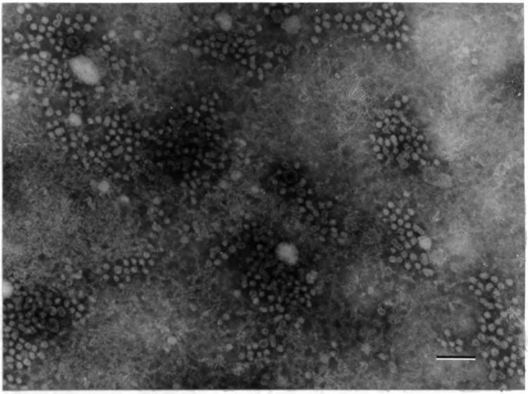

Figure 1C: Negatively stained particles in complex with spherical and tubular morphological forms of HBsAg. Bar represents approximately 100 nm.

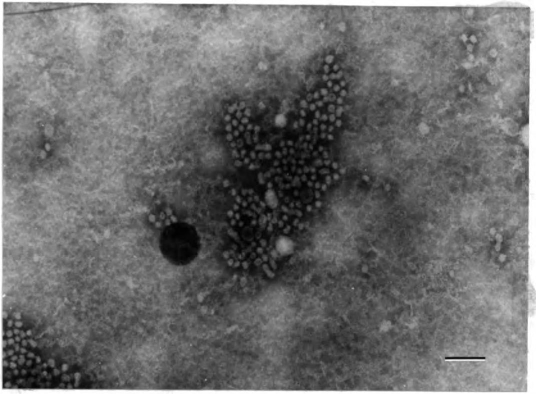

Figure 1D: Negatively stained Dane Particles in complex with spherical and tubular morphological forms of HBsAg. Bar represents approximately 100 nm.

The visual quality of specimens clarified by alternative methods of centrifugation or filtration was poor, and these techniques were not employed further.

To determine if HBV virions were pelleted during the ultracentrifugation step, a 10^{-2} dilution of serum positive for Dane particles was made in an unclarified aliquot of urine from an ESRD patient with proteinuria. This was subsequently divided into two equal volumes. One fraction was clarified as earlier described by ultracentrifugation, the other was not further processed until preparation for IEM. Examination by IEM showed that pelleting of Dane particles did not occur at the centrifugal force employed for clarification. Dane particles were detected in both preparations and virions were significantly more visible in the clarified specimen.

Since the pseudoreplica technique modified for immunoelectronmicroscopy by incubation with 10^{-1} and 10^{-2} dilutions of HBIG for one hour at 37 C, consistently allowed visualization of whole HBV virions in urines with serum positive for Dane particles added, all subsequent urines were examined by that method. A flow diagram of this protocol is shown in Figure 2.

IEM Survey of Patient Urines - Of 32 urines examined, 31 were negative for Dane particles. Urinalysis data (Appendix 3) revealed that the urine specimen positive for Dane particles by IEM contained occult blood, the probable source of virions.

2 DROPS SAMPLE + 2 DROPS HBIG

↓

INCUBATE 1 H, 37 C

↓

1 DROP SPREAD ON AGAR BLOCK, DRAINED AND DRIED

↓

1 DROP PARLODION SPREAD ON AGAR BLOCK, DRAINED AND DRIED

↓

MEMBRANE FLOATED ONTO 2% KPTA

↓

GRIDS PLACED ON MEMBRANE

↓

RETRIEVED WITH PARAFILM, DRAINED AND DRIED

↓

TRANSMISSION ELECTRON MICROSCOPY

Figure 2: Pseudoreplica Technique

DISCUSSION

HBsAg positive body fluids and excretions include urine (13,14), saliva (14), feces (14), nasopharyngeal secretions (15), menstrual blood (16), vaginal secretions (17), bile (18), breast milk (19), cerebrospinal fluid (20), sweat (21), semen (22), and synovial fluid (23), all of which have been suggested to be potentially infective. Only saliva and semen have unequivocably been shown to be infective in transmission experiments (7). Blood components, including frozen blood, are uniformly recognized as the most important vehicle of transmission (24,25,26,27).

In an assessment of the potential for transmissibility of HBV infections by urine, 51 urines from HBsAg seropositive hemodialysis or transplant patients were examined. A sensitive solid phase radioimmunoassay (RIA) enabled detection of HBe-markers in these urines. Urinary HBeAg was confirmed by specific antibody inhibition. When the urines most likely to contain Dane particles, those positive for both HBsAg and HBeAg, were inspected by immunoelectron-microscopy, whole virions were not demonstrated. The single urine in which Dane particles were observed also showed hematuria, further confirming the link between blood components and infectivity.

The presence of HBeAg in urine, here-to-fore not described, has been documented. Development of an easy, rapid and highly sensitive technique for detection of Dane particles by immunoelectronmicroscopy has been described. Incorporation of the pseudoreplica technique yielded good particle concentration while removing salts and impurities from the specimen. Use of hyperimmune hepatitis B immune globulin allowed increased specificity for HBsAg, free and virion-associated. Refinement of the system resulted in consistent visualization of Dane particles in positive control specimens.

While serum HBeAg is associated with HBV DNA polymerase and circulating Dane particles(28), HBeAg positive urine samples were negative for Dane particles using IEM. HBe-antigenuria is common among patients with end stage renal disease, both on hemodialysis and renal transplant patients. The chronic maintenance hemodialysis and transplant populations do not appear different when evaluated for HBe markers, and HBe antigenuria is both intermittent and persistent. Despite the detection of HBsAg and HBeAg in the urine samples, the absence of observable Dane particles by IEM suggests that these urines are not infectious. More definitive studies, using a primate model for experimental transmission, remain to be done.

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ABSTRACT

ABSENCE OF DANE PARTICLES IN PATIENTS' SERA
LACKING PROTECTIVE ANTIBODY TO HBV

By

C.A. Currie, T.J. Kelly, G.H. Mayor, and M.J. Patterson

Serum samples from patients with end stage renal disease (ESRD) were examined by immunoelectronmicroscopy (IEM) for the presence of Dane particles. These patients had previously been included in a statewide survey to determine prevalence, among chronic maintenance hemodialysis and renal transplant patients, of various hepatitis B virus (HBV) markers (HBsAg: hepatitis B surface antigen, anti-HBs: antibody to hepatitis B surface antigen, anti-HBc: antibody to hepatitis B core antigen, HBeAg: hepatitis B e antigen, anti-HBe: antibody to hepatitis B e antigen) detectable by radioimmunoassay (RIA). Thirty three serum samples positive for anti-HBc but lacking HBsAg and anti-HBs, were all negative for Dane particles when examined by IEM.

ABSENCE OF DANE PARTICLES IN PATIENTS' SERA

LACKING PROTECTIVE ANTIBODY TO HBV

By

C.A. Currie, T.J. Kelly, G.H. Mayor, and M.J. Patterson

INTRODUCTION

The development of solid phase radioimmunoassays (RIA) for the antigen-antibody marker systems of hepatitis B virus (HBV) infection has allowed precise interpretation of the events in this virus-host interaction (1,2,3,4). Since patients on chronic maintenance hemodialysis remain at high risk of infection with HBV, this population has provided a unique pool to study the serum prevalence of the marker systems. It has recently been reported that, utilizing RIA for five separate markers, 29.9% of hemodialysis patients had hepatitis B surface antigen (HBsAg) and/or its associated antibody (anti-HBs) in their sera, inclucing 23.8% of the population which also had antibody to hepatitis B core antigen (anti-HBc) (2). However, an additional 7.9% of these hemodialysis patients showed anti-HBc in the absence of HBsAg or anti-HBs. When hepatitis B e antigen (HBeAg) and antibody (anti-HBe) were assessed in these patients with only anti-HBc detectable in their serological profile, 8.6% had HBeAg and 25.7% anti-HBe, while 65.7% had no detectable HBe marker (2). It was important to define further the state of clinical infection and to assess potential transmission from the blood of such patients, characterized by RIAs as negative for HBsAg and anti-HBs.

and positive for anti-HBc with or without a marker of the HBe system. This study was undertaken to examine sera of these patients for the presence of circulating morphologic forms of HBV by a highly sensitive pseudoreplica immuno-electronmicroscopic technique.

MATERIALS AND METHODS

Patient and Materials - Thirty three serum samples were obtained from end stage renal disease (ESRD) patients in Michigan. These patients were part of the population previously surveyed to determine prevalence of various hepatitis B markers (HBsAg, anti-HBs, anti-HBc, HBeAg, anti-HBe) detectable by radioimmunoassay (RIA) (2,5,6).

All patients in this study were being maintained on hemodialysis. The sera examined were selected from a subpopulation demonstrating hepatitis B core antibody (anti-HBc) in the absence of HBsAg and anti-HBs. The sera were aliquoted and frozen at -20 C until use for immuno-electronmicroscopy (IEM). Immediately prior to treatment for IEM, sera were diluted 10^{-1} and 10^{-2} in 0.01 M sterile phosphate buffered saline (PBS), 0.01 M phosphate, 0.13 N NaCl, pH 6.9.

Three sera positive for HBsAg by radioimmunoassay, were used as positive controls for IEM. These sera were used in final dilutions of 10^{-1} and 10^{-2} respectively, in patient serum which was diluted in PBS as described.

Immunoelectronmicroscopy

Antiserum - Varying dilutions of goat anti-human IgG (Meloy, Springfield, VA) were used for immune complex formation. Prior to pseudoreplication, goat antiserum was added to patient sera at concentrations determined by solid phase RIA (Corab, Abbott Laboratories, North Chicago, Illinois) to remove detectable anti-HBc.

Immune Complex Reaction Mixture - Two drops of diluted goat antiserum were mixed with an equal volume of prediluted patient serum in test tubes. Tubes were sealed with parafilm and incubated for one hour, 37 C.

Grid Preparations - Grids were prepared for the pseudoreplica technique and negatively stained with 2% phosphotungstic acid (KPTA), pH 6.0-6.7 as described in detail previously (7).

Transmission Electron Microscopy - A Philips 300 TEM (80 kV) was used to examine grids. Grids were scanned (five to six squares per grid) at magnifications of approximately 26,000x and 67,000x for 20-30 minutes, for the presence of immune complexes containing morphologic subunits of HBV.

RESULTS

Determinations by solid phase radioimmunoassay of concentrations of anti-human IgG necessary to remove detectable anti-HBc from sera, were used as a guideline to incorporate goat anti-human IgG into the pseudoreplica technique. Dilutions of 10^{-1} and 10^{-2} were selected, and immune complex formation was readily achieved in all specimens prepared with HBsAg positive serum added. Dane particles occurred in clumps and singly, and appeared to have fuzzy outer boundaries. Few to moderate virions were detected in positive control sera containing high concentrations of added HBsAg positive serum, and rare to few virions were detected in the more dilute control preparations. Comparatively more virions were trapped using higher concentrations of goat anti-human IgG.

Since this modification of the pseudoreplica technique consistently allowed visualization of Dane particles in sera prepared with morphological markers, all patient sera were processed and examined by this method. The results of HBeAg and anti-HBe assays for those patients are shown in Appendix 4. All 33 sera which contained anti-HBc in the absence of HBsAg and anti-HBs and with or without e system markers, were negative for Dane particles.

DISCUSSION

With the development of highly sensitive immunologic assays for antigen-antibody markers of HBV infection, serologic profiles have emerged as correlates of the stage of clinical disease (8). When only HBsAg is detectable, the infection generally is early, often presymptomatic. Sequential serum sample studies show that HBsAg is present for 3-5 weeks prior to the appearance of the first detectable antibody, anti-HBc (9). HBsAg in the presence of anti-HBc, indicates either acute infection or the presence of the chronic carrier state. Appearance of anti-HBs signals disappearance of HBsAg and recovery from HBV infection (8). Usually anti-HBc and anti-HBs remain detectable long after infection. HBeAg appears transiently, shortly after HBsAg is detectable. HBeAg in serum is regarded as indicative of infectivity, since the concentration of HBeAg correlates closely with the number of circulating Dane particles (8,10). Persistent HBeAg is associated with continuing infectivity and chronic liver disease (8). However, more commonly anti-HBe is found shortly after the appearance of anti-HBc, during the acute phase of the clinical illness. As with the other antibody moieties associated with HBV infection, presence of anti-HBe may be prolonged in some patients.

Hemodialysis patients present a unique population with respect to HBV infection, since they remain at high risk for HBV, infection frequently results in a persistent HBsAg carrier state, and some of these patients are likely to become renal transplant recipients, subject to immunosuppressive therapy. The present study examines that particular group of hemodialysis patients whose serologic profile shows them to be negative for both surface antigen and antibody, but positive for anti-HBc, that is, a group of patients who have had HBV infection but show no protective antibody.

Presence of anti-HBc in the absence of HBsAg or anti-HBs may indicate the "immunologic window", that period during acute infection following disappearance of HBsAg and prior to detection of anti-HBs (1,11). Alternatively, this profile might represent a period long after an infection that had been appropriately terminated by anti-HBs, but at a point when anti-HBs has fallen to undetectable levels. In addition, this category can also signal the presence of persistent HBV infection where the low level of HBsAg is undetectable.

The infectivity of patients with markers of prior infection with HBV (anti-HBc with or without an e system marker) without evidence of protective antibody (anti-HBs), remain suspect with regard to infectivity. In particular, patients seropositive for anti-HBc and HBeAg may be potentially infectious. However, careful examination of sera from hemodialysis patients with previous HBV infection and without anti-HBs, by a sensitive pseudoreplica immuno-electronmicroscopic technique, shows lack of any circulating morphologic evidence of HBV.

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APPENDICES

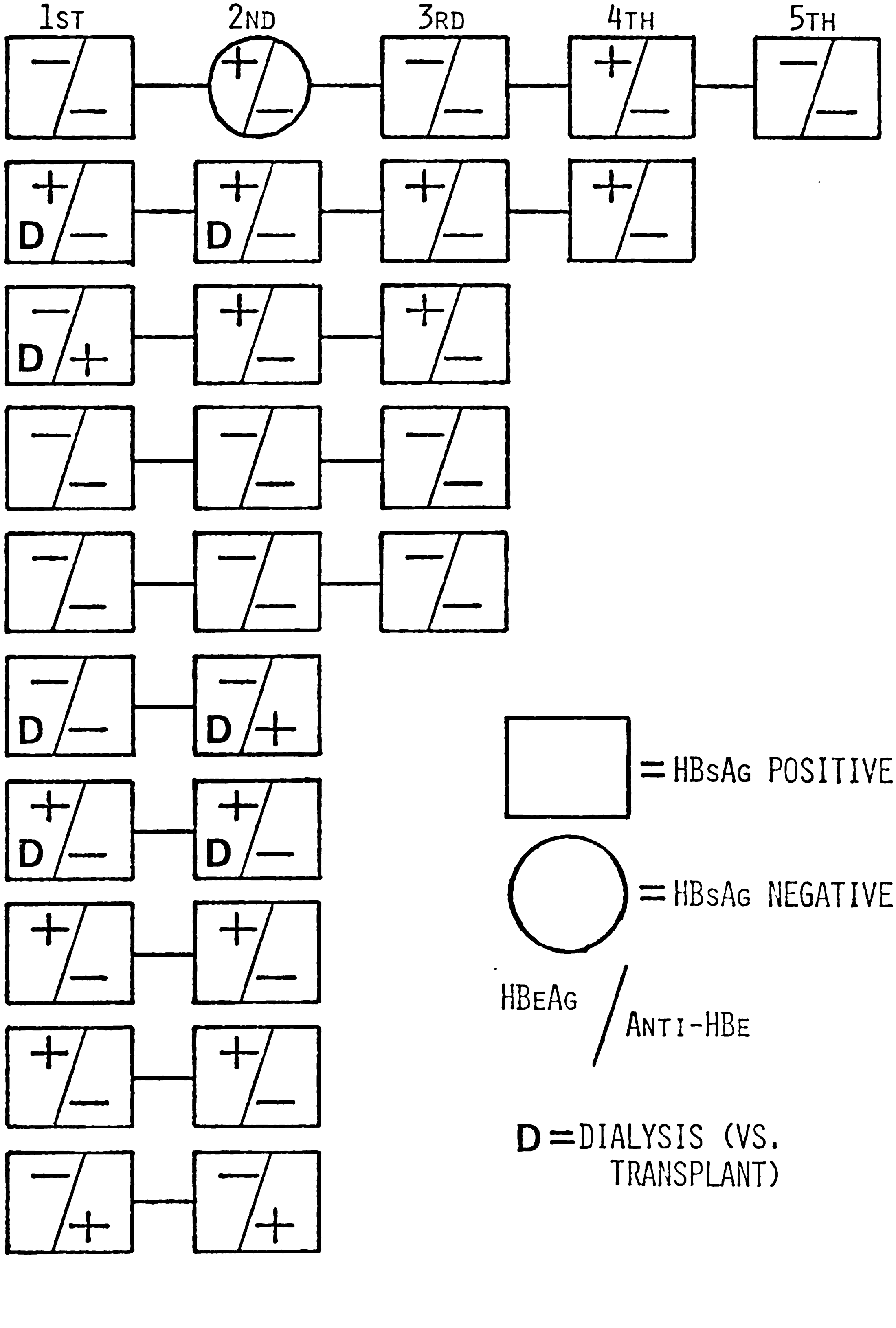
APPENDIX 1

Hepatitis B Markers in Urine From
Persistent HBsAg Seropositive ESRD Patients

		HBsAg	POSITIVE FOR HBeAg	POSITIVE FOR ANTI-HBe
ALL ESRD	44	POS-37	19(51%)	5(14%)
		NEG- 7	2(28%)	0
DIALYSIS	18	POS-12	9(75%)	2(17%)
		NEG- 6	1(17%)	0
TRANSPLANT	26	POS-25	10(40%)	3(12%)
		NEG- 1	1	0

APPENDIX 2

SERIAL URINES



APPENDIX 3

HBV Antigen-antibody System Markers Detected by Radioimmunoassay and Urinalysis Data in Urines examined by Immunoelectronmicroscopy.

	HBsAg	HBeAg	Anti-HBe	Occult Blood	Protein mg/dl
5001	-	-	-	-	100
5002	-	-	-	-	trace
5003	-	-	-	-	30
5005	-	Pos	-	-	100
5006	-	NA*	NA	-	100
5007	-	NA	NA	NA	NA
5011	-	-	-	-	100
5012	Pos	Pos	-	-	30
5013	-	NA	NA	-	100
5014	Pos	-	Pos	-	100
5015	-	NA	NA	-	100
5016	Pos	Pos	-	-	300
5017	Pos	Pos	-	-	trace
5018	-	NA	NA	-	100
5020	-	NA	NA	-	trace
5030	-	Pos	-	-	trace
5031	Pos	Pos	-	-	trace
5032	Pos	-	Pos	2+	trace
5033	Pos	Pos	-	-	30
5038	Pos	-	-	-	-
5041	Pos	Pos	-	-	30
5043	Pos	Pos	-	-	trace
5101	Pos	-	Pos	-	30
5102	Pos	Pos	-	-	30
5112	Pos	Pos	-	-	trace
5113	Pos	-	-	-	trace
5114	Pos	-	-	-	trace
5115	Pos	-	-	-	300
5116	Pos	-	-	-	100
5118	-	Pos	-	-	-
5120	Pos	-	Pos	-	-
5121	-	NA	NA	NA	NA

NA= Not assayed

Pos= Positive

APPENDIX 4

HBe Antigen-antibody System Markers Detected by Radioimmunoassay in 33 Sera (positive for anti-HBc, negative for HBsAg and anti-HBs) Examined by Immunoelectronmicroscopy.

Patient #	HBeAg	Anti-HBe
0307	-	-
0312	-	-
0905	Pos	-
0918	-	-
0920	-	-
0932	-	-
1102	-	-
1104	-	-
1111	-	Pos
1140	-	-
1163	-	Pos
1207	Pos	-
1401	-	-
1404	-	-
1505	Pos	-
1702	-	-
1921	-	-
1940	-	-
2004	-	-
2115	-	-
2134	-	Pos
2401	-	Pos
2409	-	-
2427	-	Pos
2430	-	Pos
2434	Pos	-
2603	-	-
2610	-	-
2618	-	-
2623	-	-
2704	-	Pos
2718	-	-
3212	-	Pos