IMPROVED DETECTION OF COXSACKIE B VIRUS INFECTIONS BY DETERMINATION OF VIRUS-SPECIFIC IgM ANTIBODY

A THESIS

PRESENTED TO

THE DEPARTMENT OF MEDICAL MICROBIOLOGY

FACULTY OF MEDICINE

THE UNIVERSITY OF MANITOBA

In Partial Fulfillment

of the Requirements for the Degree

MASTER OF SCIENCE

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by

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April, 1984

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

TO MY PARENTS

ACKNOWLEDGEMENTS

The author wishes to express his heartfelt gratitude to Dr. G.W. Hammond for his patience, guidance, inspiration, and encouragement throughout this research project. Many thanks are extended to the members of the Virus Isolation Unit, Cadham Provincial Laboratory, Winnipeg, Manitoba, for their timeless assistance and good humor, and to the students and staff of the Department of Medical Microbiology for their treasured support and friendship. Special thanks are extended to Dr. Ian Johnson and Drs. Khan and Verma of the St. Amant Center, Winnipeg, Manitoba, for providing the clinical material used in this project. In addition, the author would also like to thank Mr. Paul Hazelton for his technical expertise in electron microscopy and the photography for this thesis, and to Dr. C.K. Hannan for his constructive criticism and comments during the development of the CBV-IgM ELISA.

ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) utilizing the capture-antibody principle was developed for detection of coxsackie B virus (CBV) specific IgM in human serum. Detection of virus-specific IgM was a good indicator of a recent infection with a particular virus, and circumvented some of the difficulties associated with the conventional microneutralization test. The ELISA demonstrated a positivity rate of 84% in comparison to the reference standard of virus isolation. The ELISA also was more sensitive than the routine microneutralization test which demonstrated a positivity rate of 16%, and was more sensitive than neutralization tests with sucrose density gradient fractionation of human serum for IgM. The enzyme immunoassay could detect coxsackie B virus-specific IgM in sera negative for CBV IgM antibody at a dilution of 1:2.5 by microneutralization. Both homotypic and heterotypic IgM responses were observed in 19 children with a virologically confirmed CBV 4 infection. The ELISA was a highly sensitive and very rapid test which could be employed in place of more cumbersome and time-consuming tests such as microneutralization in the diagnosis of recent CBV infections. However, the ELISA probably detects group as well as type-specific IgM antibodies, both of which may be produced naturally following a CBV infection.

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INTRODUCTION

INTRODUCTION

The group B coxsackieviruses (CBV) were first identified by Melnick et al. in 1949, and were later classified with the enterovirus group (Committee on the Enteroviruses, 1957). Other members of this group include poliovirus, the oldest member of the group, which was identified in 1908 (Landsteiner and Popper), and the group A coxsackieviruses, first recognized in 1948 (Dalldorf and Sickles). Another agent was later identified that was not pathogenic for laboratory animals and could be isolated from healthy children (Hammon et al., 1957; Hatch et al., 1961; Ramos-Alvarez et al., 1954). Since the relationship of these agents to human disease was unknown, they were called "orphan" viruses or human enteric viruses which later was changed to enteric cytopathogenic human orphan viruses or ECHO viruses. Cooperative studies on the prototype strains available at the time resulted in the differentiation of 13 antigenically distinct viruses (Committee on the ECHO viruses, 1955). Since then, numerous human enterovirus types have been identified and the enteroviruses have been classed as one of the major subdivisions of the picornaviruses (International Enterovirus Study Group, 1963). All of the enteroviruses are inhabitants of the alimentary tract and as a group are associated with a wide range of clinical syndromes. The CBV were recognized as agents capable of causing human disease shortly after their identification in 1949. Moreover, this group of viruses was quickly recognized as

capable of causing serious illness in neonates and children. It was not until years later, however, that the significance of CBV in causing severe adult infections was realized (Fletcher and Brennan, 1957). Although much of the investigative work has been centered on disease in children, more attention is now being given to adult infections.

Classification of Coxsackie B Viruses

The group B coxsackieviruses are classified in the taxonomic family, Picornaviridae, genus Enterovirus (Cooper et al., 1978; Melnick et al., 1974). Currently, there are over 70 recognized enterovirus types and additional strains are being studied as possible new prototypes. The picornavirus family includes viruses of both human and animal origin (Table 1). The CBV are enteroviruses of human origin and consist of six immunotypes, which are described in Table 2.

Disease Associated with Coxsackie B Viruses

The CBV cause a wide spectrum of disease, but most often infections are asymptomatic with at least 49% being subclinical (Lerner et al., 1975). Nonetheless, this group of viruses is the most important of the enteroviruses that cause serious illness in man. CBV infections in humans range from mild and moderate infections such as undifferentiated febrile illness and upper respiratory syndromes to more life-threatening situations such as meningoencephalitis and heart disease (Table 3).

TABLE 1

The Picornaviruses

I. Picornaviruses of human origin

A. Enteroviruses

(1) Polioviruses, types 1-3
(2) Coxsackieviruses of group A, types 1-24
(3) Coxsackieviruses of group B, types 1-6
(4) Echoviruses, types 1-34
(5) Enteroviruses, types 68-71

B. Rhinoviruses (over 100 types)

II. Picornaviruses of lower animals

A. Enteroviruses (monkeys, pigs, cows, mice)

B. Rhinoviruses (horses, pigs, cows, mice)

C. Aphthoviruses (foot-and-mouth disease viruses)

D. Encephalomyocarditis virus (rats)

Table 1: From Melnick, J.L., H.A. Wenner, and C.A. Phillips, "Enteroviruses" - Chapter 15 In Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections, ed. Edwin H. Lennette and Nathalie J. Schmidt, 5th ed., American Public Health Assoc., 1979.

TABLE 2

Prototype Strains of Coxsackie B Viruses Types 1-6

Type	Prototype Strain	Geographic Origin	Illness in Person Yielding Prototype Strain	Investigator
1	Conn-5	Connecticut	Aseptic meningitis	Melnick
2	Ohio-1	Ohio	Summer grippe	Melnick
3	Nancy	Connecticut	Minor febrile illness	Melnick
4	JVB	New York	Chest and abdominal pain	Sickles
5	Faulkner	Kentucky	Mild paralytic disease	Steigman
6	Schmitt	Philippine islands	None	Hammon

* All isolates were from stools.

Table 2: From Melnick, J.L., H.A. Wenner, and C.A. Phillips, "Enteroviruses" - Chapter 15 In Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections, ed. Edwin H. Lennette and Nathalie J. Schmidt, 5th ed., American Public Health Assoc., 1979.

TABLE 3

Clinical Syndromes Associated with Group B Coxsackieviruses

Syndromes	Associated CBV Types
Pleurodynia	1 - 5
Aseptic meningitis	1 - 6
Paralysis (infrequent)	2 - 5
Severe systemic infection in infants, meningoencephalitis, and myocarditis	1 - 5
Pericarditis, myocarditis	1 - 5
Upper respiratory illness and pneumonia	4 and 5
Rash	5
Hepatitis	5
Undifferentiated febrile illness	1 - 6

Table 3: Adapted from Melnick, J.L., H.A. Wenner, and C.A. Phillips, "Enteroviruses" - Chapter 15 In Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections, ed. Edwin H. Lennette and Nathalie J. Schmidt, American Public Health Assoc., 1979.

The spectrum of CBV infections is wide and variable, and includes a number of illnesses that are severe and life-threatening. In a 10 year review of diseases associated with enteroviruses in the United States, CBV have been associated with a disproportionately large number of cases of pericarditis and myocarditis (63%), in addition to cases of meningitis, encephalitis, respiratory illness, rash, paralysis, and non-specific febrile illness (Moore, 1982). The role of CBV in other diseases such as chronic heart disease and diabetes mellitus will be an important area of investigation in the future.

Pathology of CBV Infections

In the newborn and older infants and in young children, the enteroviruses may leave evidence of cell injury during infections. The CBV in newborn infants cause a severe generalized disease characterized by pathologic changes consisting of focal necrosis as well as infiltration of lymphocytes and polymorphonuclear leukocytes (PMNL). Lesions are found primarily in the heart, but can also be present in the brain, spinal cord, liver, kidney, and adrenals. The myocardial muscle fibers demonstrate necrosis and peripheral inflammatory response. The early lesions produced consist of focal necrosis with pyknosis and karyorrhexis of myocardial nuclei with PMNL in the interstices. Late lesions are characterized by profound myocardial injury with mononuclear cells and histiocytes predominating in the injured myofibrils.

In central nervous system (CNS) infections, both the white and gray matter of the CNS may be involved resulting in features of meningoencephalomyelitis. There appears to be a predilection of this disease for the brain stem, particularly in infants. The anterior horns of the spinal cord are not involved constantly, nor as severely as in poliomyelitis.

Pleurodynia patients yielding a CBV demonstrate severe inflammatory infiltration and degeneration of muscle fibers, or both, as observed in biopsied muscle from these patients.

Other symptoms associated with CBV infections also demonstrate characteristic pathologic changes. Pancreatic lesions in the newborn consist of parenchymal infiltrates of PMNL, necrobiosis of acinar tissue, and destruction of islet cells. The testes also occasionally show nonspecific subacute inflammation. Electron micrographs of biopsied muscle from polymyositis patients have shown the presence of CBV aggregates at the site of muscle damage (Gyorkey et al., 1978).

The factors which predispose the human neonate to enteroviral infections are not known. However, in suckling mice, several factors have been suggested. First, cells of immature animals are not capable of elaborating as much interferon as cells of mature animals (Heineberg et al., 1964). Second, it has been shown that suckling mice have increased concentrations of transplacentally acquired adrenocortical hormones (Behbehani et al., 1962), which have deleterious effects in coxsackievirus infections (Kilbourne et al., 1956). Third, a variety of tissues in newborn mice bind CBV, whereas tissues in adult mice are virtually inactive in this respect (Kunin, 1962). Consequently, a

greater number of active cell receptors for CBV may account for the greater severity of these infections in infants.

Epidemiology of Coxsackie B Viruses

Enteroviruses are found in persons living in all regions of the world, and they are ubiquitous in tropical and semitropical zones. The mode of transmission of CBV is hypothesized as a fecal-oral route and/or respiratory route. That is, the virus can be spread by fecal contamination (fingers, table utensils, foodstuffs, milk), by respiratory droplets, or by flies and cockroaches which act as mechanical carriers. Transplacental spread does occur and in infants, the mortality of cases of acute infectious myocarditis is approximately 50% (Lerner et al., 1975). CBV types 1, 3, and 4 are highly endemic with occasional outbreaks whereas CBV type 2 is rarely an endemic infection, although extensive dissemination occurs occasionally. CBV type 5 has strong preference for primary infection in very young children with periods of quiescence between outbreaks (Lerner et al., 1975; Marier et al., 1975). The lowest endemicity of the group occurs with CBV type 6 (Sato et al., 1972). Urbanization and improved hygiene, however, has induced an epidemiological shift from infants to a greater number of cases of CBV infections in adults (Lerner et al., 1975).

The incubation period between implantation of the virus and clinical expression of the infection varies widely from 2 to 35 days. Virus excretion, which peaks in the late summer and fall, may persist

for as long as 70 to 90 days. Figure 1 illustrates a possible route of CBV infections in man.

Detection and Diagnosis of CBV Infection

The present methods of detection of CBV infections include virus isolation and serological techniques. Virus isolation has been the method of choice as the "gold standard" in identification of CBV since a positive virus isolate is definitive evidence of an infection. The Cultivation of CBV in tissue culture is usually done from fecal specimens (feces or rectal swabs) and/or throat swabs or washings. Best results are obtained when the specimen is collected as soon after the onset of symptoms as possible. In cases of central nervous system involvement, cerebrospinal fluid (CSF) is the most desirable specimen for CBV isolation; however, isolation from CSF is infrequently achieved (McCracken and Newman, 1975). Pleural and pericardial fluid or biopsy material is also occasionally valuable material for diagnosing a CBV infection. Identification of the virus isolate is accomplished by serum neutralization tests. The specific identification of the infecting serotype uses intersecting pools of hyperimmune viral antisera (Lim and Benyesh-Melnick, 1960).

Virus isolation procedures, however, have been supplanted by other methods in recent years due to several factors. Isolation and identification of a virus in cell culture is time-consuming, resulting in decreased clinical relevance with the delay. Also, in many instances of viral infections, the viremic phase of infection and virus excretion

FIGURE 1

Pathogenesis of Coxsackievirus B Infections in Man

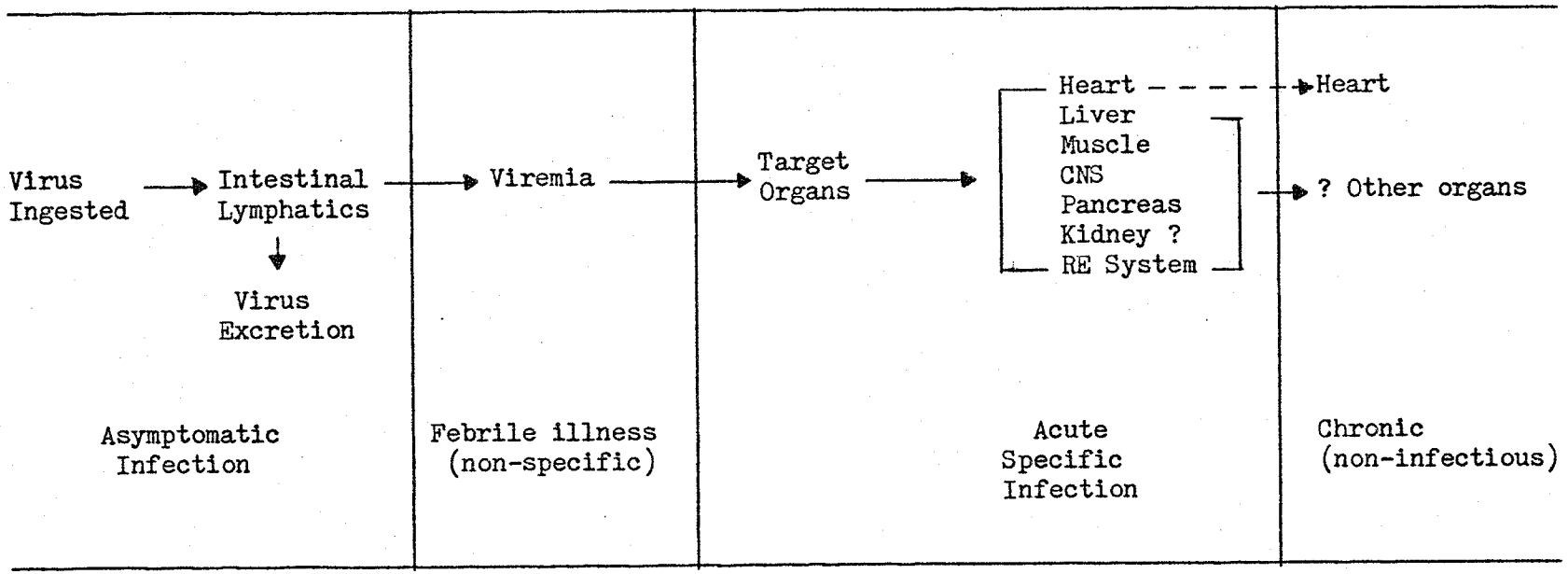


Figure 1: From McCracken, A.W. "Coxsackievirus B Infections in Adults." Texas Medicine 72: 77-83, 1976.

from other sites may have ceased by the time symptoms appear, making virus isolation impossible. Conversely, because of prolonged or asymptomatic enterovirus excretion, isolation of virus may not be specific to illness at that time. Consequently, there has been a change in emphasis towards immunological methods and serological tests. These tests alone or in conjunction with virus isolation, however, may be sufficient to provide good presumptive evidence that a virus is the causal agent of an infection.

The neutralization test is one test that has been used in the diagnosis of enterovirus infections. Past work has been centered around CBV and polioviruses since there are only a limited number of serotypes. The neutralization test requires obtaining acute and convalescent serums from the patient. The virus-disease association is then based on demonstrating a four-fold or greater elevation in neutralizing antibody titers, i.e., a seroconversion, between the acute and convalescent sera. Lennette et al. (1961) demonstrated that the neutralization test was specific for measuring antibodies against a particular strain of CBV causing infection. However, the present serological tests for diagnosis of CBV infections have several disadvantages. The CBV neutralization test is cumbersome and somewhat time-consuming when considering the time involved from collection of the patient serum samples to obtaining of test results. This test also has a low positivity rate, i.e., a low number of seroconversions, due to the fact that in many instances, the acute serum is obtained at a time when antibody levels are already elevated and/or the convalescent serum is obtained at a time when antibody levels are beginning to decline.

Other problems include heterotypic antibody responses resulting from a "booster effect" by later infections with other CBV serotypes, and high static antibody levels arising from past, frequently subclinical, infections.

Although other serological tests such as hemagglutination inhibition (HI) and complement fixation (CF) have been used in the identification of some enteroviruses, their usefulness in diagnosing CBV infections is limited. HI for CBV identification cannot be used as only CBV types 1, 3, 5, and 6 hemagglutinate (Schmidt et al., 1966). CF has limited usefulness as many individuals do not develop homotypic antibodies and there are major heterotypic cross-reactions among the various antigens (Kraft and Melnick, 1952). Also, many of the serological tests are cumbersome and time-consuming in addition to their lesser sensitivity. Consequently, there has been much research conducted into obtaining more rapid and sensitive techniques. Since antibodies of the IgM class are produced early in primary viral infections, it was suggested that a method for detecting enterovirus-specific IgM antibodies would be of more value in diagnosis of recent infection as well as differentiating between primary and secondary immune responses (Schluederberg, 1965).

While many researchers were investigating alternatives to the available immunological and serological methods for detection of CBV infections, others were simultaneously attempting to improve on the virus antigen detection methods. In 1980, Yolken and Torsch developed an enzyme-linked immunosorbent assay (ELISA) for the detection of CBV in tissue cultures and clinical specimens. The assay involved

adsorbing anti-CBV serum on a solid support to react with CBV in the specimen followed by reaction with another anti-CBV serum from a different animal species. Bound virus antigen and antisera were subsequently detected by an alkaline phosphatase enzyme-labelled antibody. The authors found that the ELISA could detect and distinguish all CBV types 1-6 at concentrations one hundredfold to ten thousandfold less than could by detected by CF tests. Furthermore, the assay correctly identified the presence of CBV in 19/21 tissue culture fluids and 5/9 rectal swab specimens. The ELISA did not react when presented with other antigens such as ECHO virus, coxsackie A virus, rhinovirus, rotavirus, or Norfolk virus.

Although this assay was very rapid and sensitive when compared to other diagnostic tests, it was not as sensitive as virus isolation, the "gold standard", as the experimental results indicated. The assay, however, may be implemented in addition to a standard technique and in this regard, offers potential for rapid identification of CBV in clinical specimens. One must keep in mind, though, of the nature of CBV infections. In many cases, as stated previously, CBV infections are often asymptomatic or viremia has occurred already by the time symptoms appear. Therefore, identification of CBV in a clinical specimen may be impossible. Consequently, it would be or more use if an ELISA for detection of CBV-specific IgM could be developed. A positive result from this type of assay would be a strong indication of a recent CBV infection.

IgM Assays for Coxsackie B Virus Infections

The validity of the hypothesis that an acute viral infection can be diagnosed by demonstrating the presence of virus-specific IgM antibodies in a single serum specimen taken at an early stage of illness depends on several factors. These factors are that the IgM antibody response should be reliably measurable, specific to a particular virus infection, a constant phenomenon in all acute viral infections, transient, and sufficiently uniform from individual to individual (Meurman, 1983). Some of the methods used in the determination of IgM antibodies are listed in Table 4. The list of methods is quite extensive at present, and several modifications of these techniques as well as combinations of some of these methods have been introduced.

The first test evaluated for CBV-specific IgM was an immunodiffusion technique devised by Schmidt et al. (1962). Virus-specific IgM was detected in cases of pericarditis (27% of 148 patients), myocarditis (25% of 92), and pleurodynia (37% of 19) (Schmidt et al., 1973). It was also demonstrated that specific IgM antibody was not detected in any specimen from a patient more than 42 days after the onset of illness. Virus-specific IgM was also detected in 21/259 control patients, but on closer examination, 6 of these had some evidence of cardiac or central nervous system disease. However, 56/70 (80%) of the IgM-positive sera demonstrated a positive result with only one CBV type; 11/70 (16%) were positive for two CBV types; one serum was positive with three, another with four, and another with five types.

Minor et al. (1979) utilized a counterimmunoelectrophoresis (CIE)

TABLE 4

Methods Used For IgM Antibody Determination

1. Comparison of antibody titers before and after inactivation of IgM antibodies:
   - Alkylation-reduction by mercaptans

2. Antibody determinations from the isolated IgM fraction after physicochemical separation of IgM:
   - Sucrose density gradient fractionation
   - Gel filtration
   - Ion-exchange chromatography
   - Affinity chromatography

3. Antibody determination from serum after removal of IgG (and IgA) antibodies:
   - Protein A absorption
   - Anti-IgG (and anti-IgA) absorption

4. Indirect solid-phase immunoassays using labelled anti-human IgM antibodies:
   - Immunofluorescence
   - Immunoperoxidase assay
   - RIA
   - EIA

5. Solid-phase anti-IgM assays:
   - RIA
   - EIA
   - Solid-phase immunosorbent hemagglutination inhibition
   - Solid-phase immunosorbent hemadsorption

6. Other assays used for the determination of IgM antibodies:
   - Immunodiffusion
   - Counterimmunoelectrophoresis
   - Radioimmunodiffusion
   - Radioimmunoprecipitation
   - Anti-IgM blocking RIA
   - Latex-IgM agglutination
   - Microimmunobead-IgM hemagglutination reduction
   - Anti-IgM hemagglutination

Table 4: From Meurman, Olli. "Detection of antiviral IgM antibodies and its problems - A Review." Curr. Topics Microbiol. Immunol. 104: 101-132, 1983.

test for detecting virus-specific IgM in patients who had a CBV isolated and who had exhibited a seroconversion in neutralizing antibody titers. In 20/22 (91%) of the patients, virus-specific IgM was detected with 19 having antibody to the infecting serotype and one patient infected with CBV 5 demonstrating heterologous IgM antibody. However, the primary disadvantage of both the immunodiffusion and CIE tests is the occurrence of heterologous reactions making identification of the infecting serotype very difficult.

In the search for a more rapid and sensitive diagnostic test for CBV infections, the era of solid phase immunoassays was entered. In this new type of assay, the IgM antibodies are separated from other serum components by immunoadsorption to an anti-human IgM antibody or an antigen bound to a solid phase support. Bound IgM is detected by either radio- or enzyme-labelled antibody or antigen depending on the particular system used. These immunoassays in general are more rapid, sensitive, and specific than conventional serological or immunological techniques. Consequently, the potential for their use, particularly in virus diagnostic laboratories, is rapidly expanding.

Thus, the aim of the research conducted in this thesis is to develop and evaluate an ELISA for the detection of virus-specific IgM antibody in CBV infections.

I. LITERATURE REVIEW

I. LITERATURE REVIEW

The Coxsackievirus Virion

(A) Physical Characteristics

The coxsackieviruses have a positive single-stranded ribonucleic acid (RNA) core enclosed by a nucleocapsid (protein coat) demonstrating cubic symmetry. The virion is between 15-30 nm in diameter and is non-enveloped, thus, making its infectivity resistant to lipid solvents such as ether and chloroform. The virion RNA is a single, infectious molecule (Colter et al., 1957) with a molecular weight of approximately 2.5 - 2.8 x 10^6 daltons, and the nucleic acid comprises between 20-30% of the virus particle. The RNA, when it is freed from the nucleocapsid and its surface protein antigens, cannot be neutralized by viral antiserum. In the presence of $MgCl_2$ and other divalent cations, the coxsackieviruses are heat stable and are not inactivated at 56° C for one hour (Wallis and Melnick, 1962). The viruses are stable at freezing temperatures for many years, at refrigerator temperatures (4° C) for weeks, and at room temperature for days. NaCl protects virus activity at 50° C, but markedly increases the rate of inactivation at 37° C (Wallis and Melnick, 1961; Wallis and Melnick, 1962). These viruses are also inactivated by ultraviolet (UV) light and usually by drying. Coxsackieviruses can also crystallize and the

crystals contain infectivity (Mattern and DuBuy, 1956). In CsCl gradients, CBV have a density of 1.34 g/cm^3.

(B) Biological and Biochemical Characteristics

The group A and B coxsackieviruses differ from each other in that many of the coxsackie A viruses do not grow in tissue culture, whereas coxsackie B viruses grow readily in primate epithelial cells, i.e., monkey kidney cells, human amnion cells, embryo kidney cells, HeLa cells. The CBV replicate poorly or do not grow at all in RD cells, a cell line derived from a human rhabdomyosarcoma, whereas a number of the coxsackie A viruses do grow in this cell line. Newborn mice up to seven days old are very susceptible to both coxsackievirus groups and the pathological changes in the mice demonstrate a great similarity to pathological changes which occur in humans. Therefore, the mouse is an excellent animal model system for these types of virus infections. The CBV cause tremors, spasticity, and spastic paralysis in newborn mice as well as focal degeneration of skeletal muscles, necrosis of brown foot pads, myocarditis, hepatitis, and other symptoms. In contrast, the coxsackie A viruses cause a flaccid paralysis with severe and extensive degeneration of skeletal muscles.

The growth of enteroviruses in tissue culture is associated with a characteristic cytopathic effect (CPE). In tissue culture, the CPE caused by CBV involves rounding and shrinkage of infected cells, followed by nuclear pyknosis with the cells then becoming refractile

and eventually detaching from the surface of the tissue culture flask. Under agar overlay, plaques are formed by some of the enteroviruses in cultures of susceptible cells. The CBV produce plaques similar to those of polioviruses, i.e., circular plaques with clear centers and sharp boundaries, but the plaques produced by CBV have diffuse boundaries and their appearance may be delayed. The growth of all CBV is inhibited by compounds such as 2-α-hydroxybenzimidazole (HBB) and guanidine hydrochloride, drugs that inhibit formation of viral RNA polymerase.

The CBV virion consists of four polypeptides, virion proteins (VP) 1-4. Each virion contains equimolar amounts of the four virus polypeptides and the complete virus particle contains 60 monomers of each. CBV 3 has been the most extensively studied of the coxsackieviruses, and it has been determined that VP 4 has a molecular weight (M.W.) of 5500 daltons, VP 2 has a M.W. of 25,000, and VP 1 plus VP 3 have a M.W. of 50,000. The M.W. of the four virion polypeptides is approximately 80,000 with the total M.W. of the virion estimated to be between 4.8 - 5.4 x 10^6 daltons (Phillipson et al., 1973).

Within the enteroviruses, there is approximately 20% homology of their nucleotide sequences, while the CBV demonstrate 30-50% homology among the six types (Young, 1973).

(C) Antigenic Characteristics

CBV 1-5 and coxsackie A9 virus have been shown to have a common group antigen (Plummer, 1965). A type-specific antigen is also present in the complete virion. Two precipitation antigens have

been differentiated with CBV 1, 3, 5, and coxsackie A9 which are a "group" and "type-specific" precipitating antigen as detected by gel diffusion studies (Schmidt and Lennette, 1962). The group antigen corresponded to the CF antigen which seemed to be responsible for much of the heterotypic activity of some human sera. Conversely, the type-specific antigen of these viruses appear to be immunologically distinct from each other.

The early neutralizing and HI antibodies of humans induced by the CBV are primarily 19S immunoglobulins which are later replaced by 7S immunoglobulins in the convalescent phase of infection (Schmidt et al., 1968). Type-specific antibodies usually appear in the bloodstream within a week after onset of infection in humans, and maximal titers are reached by the third week (Hirschman and Hammer, 1974). Neutralizing antibody can apparently be present for several years or even a lifetime. CF antibodies begin to decrease after 2-3 months and then diminish rapidly. The group antibodies to CBV appear earlier, reach higher levels, and persist longer than the type-specific antibodies (Schmidt and Lennette, 1962).

There exists some disparity in the literature concerning the virion protein which induces neutralizing antibodies in the infected host. Katze and Crowell (1980), using urea-disrupted virions, found that the group-reactive antigen was the VP 1 polypeptide. Type-specific antibodies were detected by ELISA to the VP 2 and VP 3 polypeptides , and it was revealed that the VP 2 protein induced the neutralizing antibodies with CBV 3. Beatrice et al. (1980) also found that the VP 2 protein was the type-specific immunogen with CBV 3. In

contrast to these results, Dörries and ter Meulen (1983) noted that both type- and group-specific antigenic determinants were located on VP 1 by immunoblot analysis. In comparison, the VP 1 polypeptide is the neutralizing antigen in foot-and-mouth disease virus, another picornavirus (Kleid et al., 1981), and also in poliovirus (Emini et al., 1983).

ELISA: Principles

The search for new and better diagnostic aids over the past ten to twenty years has led to the development of the enzyme immunoassay. This assay combines the specificity of the antigen-antibody reaction with the sensitivity of an indicator system attached to the antibody. For high sensitivity, the antigen, hapten, or antibody is labelled in some way. Historically, the most commonly employed label has either been a fluorescent dye or radioisotope (O'Beirne and Cooper, 1979). Although the sensitivity imparted by use of radioisotopes approaches the 10^{-11} to 10^{-17} mole range, the biological hazards associated with these compounds make them less desirable (Scharpe et al., 1976). Since the sensitivity of enzyme systems also approaches comparable levels to those of radioisotopes, the use of enzymes as labels in immunoassays was investigated. Enzyme immunoassays (EIA) or enzyme-linked immunosorbent assays (ELISA) overcome some of the limitations of radioimmunoassays (RIA) and have several advantages (Wisdom, 1976):

(1) Specific and sensitive assays of wide applicability can be developed.

(2) Equipment required is relatively inexpensive and widely available.

(3) Reagents are relatively inexpensive and have a long shelf-life.

(4) Manipulations are simple.

(5) Assays may be very rapid.

(6) A separation step of immunoglobulins may not be required.

(7) The variety of labels available may allow multiple, simultaneous assays to be performed.

(8) There is potential for automation.

(9) There are no radiation hazards.

A variety of enzymes are available for EIA's and these are compared in Table 5.

The two basic types of ELISA's are assays for detection and quantitation of antigen and haptens, or assays for detection and quantitation of antibody. An excellent review of the basic principles of both types of immunoassays is offered by Ekins (1981). In general, ELISA can detect antigens in the 1-10 ug/liter range (Wisdom, 1976), and antibodies at < 1 ug/liter (Engvall and Perlmann, 1972). An extensive review of ELISA for antigen has been offered by Yolken (1982), and a review of ELISA for antibody by O'Beirne and Cooper (1979).

(i) ELISA for IgM Antibodies

The nomenclature of enzyme immunoassays is an area which is still not standardized. Often, authors assign a name of their own

TABLE 5

Comparison of Enzymes Available for EIA

Enzyme	Source	pH optimum	Molecular weight	Visual Substrates Available[1]
Alkaline phosphatase	Calf intestine	8-10	100,000	$NP\text{-}PO_4$
Peroxidase	Horseradish	5-7	40,000	H_2O_2 + 5-AS H_2O_2 + OPD
B-galactosidase	Escherichia coli	6-8	540,000	NP-Gal
Glucose oxidase	Aspergillus niger	4-7	160,000	Glu + 5-AS Glu + NBT Glu + MTT
Catalase	Calf liver	6-8	250,000	H_2O_2 [2]

[1]Abbreviations: $NP\text{-}PO_4$ = p-nitrophenyl phosphate; 5-AS = 5-aminosalicylic acid; OPD = o-phenylene diamine; NP-Gal = nitrophenyl galactose; Glu = Glucose; NBT = p-nitroblue tetrozolium chloride; MTT = thiazolyl blue

[2]Measured spectrophotometrically at 240 nm.

Table 5: Adapted from Yolken, R.H. "Enzyme immunoassays for the detection of infectious antigens in body fluids: Current limitations and future prospects." Rev. Infect. Dis. 4(1): 42, 1982.

to the enzyme immunoassay published, and this results in some confusion when one attempts a comparison of assays that appear to be different upon initial inspection. In addition to "solid-phase anti-IgM assay", designations such as "direct immunoassay", "reverse immunoassay", "IgM antibody capture assay", "double sandwich IgM assay", and "enzyme-labelled antigen IgM assay" have been used with ELISA applications. Consequently, this can be confusing when examining literature on the subject of ELISA.

The solid-phase immunoassays for IgM antibody have proven to be very specific and sensitive. These assays utilize an anti-IgM bound to the solid phase to react with IgM in the serum. The bound IgM is usually detected by addition of the virus antigen followed by an enzyme-labelled detector antibody directed against the antigen. The ELISA for IgM is more sensitive than assays based on sucrose density gradient fractionation (Mortimer et al., 1981; Roggendorf et al., 1980). Since the first step in these assays is the separation of IgM from other serum components, there is no competition between IgM and IgG. Consequently, unlike the indirect immunoassays where antigen is first bound to the solid phase, the sensitivity of the anti-IgM assays is not influenced by the ratio of antigen-specific IgG to IgM. However, in these assays, antigen-specific IgM will compete with other IgM for the limited number of binding sites on the solid phase; therefore, the sensitivity of the ELISA will be governed by the ratio of specific IgM antibodies to total IgM (Heinz et al., 1981; Vejtorp, 1981).

In general, the advantages of the anti-IgM assays over the indirect immunoassays include lack of competition between IgM and IgG,

a higher sensitivity, and decreased interference by rheumatoid factor (Meurman, 1983). The disadvantage of the test is that a conjugated antigen or antiserum for each antiviral IgM antibody is required.

The ELISA that was first applied to the detection of antiviral IgM antibodies utilized an antigen-coated phase to capture the antiviral IgM (Voller and Bidwell, 1976). Detection of the reaction was by an anti-human immunoglobulin antibody labelled with an enzyme. The first IgM ELISA reported that utilized the capture anti-IgM principle was for detection of IgM antibodies to hepatitis A virus (Duermeyer and van der Veen, 1978). Since then, several ELISA's for detection of IgM antibodies to viruses have been reported (Table 6).

The difficulties associated with the IgM ELISA's are of two types: "technical problems", which are method dependent, and "biological problems", which can be encountered in any type of assay. Technical problems in the immunoassays include primarily background activity caused by nonspecific binding, and the presence of rheumatoid factor. Biological problems range from cross-reactive antibodies resulting in false-positive tests, to individual variation in IgM response or even lack of an IgM response. Since the sensitivity of an immunoassay is dependent on the level of background activity, i.e., the degree of absorbance observed with negative specimens, these problems are of primary concern in the development of ELISA's. A high background level of activity makes interpretation of positive or negative results difficult and increases the number of "borderline" cases. For a review of some of the difficulties associated with anti-IgM immunoassays, see Meurman (1983).

TABLE 6

ELISA's for Detection of IgM Antibody

Duermeyer et al. (1979)	--	Hepatitis A
Moller and Mathiesen (1979)	--	Hepatitis A
Roggendorf et al. (1980)	--	Hepatitis A
Gerlich and Luer (1979)	--	Hepatitis B core antigen (HB_cAg)
Kryger et al. (1981)	--	Hepatitis B core antigen
Lemon et al. (1981)	--	Hepatitis B core antigen
Mortimer et al. (1981)	--	Hepatitis B core antigen
Vejtorp (1981)	--	Rubella
Diment and Chantler (1981)	--	Rubella
Roggendorf et al. (1981)	--	Flaviviruses
Heinz et al. (1981)	--	Flaviviruses
Tedder et al. (1981)	--	Herpes zoster virus
Schmitz et al. (1980)	--	Cytomegalovirus
Yolken and Leister (1981)	--	Cytomegalovirus
El-Hagrassy et al. (1980)	--	Coxsackie B virus
Dorries and ter Meulen (1983)	--	Coxsackie B virus
Banatvala et al. (1983)	--	Coxsackie B virus

ELISA for Coxsackie B Virus IgM: A Literature Critique

In 1980, Katze and Crowell tested the principle of the enzyme immunoassay for diagnosis of CBV infections by using an ELISA for detection of IgG antibody to CBV. The authors found that type-specific detection of antibody was equal to or greater than the sensitivity of the neutralization test, and over fifty times higher than that of the complement fixation test. The enzyme immunoassay utilized virus adsorbed to the solid phase to detect CBV antibodies (IgG) in hyperimmune rabbit antiviral serum, with a peroxidase enzyme-labelled antibody as the detector. The ELISA was rigidly controlled with the cut-off level defined as the highest serum dilution giving an absorbance value of 0.25 absorbance units above control levels. Their results indicated that a type-specific reaction depended on the presence of intact whole virions. Disruption of the virus by urea revealed the group-reactive antigen and that it was the VP 1 polypeptide. This group reactivity, however, was limited to the CBV and did not extend to coxsackie A2, poliovirus type 2, echovirus type 6, or human rhinovirus 2. This report described a rapid and sensitive assay for detection of antibodies to CBV, and indicated its potential for diagnosis and serological studies of CBV infections in humans. However, the indirect system used by the authors would be subject to interference by rheumatoid factor in human sera, and would not be particularly useful in diagnosing a recent CBV infection as it measured IgG antibody which appears later in an infection. Detection of IgM may help to clarify diagnosis of a recent CBV infection because the frequency of development of heterotypic

antibodies, usually IgG in nature, increases with age. Consequently, the authors did suggest that an ELISA for the measurement of IgM antibody might prove more useful.

The first solid phase immunoassay for the detection of IgM to CBV was described by El-Hagrassy et al. (1980). The assay procedure consisted of adsorbing an anti-human IgM to polyvinyl microtiter plates to capture CBV-specific IgM. The antigen, which was a crude preparation of pooled CBV 1-5 viruses, was added followed by addition of the detector antibody, an alkaline phosphatase conjugated pool of CBV 1-5 antisera. In the assay, each sample was tested in duplicate with three positive and three negative control sera as well as positive and negative antigen controls. The cut-off level for samples being positive was determined as twice the mean of the negative controls when reacted with positive antigen. Sera that were found positive on screening at a 1:100 dilution were titrated at dilutions from 1:200 to 1:10,000, and also tested for rheumatoid factor by latex agglutination and the Rose Waaler test.

Sera from 12 patients with a confirmed CBV infection by virus isolation and/or greater than or equal to four-fold rise in neutralizing antibodies were tested and 83% (10) of these patients had detectable IgM to a CBV. Another 10 sera fractionated for IgM by sucrose density gradient centrifugation were shown to contain CBV-specific IgM by neutralization tests. Positivity rates with patients with CBV related disease were 37.5% (24/64 patients) with acute pericarditis, 36% (14/38) with acute myocarditis, 13.3% (4/30) with acute ischaemic heart disease, 7.1% (2/28) with congestive cardiomyopathy, and 36.8% (21/57) with

Bornholm disease. A control group of 46 patients with recent infections other than CBV (M. pneumoniae - 12, influenza A - 4, influenza B - 8, Chlamydia psittaci - 5, adenovirus - 4, parainfluenza viruses - 3, and varicella-zoster virus - 2) were tested. All of these sera were negative in the ELISA for CBV-specific IgM. CBV-specific IgM was found to persist for 6-8 weeks after the onset of symptoms in the 10 patients from whom sequential samples were available. False positive results did not occur with sera containing high titers of rheumatoid factor.

This ELISA was the first described for detection of CBV-specific IgM and as such was of great value to diagnostic virology. The authors presented work that contained adequate controls, and utilized at least some serum samples that were supported by virus isolation. The fractionation of some sera by sucrose density gradients, which is an accepted method of IgM determination, with subsequent testing by microneutralization to establish that virus-specific IgM was present in the serum samples was also sound scientific procedure. The assay also utilized a crude preparation of antigens. As it has been shown in the past (Katze and Crowell, 1980), a type-specific reaction is dependent on the presence of whole intact virions, which may require a more purified preparation of antigen. However, the objective of the authors was only to develop an ELISA for group-specific IgM responses to CBV infections. The potential usefulness of this type of assay for diagnosis of CBV infections was very apparent, nonetheless, and a group-specific ELISA for CBV IgM cannot be understated in its importance for clinical purposes. However, if a type-specific assay

could be developed, it would be of more value in epidemiological studies.

Another indirect solid phase enzyme immunoassay was later described by Dörries and ter Meulen (1983). In this assay, viral antigen was adsorbed to the solid phase to react with test serum, with the indicator being an alkaline phosphatase labelled anti-human IgM. The cut-off level was determined in the assay as being the last serum dilution that gave a net absorbance of > 0.25, a value which represented the mean net absorbance plus three standard deviations of a 1:100 dilution of 40 sera from healthy newborn children. Serum samples were also tested for rheumatoid factor. Results obtained by these authors suggested three patterns of human IgM response: homotypic, type-predominant with weak heterotypic reaction, and heterotypic. The technique of immunoblot analysis, or Western blotting, was utilized to investigate the problem of homotypic and heterotypic reactions. By this technique, the antigenic determinants of the virus-specific IgM antibodies were detected. They found that their antigen preparations consisted of both intact infectious virus and incomplete virions on the basis of the presence of large amounts of VP 0, VP 1, and VP 3 and small amounts of VP 2 and VP 4 after sucrose density gradient centrifugation and SDS-polyacrylamide gel electrophoresis. Reaction of the separated virus polypeptides with patient sera exhibiting heterotypic or homotypic responses demonstrated that the type- and group-specific antigenic determinants were located on VP 1.

The ELISA presented by the authors has several disadvantages. The virus preparations utilized in the assay were not very pure as indicated by their own analysis of the relative amounts of the four

virion proteins. Consequently, the reactions obtained by ELISA varied from homotypic to heterotypic reactions. As Katze and Crowell (1980) have shown and the results of Dörries and ter Meulen (1983) have implied, a type-specific reaction in these immunoassays is dependent on the presence of intact whole virions, thus, a more purified preparation of antigen. Also, in this solid phase antigen-bound system, as with the indirect ELISA of Katze and Crowell (1980), rheumatoid factor in human sera could result in false positive results. Therefore, an ELISA utilizing the capture antibody principle would be more advantageous.

Morgan-Capner and McSorley (1983) established an antibody capture RIA for detection of CBV 4 and 5 specific IgM. The assay was not an ELISA, but is worth considering for the experimental data obtained and for comparison of similar immunoassay systems. Their assay involved a negative serum control, a positive serum control, and a low positive serum control obtained by diluting the positive serum 1:20 in negative control serum. The authors set very strict criteria for a positive reaction. Only sera exhibiting a reaction greater than the mean plus three standard deviations of sera from 100 healthy adults were considered positive. This value was equivalent to a positive/ negative control serum (T/N) ratio of 2.5 for the CBV 4 assay, and 2.9 for the CBV 5 assay.

Sera taken from patients who had CBV 4 (4 cases) or CBV 5 (6 cases) isolated were positive in the homotypic IgM assay. Heterotypic responses were also detected in the early convalescent sera and in 10/20 cases of CBV 1, 2, 3, 5, or 6 infection, there was group

reactivity in the CBV 4 IgM assay. In 7/18 (39%) cases of CBV 1, 2, 3, 4, and 6 infections, sera reacted in the CBV 5 assay. Moreover, sera from 9/13 (69%) patients from whom coxsackie A or echoviruses had been isolated also resulted in a positive reaction in both the CBV 4 and CBV 5 IgM assays. Positive reactions were not found with sera from 41 cases of infection due to viruses other than enteroviruses or 5 cases of Mycoplasma pneumoniae infections. Twelve rheumatoid factor containing sera were also negative in both assays.

Although this immunoassay was limited to only CBV 4 and 5, some general information was derived from the study. The authors have confirmed the fact that IgM-capture antibody assays are relatively unaffected by interference with rheumatoid factor in human sera. Consequently, for IgM detection, future assays should be of this type to minimize nonspecific reactions. The RIA presented by these authors also did not utilized highly purified antigen. The question arises concerning specificity of reaction in the assay using crude virus preparations. Crude virus preparations will contain both intact and incomplete virions. Therefore, such a preparation will result in the exposure of some group determinants in the immunoassay in the form of incomplete virions, and possibly decrease the type-specificity of the assay. However, as the authors have indicated, the difficulty of developing a type-specific ELISA may also relate to heterotypic IgM responses in CBV infections in humans. If this occurs, it may be impossible to develop a homotypic IgM enzyme immunoassay.

Banatvala et al. (1983) developed an IgM ELISA for analyzing the CBV-specific IgM responses in children with insulin-dependent

(juvenile-onset; type 1) diabetes mellitus (IDDM). The assay utilized a pool of CBV 1-6 antigen and a pool of CBV 1-6 antisera. The ELISA utilized was an improved system of an earlier enzyme immunoassay for detection of CBV-specific IgM (El-Hagrassy et al., 1980). The improved assay involved the use of monovalent CBV 1-6 virus reagents, and a peroxidase labelled antibody as the detector. The negative cut-off value was taken as three standard deviations above the mean value of sera negative for CBV-specific IgM. Each test also included reference positive and negative sera, and samples were tested in triplicate at a 1:400 dilution. Their results were standardized by correcting the sample readings back to the positive reference to compensate for day-to-day variations. All sera giving positive reactions by ELISA were tested for rheumatoid factor, and confirmed by a blocking test in which the reaction of their mouse anti-CBV sera was blocked by rabbit anti-CBV sera.

In 11/28 (39%) children aged 3-14 years who developed IDDM, CBV 1-6 IgM responses were detected. Five patients had a homotypic response to CBV 4 and one had a homotypic response to CBV 5. Of 290 children in the control group, only 16/290 (5.5%) had CBV-specific IgM responses. Also, only 6/18 sera were positive for CBV-specific IgM whereas 15/18 of these sera were positive for islet-cell cytoplasmic antibodies (IgG) and complement fixing islet-cell antibodies, therefore, suggesting that CBV and islet-cell antibodies were not cross-reactive. Upon examination of sera from children with virologically confirmed CBV infection (either by virus isolation and/or four-fold rise in neutralizing antibody titer), the development of heterotypic or homotypic

antibodies in CBV infections was shown to be age-related. 29/36 (81%) children aged 6 months to 4 years had a homotypic response, whereas 44/57 (77%) persons aged 15 years had a heterotypic response. 74/80 (92.5%) of these patients had CBV-specific IgM by ELISA.

The results presented by these authors implied that a type-specific IgM ELISA was possible to develop. The experiments were adequately controlled and the results obtained are important as preliminary data for the association of IDDM with CBV infections. However, the purity of the antigen preparations, and the methods for quantitation and standardization of the CBV 1-6 antigens and antisera were not clearly defined in the publication. Since the antigens and antisera used by these authors were obtained from a commercial source, investigation into this source, however, revealed that the antigens and antisera were actually CF reagents. Therefore, the antigen preparations used by the authors were not pure preparations of virus as they were only CF antigens. Consequently, a type-specific reaction obtained with the crude virus preparations in this ELISA for CBV-specific IgM does not support the data presented by Katze and Crowell (1980) and by Dörries and ter Meulen (1983). A possible explanation for the differences in experimental results is that the ELISA of the latter two groups is an indirect ELISA system where the antigen is directly attached to the solid phase. In these enzyme immunoassays, there can be competition between IgM and IgG for the antigenic sites, which may possibly be altered during the attachment to the solid phase. Consequently, there may be exposure of group determinants due to some conformational rearrangement of proteins or some other similar

disturbance. In a capture IgM system, these occurrences may not occur since antigen is not directly attached to the solid phase.

To date, there appears to be general agreement that the IgM response in CBV infections do not occur as a consequence of virus infections other than those due to enteroviruses. Only one case (from an influenza A infection) was positive for CBV IgM out of the combined total of 142 sera from non-enteroviral infections studied by Minor et al. (1979), El-Hagrassy et al. (1980), and Morgan-Capner and McSorley (1983). It is highly probable, though, that in the case of of influenza A infection from Minor et al. (1979), the patient had contracted a CBV infection shortly before the confirmed influenza A infection rather than a CBV-specific IgM response produced as a result of an influenza A infection (Pattison, 1983). From the previous reports, it appears that obtaining a type-specific ELISA for CBV-specific IgM is much more difficult than developing a group-specific test. For diagnostic purposes, though, a group-specific test is most invaluable. The ELISA developed by Banatvala et al. (1983) appears to be the first type-specific IgM assay for CBV, therefore, indicating that a specific assay is possible. Nevertheless, definitive conclusions regarding the extent of heterotypic reactions to other viruses in CBV infections cannot be made from the studies that have be conducted thus far. Of possible future interest is the examination of sera from cases of hepatitis A, as there is some consideration in classification of the hepatitis A virus as an enterovirus (Melnick, 1982). The present course of the solid phase immunoassay, though, appears to require the antibody capture principle.

II. MATERIALS AND METHODS

II. Materials and Methods

A. Culture and Purification of Coxsackie B Viruses

(i) Growth of CBV

Reference strains of CBV 2-5 were obtained from the American Type Culture Collection (ATCC), Rockville, Md. The cell line chosen to culture the CBV was a continuous African green monkey kidney cell line, designated BGM, which has been demonstrated to be a very sensitive cell line for culturing CBV (Barron et al., 1970; Menegus and Hollick, 1982).

The BGM cells (passage 74) were obtained from Flow Laboratories, McLean, Va., and were grown in medium consisting of Eagle's minimal essential medium (MEM) (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% inactivated fetal bovine serum (Gibco), 50 ug/ml of gentamycin (Roussel Canada, Montreal, Que.), and buffered to pH 7.4 with 7.5% sodium bicarbonate solution (Gibco). The BGM cells were cultured in 490 cm^2 roller bottles (Corning Glass Works, Corning, N.Y.) at 35° C until confluency. The cells were washed three times with phosphate-buffered saline (PBS), and then infected with one of CBV types 2-5. The roller bottles were incubated at 35° C until a 4+ cytopathic effect (CPE) was observed. The CBV was harvested by freeze-thawing the roller bottles three times at -70° C.

(ii) Purification of CBV

The initial purification of the harvested CBV involved differential centrifugation where a series of clarification steps separated the cellular debris from the viruses. The crude CBV was first clarified by centrifugation at 3000 rpm in a Beckman TJ-6 centrifuge for 20 minutes. The supernatant was then centrifuged at 15,000 rpm for 1 hour with a Type 35 rotor (Beckman) in a Beckman L5-65 ultracentrifuge. Clarified virus (supernatant) was pelleted by centrifugation at 30,000 rpm for 5 hours with a Type 35 rotor in a Beckman L5-65 ultracentrifuge. The virus pellet was resuspended in 1% NaCl to 1/100th the original volume. The concentrated virus was then purified by cesium chloride density gradient centrifugation.

Briefly, four CsCl solutions (1.4969 g/cm^3, 1.4196 g/cm^3, 1.3110 g/cm^3, and 1.2055 g/cm^3) were made with 0.01M Tris-HCl buffer, pH 9.0 (Griffiths, 1976). The CsCl gradients were made in 5.0 ml cellulose nitrate tubes (Beckman #305050) by successively layering 1.0 ml of each of the four CsCl solutions starting with the most dense solution at the bottom of the tubes. 0.5 ml of the concentrated crude preparation of virus was then layered on top of the formed gradient, followed by 0.5 ml of mineral oil on top of the virus and gradient. The CsCl gradients were centrifuged with a Beckman SW 50.1 rotor in a Beckman L5-65 ultracentrifuge for 20 hours at 40,000 rpm. To collect the purified virus fractions, the cellulose nitrate tubes were punctured at the bottom and fractions of 0.3 ml were collected using a Beckman micro-fractionator. The refractive index of each fraction was determined utilizing a Bausch and Lomb refractometer. The densities were computed

from the refractive indices using the relationship $p^{25} = 10.2402\ n_D^{25} - 12.6483$ for densities between 1.00 and 1.38, and $p^{25} = 10.8601\ n_D^{25} - 13.4974$ for densities above 1.37 (Bruner and Vinograd, 1965). Fractions banding at approximately 1.34 g/cm^3 were examined by electron microscopy (EM) and assayed for infectivity to confirm the location of the purified virus in the gradient. The fractions containing the purified intact virus were pooled and stored at 4° C.

Purity of the virus preparations was examined only by electron microscopy utilizing the procedure of Hammond et al. (1981). Figure 2 is a diagrammatic representation of the culturing and purification process of the CBV.

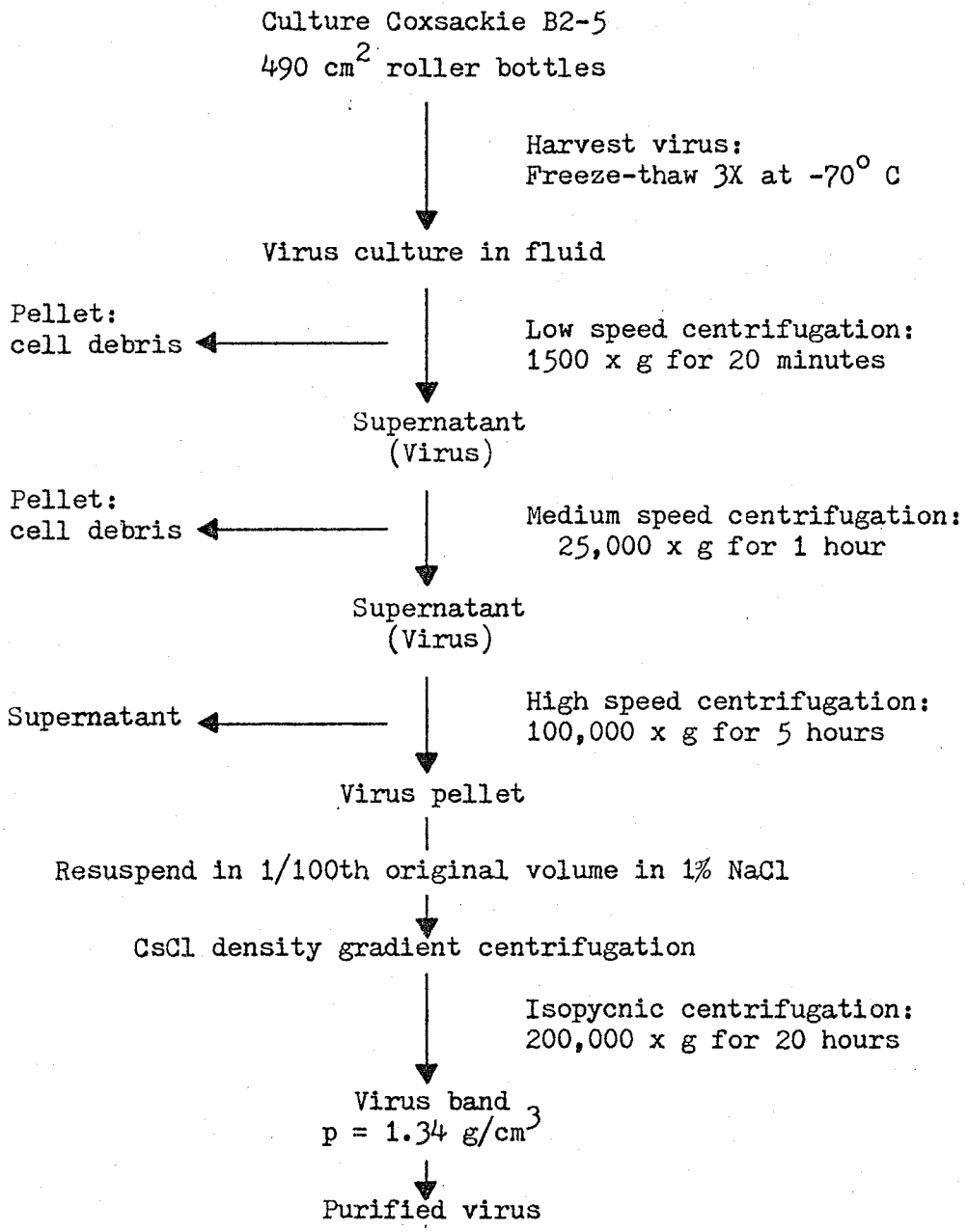
(iii) Quantitation of CBV

(a) Protein determination

The purified CBV was assayed for total protein content by the microprotein determination method of Schaffner and Weismann (1973). Several minor modifications of the method were employed for determination of the total protein of the virus preparations. For the washing steps in the method, 10% trichloroacetic acid (TCA) was used to rinse the tubes in which the protein had initially been precipitated with 60% TCA. Both the tubes and the filter paper strips (where the precipitated protein was spotted) were rinsed three times with 10% and 6% TCA, respectively. Also, the time in which the stained filter paper spots (protein) were in contact with the eluant solution was increased from 10 minutes to 30 minutes.

FIGURE 2

Growth and Purification of Coxsackie B Viruses



(b) Virus titration

Virus titrations of either crude or purified virus preparations were performed utilizing microtiter plates. Serial dilutions of the virus from 10^{-1} to 10^{-9} were made in tubes with diluent consisting of Eagle's MEM buffered with 0.02M HEPES and supplemented with penicillin (Glaxo Laboratories, Toronto, Ont.) (100 IU/ml), streptomycin (Allen and Hanburys, Toronto, Ont.) (100 ug/ml), and Nystatin (E.R. Squibb & Sons, Princeton, N.J.) (50 ug/ml). After 0.025 ml of the diluent was added to the appropriate wells of 96-well microtiter plates (Becton Dickinson, Oxnard, Calif.), 0.025 ml of the virus dilutions were then added to the same wells of the microtiter plates. Another 0.050 ml of diluent was added to the wells to bring them to a volume equivalent to that of microneutralization test. 0.1 ml of BGM cells at a concentration of 200,000 cells/ml was then added to the wells. A cell control was included with each plate. The microtiter plates were then placed in plastic bags (to prevent evaporation) and incubated for 4 days at 35° C. Each of the virus dilutions was tested in quadruplicate and after 4 days, wells were scored as (+) or (-) CPE. The 50% tissue culture infective dose ($TCID_{50}$) was then determined by the Karber method.

B. Patient Sera

Sera from 24 children were obtained from the St. Amant Center, Winnipeg, Manitoba, during a CBV 4 outbreak at this center. Of these individuals, 19 had isolation of CBV 4 either from a stool sample and/or

throat swab which were taken upon the appearance of symptoms (fever or pharyngitis) in these children. The other 5 children were negative for CBV isolation. The acute and convalescent serum samples were obtained approximately two and four weeks, respectively, after the appearance of symptoms in these children.

C. Microneutralization Test

The neutralizing antibody titers of the patient sera described in (B) were determined by microneutralization for a later comparison to ELISA for sensitivity and specificity. Neutralizing antibody titers to CBV 2-5 were determined using a method based on the procedure described by Rosenbaum et al. (1963).

Patient sera were diluted 1:5 with Eagle's MEM buffered with 0.02M HEPES and supplemented with 100 IU/ml penicillin (Glaxo Lab., Toronto, Ont.), 100 ug/ml streptomycin (Allen and Hanburys, Toronto, Ont.), and 50 ug/ml Nystatin (E.R. Squibb & Sons, Princeton, N.J.). The diluted sera were then heat inactivated at 56° C for 30 minutes. 0.025 ml of MEM was dropped into every well of test, positive and negative serum control plates, and 0.050 ml into cell control wells of 96-well microtiter plates (Becton-Dickinson, Oxnard, Calif.). Next, 0.025 ml of diluted sera was added to duplicate wells to the first wells of the appropriate plates. Microdiluters (0.025 ml) (Linbro Scientific, Hamden, Conn.) were used to serially dilute the sera to the end of the plates (8 wells), thus, giving serum dilutions ranging from 1:10 to 1:1280. Virus was added next to all wells of the appropriate plates at a

concentration of 100 $TCID_{50}$ per well (0.025 ml). The virus was then further log diluted for a back titration and 0.025 ml of each dilution was added to four wells of the virus control plate. The plates were then shaken on a mechanical shaker for 30 seconds, and incubated at 35° C for 1 hour. Following the incubation period, 0.050 ml of MEM was added to all wells. BGM cells were then prepared at a concentration of 200,000 cells/ml in MEM supplemented with 3% heat inactivated fetal bovine serum. 0.1 ml of the cells were added to all wells. Plates were placed in plastic bags (to prevent evaporation) and incubated at 35° C until the virus control reading was 100 $TCID_{50} \pm 0.25$, and the serum control read the correct titer ± one well.

A four-fold or greater rise in neutralizing antibody titer between the acute and convalescent sera represented a seroconversion. A permanent record of the microneutralization tests can be made by staining of the microtiter plates by the method of Hierholzer and Bingham (1978).

D. Sucrose Density Gradient Centrifugation

Sucrose density gradient centrifugation was performed on the patient sera described in (B) to fractionate the IgM from other serum components. Only those sera with sufficient volume (at least 0.3 ml) were fractionated by sucrose gradients. An adaptation of the method of Palmer et al. (1977) was used.

Working dilutions of sucrose (31.25%, 25.0%, 18.75%, 12.5%) were made up from a stock solution of 37.5% sucrose utilizing PBS, pH 7.2.

The gradients were made by successively layering the sucrose solutions in 5.0 ml cellulose nitrate tubes (Beckman #305050), starting with the most dense solution first. The gradients were allowed to sit at 4° C for an hour to become continuous. 0.3 ml of patient sera was then layered on top of the sucrose gradient. Using a Beckman SW 50.1 rotor, the gradients were centrifuged for 16 hours at 35,000 rpm in a Beckman L5-65 ultracentrifuge. When the centrifugation was complete, fractions of approximately 0.3 ml were collected using a Beckman microfractionator. Fractions 1 to 4 were tested for the presence of IgM and IgG by radial immunodiffusion (RID) using RID plates obtained from Kallestad Laboratories, Austin, Tex. Fractions containing IgM were pooled and dialysed overnight against PBS, changing the dialysate after the first 6 hours. The dialysed IgM was then concentrated to approximately the original volume using an Amicon S125 serum concentrator. Fractionated IgM was then titreated for CBV-specific IgM by microneutralization tests.

E. ELISA: Optimization of Reagents

A series of experiments were designed to evaluate the various reagents for the IgM ELISA, and to optimize these reagents for the assay.

(a) Optimization of Enzyme-Conjugates

Several enzyme conjugates were optimized and tested during the development of the IgM ELISA. The sensitivity and optimization of the peroxidase conjugated F(ab')2 fragment goat anti-

horse IgG (heavy and light chain specific) (Cappel Laboratories, Cochranville, Pa.) and peroxidase conjugated F(ab')2 fragment goat anti-rabbit IgG (heavy and light chain specific) (Cappel) was performed by checkerboard titration. The anti-horse IgG conjugate and the anti-rabbit IgG conjugate were titrated at different dilutions with various concentrations of horse IgG and rabbit IgG, respectively. Horse IgG was obtained by ammonium sulfate precipitation of normal horse serum followed by purification by DEAE-cellulose ion-exchange column chromatography.

The gamma globulin fraction from normal horse serum was obtained by precipitation with an optimum concentration of ammonium sulfate as determined by Hebert et al. (1973). Three precipitations of the horse serum were performed with an ammonium sulfate concentration of 30% at 4° C. The fractionated gamma globulins were then dialysed extensively at 4° C against 0.85% NaCl in distilled water (pH 8.0) until the dialysate was free of sulfate ions.

For the purification of the gamma globulins, a DEAE-cellulose column (2 x 30 cm) was prepared and equilibrated with 0.01M phosphate buffer, pH 8.0 (Sternberger, 1979; Helms and Allen, 1970). The horse gamma globulin fraction was then applied to the ion-exchange column. Utilizing a LKB fraction collector (Pharmacia), 50 fractions of 3.5 ml each at a rate of approximately 1 drop/5 seconds were collected. To locate the purified horse IgG fractions, the absorbance of each fraction was read at 280 nm in a U.V. spectrophotometer. Fractions having an absorbance greater than 0.10 were pooled. Purity of the IgG from other contaminants, i.e., IgM or albumin, was checked by slide immunoelectro-

phoresis.

Slide immunoelectrophoresis was performed as follows. Precleaned slides (Bev-L Edge slides, Fisher Scientific) were precoated with 2.0 ml of 0.5% agarose (Pharmacia) per slide. After allowing the slides to set for 10 minutes and then drying overnight at 35° C with #1 Whatman filter paper covering them, the slides were coated with 3.0 ml of 2% agar (0.6 g Noble agar (Difco), 15.0 ml 0.05M barbital buffer, pH 8.6, 15.0 ml distilled water) per slide. Slides were then allowed to harden overnight at 4° C in a moist chamber. Wells in the slides were cut with a Culliford cutter (wells = 2mm, trough = 60 x 2 mm) and 2-4 ul of the sample (purified horse IgG or gamma globulin) was loaded into the wells using a hematocrit capillary tube. Slides were placed in the electrophoresis apparatus, 0.05M barbital buffer (pH 8.6) was added to the two chambers of the apparatus, and paper wicks (#1 Whatman filter paper) were placed in a position such that the slides were in contact with buffer. The slides were electrophoresed for 1 hour using 5 mA per slide. Following this, the trough was loaded with the appropriate antiserum, and the slides were then incubated overnight at room temperature in a moist chamber to allow the precipitin lines to develop. Slides were then washed in 0.85% saline overnight, distilled water for 4 hours, and then dried overnight at room temperature with filter paper covering them. The dried slides were then stained with Amido Black (1.0 g Amido Black (Sigma) , 25.9 ml glacial acetic acid, 6.8 g sodium acetate) for 10 minutes. Slides were destained by 3 washes of 10 minutes each with destaining solution (980 ml methanol, 20 ml glacial acetic acid, 300 ml distilled water), and then air dried.

The purified horse IgG was concentrated by using an Amicon S125 serum concentrator. Rabbit IgG was purified from normal rabbit serum by similar methods (Greg James, M. Sc. Thesis, University of Manitoba, Winnipeg, Mb.). The total protein content of the purified horse and rabbit IgG was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.).

Polystyrene beads (6.4 mm diameter) (Precision Plastic Ball Co., Chicago, Ill.) were coated overnight at 4° C in 0.06M carbonate-bicarbonate buffer, pH 9.6 with concentrations of horse IgG or rabbit IgG ranging from 1 pg to 1000 ng. Beads were post-coated with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.3, for 1 hour at room temperature. The beads were then placed in the appropriate wells of 20-well polystyrene reaction trays (Abbott Laboratories, Chicago, Ill.) and washed once with PBS containing 0.05% (v/v) Tween 20 (PBST) using an Abbott pentawash-aspirator device (Plate 1). Dilutions of the appropriate conjugate (either anti-horse or anti-rabbit IgG) ranging from 1:2000 to 1:10,000 were made with phosphate-buffered saline containing 2% BSA (w/v) and 0.15% Tween 20 (v/v) (PBS-BSAT), and 0.2 ml of each dilution was added in duplicate to the beads and allowed to incubate for 1 hour at 37° C. Beads were washed 5 times with PBST and transferred to another tray before addition of the enzyme substrate, o-phenylenediamine (OPD) plus hydrogen peroxide (H_2O_2). After a 30 minute incubation period, the enzyme reaction was terminated with 0.1 ml of 8N sulfuric acid (H_2SO_4). The reaction was quantitated by optical density (OD) at 492 nm. Controls included beads not coated with horse or rabbit IgG, and substrate control wells.

PLATE 1

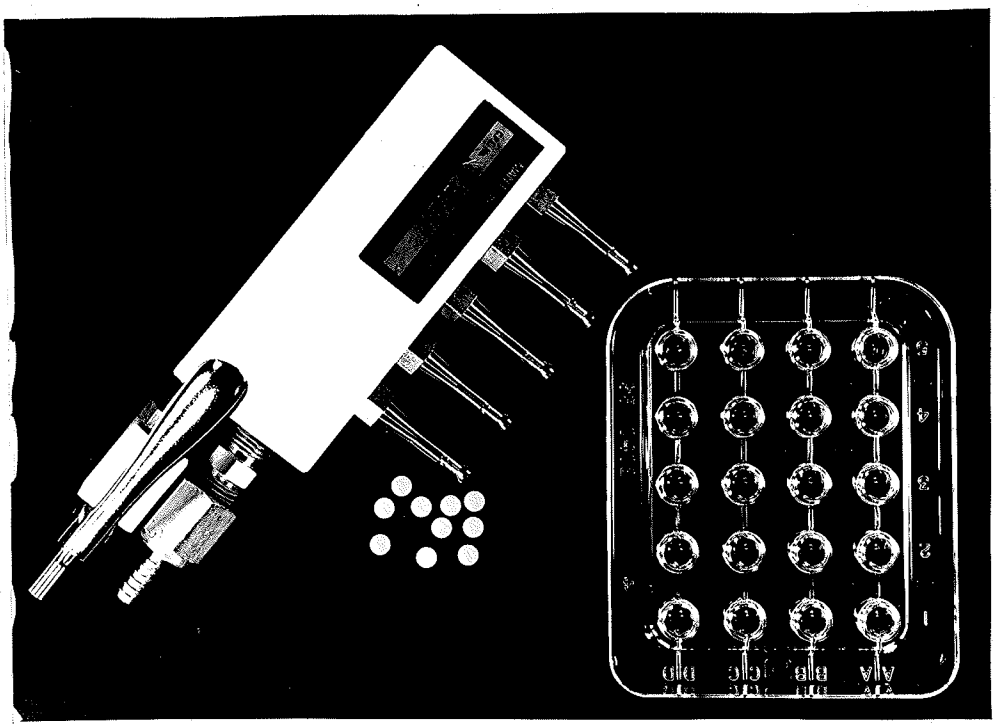


Plate 1: Photograph of equipment used in ELISA. From left to right: Abbott Pentawash-aspirator; Polystyrene beads (6.4 mm); 20-well polystyrene reaction trays.

The negative cut-off levels were determined as 2X the OD values of the background activity due to the nonspecific binding of the conjugate.

Peroxidase conjugate F(ab')2 fragment goat anti-human IgM (u-chain specific) (Cappel) was optimized by checkerboard titration with human IgM. Purified human IgM was obtained by sucrose density gradient centrifugation of normal human serum as described earlier in Materials and Methods. The fractionated IgM was tested for purity by radial immunodiffusion (RID). RID plates for detection of IgM (6-50 mg/dl) and IgG (2.0-25.5 mg/dl) were obtained from Kallestad Laboratories, Austin, Tex. The protein content of the purified human IgM was determined by the Bio-Rad protein assay. Concentrations of purified human IgM ranging from 10 pg to 10 ug were utilized to determine the sensitivity and the optimal dilution of the anti-human IgM conjugate. The assay test procedure was performed as described earlier.

The optimal conjugate dilution that was chosen for the ELISA was the concentration that offered the best balance between sensitivity, the level of background activity, and economy of use.

(b) Optimization of Capture Antibody

Three types of anti-human IgM, or capture antibody, were evaluated for the IgM ELISA. These capture antibodies were a goat anti-human IgM (u-chain specific) (Cappel Laboratories, Cochranville, Pa.), two affinity purified goat anti-human IgM (u-chain specific) antibodies (Cappel Laboratories and Dakopatts Antibodies, Westbury, N.Y.), and a mouse monoclonal anti-human IgM (u-chain

specific) (Cappel). The sensitivity and optimal coating concentrations were determined by checkerboard titration with purified human IgM. IgM bound by the capture antibodies was detected using the peroxidase conjugated F(ab')2 fragment goat anti-human IgM which was optimized in previous experiments. Briefly, capture antibody was coated onto polystyrene beads at a concentration deemed to saturate the support surface. These concentrations were 5 ug/bead for goat anti-human IgM, 1 ug/bead for the affinity purified anti-human IgM's, and 1 ug/bead for the monoclonal anti-human IgM. To determine the sensitivity of the capture antibodies, concentrations of human IgM ranging from 1 ng to 10 ug were utilized, with the capture IgM detected by the anti-human IgM conjugate. The assay procedure was performed as described previously. Cut-off levels were determined as 2X the background activity due to the nonspecific binding of the conjugate.

The capture antibody chosen for the ELISA was the antibody which demonstrated the greatest IgM binding ability at an economical concentration.

(c) Specificity of CBV Antisera

The specificity of two commercial antisera to CBV 2-5 was assessed by neutralization tests and by ELISA. Horse CBV 2-5 antisera (ATCC, Rockville, Md.) and rabbit CBV 2-5 antisera (Microbiological Associates, Walkerville, Md.) were tested for specificity to the homologous CBV prototype strain by microneutralization tests. The horse antisera were tested at dilutions from 1:20 to 1:40,960 and the rabbit antisera from 1:10 to 1:20,480.

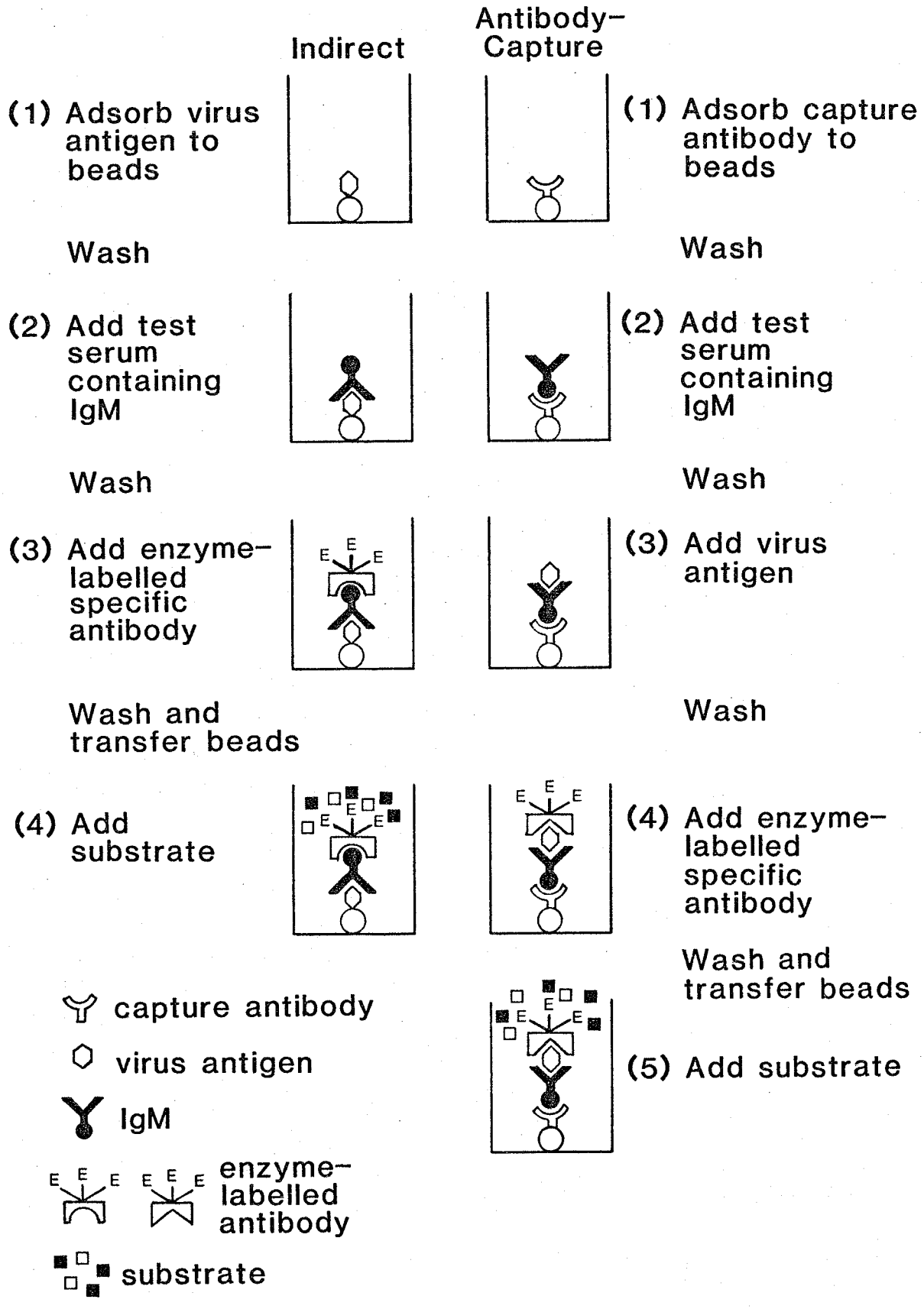
The effect of varying the concentrations of CBV antigen and antisera by ELISA was also examined. Experiments were performed to evaluate the effects of variation of CBV horse antisera concentrations with varying concentrations of both pure and crude virus preparations. An indirect ELISA method (Figure 3) was utilized for the experiments. Virus antigen (either purified or crude) was coated on polystyrene beads overnight at 4° C in 0.06M carbonate-bicarbonate buffer, pH 9.6. The horse antisera was added next followed by the peroxidase conjugated anti-horse IgG. The OD readings obtained were corrected for background activity by subtraction of the the absorbance due to nonspecific binding of each antiserum concentration and conjugate to the beads. Test conditions were the same as outlined previously. Virus antisera were titrated from 5 U to 200 U with one unit (U) of antibody defined as the neutralizing antibody titer of the antisera. Virus antigen was tested from 10^4 to 10^7 $TCID_{50}$ using purified titrated virus preparations. Virus antisera concentrations were titrated from 1 U to 50 U and virus antigen from 1 to 100 ug protein when evaluating crude virus preparations. The OD readings in these experiments were obtained after subtraction of absorbance due to background activity caused by nonspecific binding at each antiserum concentration and conjugate with control antigen (mock-infected cell supernatant).

The specificity of the horse CBV antisera by an indirect ELISA method was also evaluated utilizing both crude and purified preparations of CBV. Crude CBV 2-5 preparations were coated on the polystyrene beads at an equal concentration of 50 ug/bead, and purified CBV 2-5 antigen

was coated at a concentration of 1 ug/bead. The amount of each CBV antisera used was 50 U. The reaction was detected by use of the peroxidase conjugated anti-horse IgG. All optical density values were corrected for background activity as described earlier.

FIGURE 3

ELISA For Measuring IgM Antibody



F. ELISA: Test Procedure

A schematic representation of the basic principles of the capture antibody ELISA is shown in Figure 3. The IgM ELISA in this thesis utilized the following established procedure. Polystyrene beads were coated overnight at 4° C with 1 ug/bead of affinity purified goat anti-human IgM in carbonate-bicarbonate buffer, 0.06M, pH 9.6. The beads were then post-coated with 1% BSA in PBS, pH 7.3, for 1 hour at room temperature. Coated beads were then placed in the wells of 20-well polystyrene reaction trays and washed once with PBST. Patient serum was diluted 1:100 in PBS-BSAT and 0.2 ml of the diluted serum was added in duplicate to the appropriate wells in the reaction trays. The serum was incubated with the capture antibody coated beads for 1 hour by placing the reaction trays in a 37° C water bath. Following this incubation period, the beads were washed 5 times with PBST. Crude CBV 2-5 antigen was then added separately to the appropriate wells at an equal concentration of 50 ug/0.2 ml diluted in PBS, pH 7.3. Control antigen wells received 0.2 ml of a mock-infected cell supernatant prepared in the same way as the virus antigen. Antigen was then incubated with the beads for 1 hour at 37° C or overnight for 16-24 hours at 4° C. After this incubation, the beads were washed 5 times with PBST. A modification of the capture antibody protocol outlined in Figure 3 was incorporated at this point. CBV 2 and 5 antisera (horse) diluted in PBS-BSAT to concentrations of 25 U/0.2 ml and CBV 3 and 4 antisera diluted to 5 U/0.2 ml were added to the appropriate wells, and incubated with the beads for 1 hour at 37° C. Following this

incubation period, beads were washed 5 times again with PBST. Peroxidase conjugated F(ab')2 fragment goat anti-horse IgG was then diluted 1:6000 in PBS-BSAT and 0.2 ml was added to the wells. The incubation period for the conjugate was for 1 hour at 37° C. After the incubation with the conjugate, the beads were washed 5 times with PBST and transferred to a new plate. The substrate solution consisting of 40 mg of OPD and 0.040 ml of 30% H_2O_2 in 100 ml of 0.01M citrate-phosphate buffer was made immediately before use. 0.4 ml of this substrate solution was added to each well of the reaction trays. Following an incubation of 30 minutes at 37° C, the enzymatic reaction was terminated with 0.1 ml of 8N H_2SO_4. The color reaction was then quantitated by optical density at 492 nm in a Gilford Stasar III spectrophotometer.

Each patient serum was utilized as its own control with duplicate wells containing virus antigen, and duplicate wells containing control antigen which was mock-infected cell supernatant prepared in the same was as the virus antigen.

The optical density (OD) readings of the ELISA tests were calculated with the following equation: Mean net OD = mean OD of virus antigen wells - mean OD of the control antigen wells. The negative cut-off value for the ELISA was established by testing the cord bloods from 42 newborn infants known to be seronegative for antibody to CBV. The cut-off OD was determined as the mean net OD plus three standard deviations of the 42 cord bloods. Other controls included an antiserum control (bead plus antiserum and conjugate only), a conjugate control (bead plus conjugate only), and a substrate control (bead plus substrate only)

which served as a blank for the spectrophotometer readings. All tests were performed in duplicate.

The sensitivity of the ELISA was examined by testing doubling dilutions of two patients' sera: one serum with a high titer of CBV-specific IgM after sucrose density gradient fractionation and micro-neutralization tests, and one serum with a low titer of IgM. The optimal serum dilution for the IgM ELISA was derived from this experiment.

A blocking assay was also performed to test specificity of the detector antisera (horse CBV antisera) in the ELISA, utilizing rabbit antisera (Microbiological Associates, Walkerville, Md.) to block the reaction of the horse antisera. Rabbit CBV 2-5 antisera were used to block the horse CBV 2-5 antisera, respectively, and rabbit CBV 4 antiserum was also used to attempt to block the reaction of all four CBV horse antisera types in the IgM ELISA. The concentration of rabbit antisera used was 50 U.

To test day to day variability of the ELISA, five IgM positive and two IgM negative sera were tested in duplicate on separate days.

III. RESULTS

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A. Culture and Purification of Coxsackie B Viruses

(i) Growth of CBV

The BGM cell line grew readily in roller cultures, and the CBV produced a very distinctive cytopathic effect (CPE) in these cells involving rounding and shrinkage of infected cells, followed by the cells becoming refractile and eventually detaching from the tissue culture flask surface. The growth of the virus in the BGM cells was quite rapid when infected with high titered virus, and CPE began to appear approximately 6 hours post-infection with complete involvement of the cell monolayer in 12-24 hours. Virus titers varied between different virus preparations, and in summary, the range of CBV titers were ($TCID_{50}$/0.025 ml):

CBV 2 = $10^{6.0}$ - $10^{6.5}$ CBV 3 = $10^{4.5}$ - $10^{5.25}$

CBV 4 = $10^{5.5}$ - $10^{6.75}$ CBV 5 = $10^{6.0}$ - $10^{7.75}$

Crude virus preparations, obtained by freeze-thawing the infected cells, and clarifying at 1500 x g and 25,000 x g, were determined to have protein contents ranging as follows:

CBV 2 = 120 - 250 ug/ml CBV 3 = 140 - 240 ug/ml

CBV 4 = 300 - 315 ug/ml CBV 5 = 150 - 240 ug/ml

Plates 2a and 2b illustrate normal uninfected BGM cells and cells infected with CBV 4.

PLATE 2a

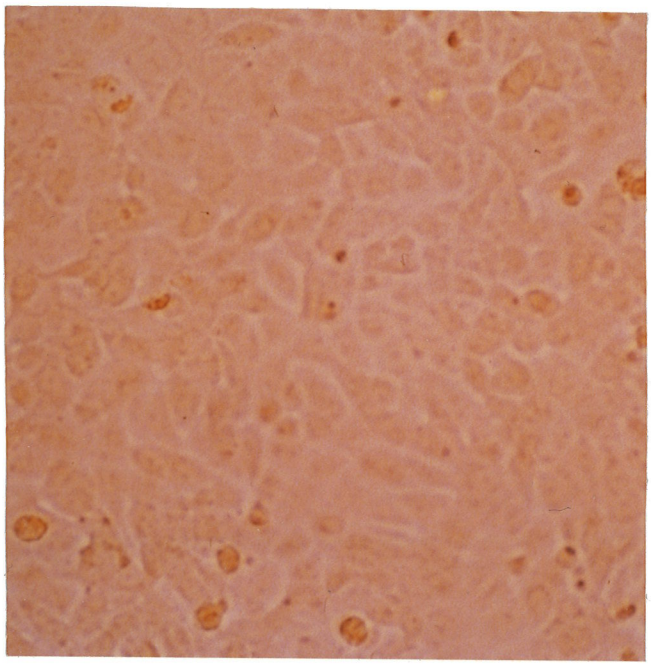


Plate 2a: Photomicrograph of uninfected BGM cells. (Magnification: 64X)

PLATE 2b

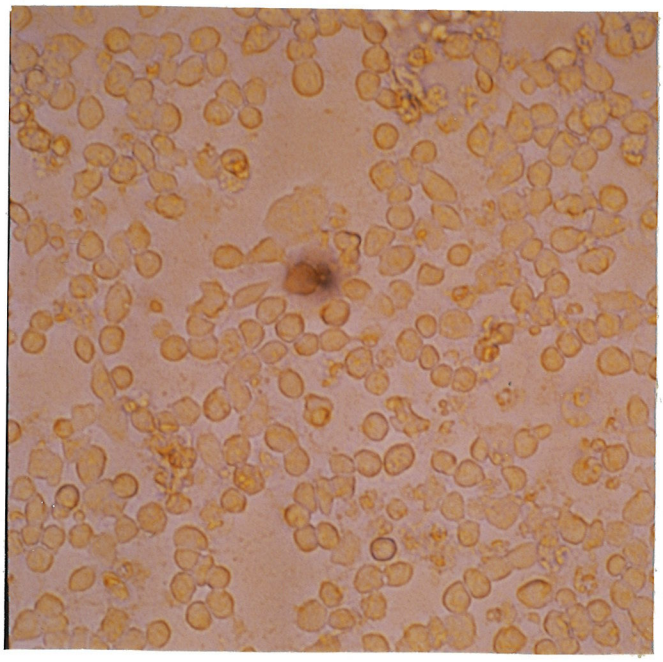


Plate 2b: Photomicrograph of BGM cells infected with CBV 4. Cytopathic effect at 16 hours post-infection. (Magnification: 64X)

(ii) Purification of CBV

By CsCl density gradient centrifugation, purified whole CBV was obtained consistently from fractions 8 and 9 as observed by EM. Infectivity profiles showed that infectious (whole) CBV was obtained only from fractions 8 and 9, therefore, these fractions were pooled. The purified virus banded at a density of 1.34 g/cm^3 and was examined for purity only be EM after negative staining with 1.5% phosphotungstic acid (Plate 3). Fractions following 8 and 9 contained incomplete and defective virus particles as well as some extraneous cell debris as observed under EM. Whole CBV from fractions 8 and 9 had infectivity titers that varied between virus preparations, and between CBV types. The ranges of infectivity titers observed were as follows ($TCID_{50}$/0.025 ml):

CBV 2 = $10^{6.0}$ - $10^{8.0}$ CBV 3 = $10^{4.75}$ - $10^{6.0}$

CBV 4 = $10^{6.0}$ - $10^{8.25}$ CBV 5 = $10^{6.25}$ - $10^{8.75}$

The protein contents of the purified preparations of CBV also varied and by microprotein assay, the following ranges were observed:

CBV 2 = 12 - 23 ug/ml CBV 3 = 6 - 12 ug/ml

CBV 4 = 33 - 40 ug/ml CBV 5 = 12 -20 ug/ml

The purified virus preparations were stored in the CsCl solutions at 4° C with no loss of infectivity observed over a period of four months.

PLATE 3

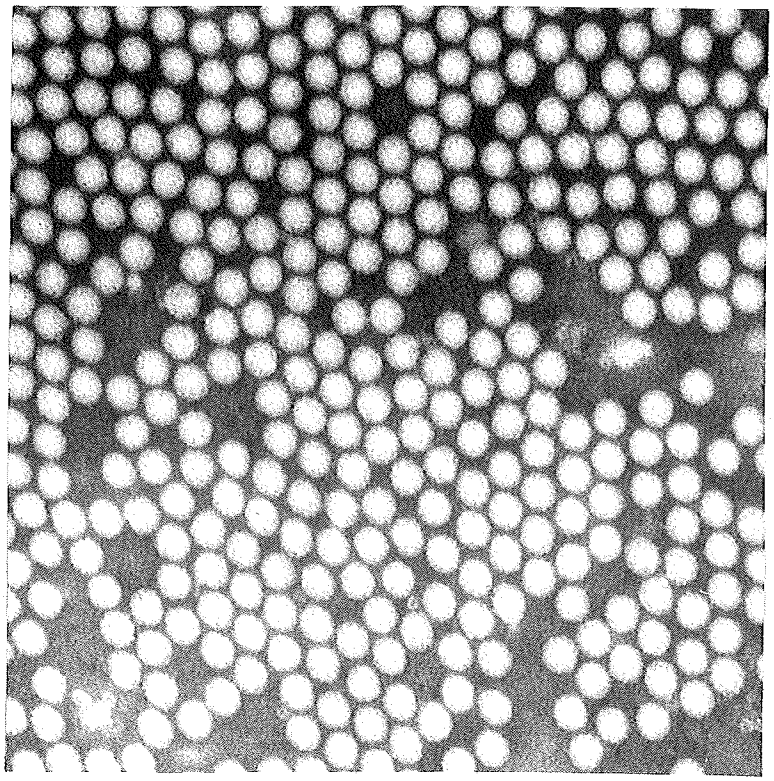


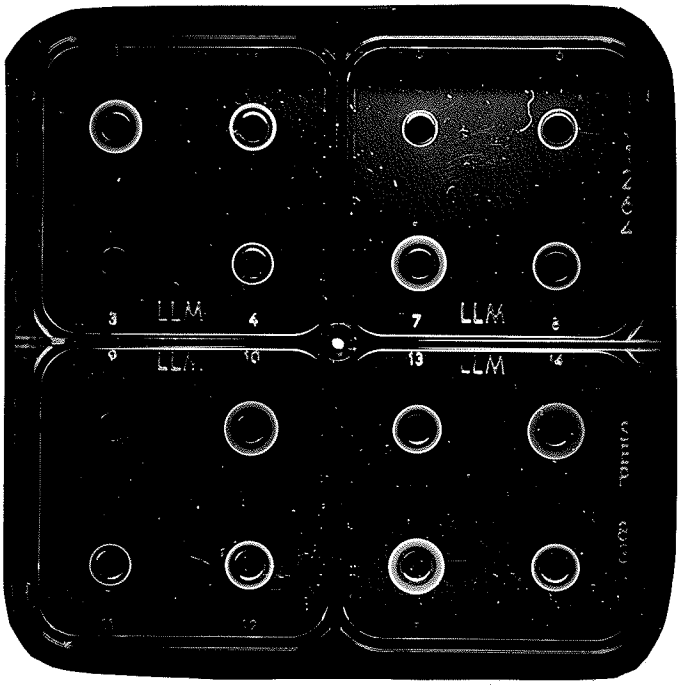
Plate 3: Electron micrograph of purified coxsackie B virus. (Magnification: 200,000 X)

B. Sucrose Density Gradient Centrifugation

Sucrose density gradient fractionation of human sera resulted in the collection of the IgM component from fractions 1 and 2 in the gradient. Testing of the sucrose gradient fractions by radial immunodiffusion (RID) demonstrated that the IgM was present in fractions 1, 2, and 3, and that the IgG started to appear in fraction 3 with the majority of the IgG in the following fractions (4 and 5). Of the panel of sera described in (B) of Materials and Methods, only those sera with sufficient volume were fractionated for IgM by sucrose gradients. Therefore, only 41/47 sera (acute and/or convalescent) were fractionated from the panel of sera from the 24 children described in (B) of Materials and Methods.

An example of the RID method of quantitating the purified IgM is illustrated in Plate 4. The diameter of the precipitin rings formed by the IgM standards was measured and plotted on a graph. The test (IgM) samples' rings were measured and the concentration of IgM determined by interpolation on the graph.

PLATE 4



Standards

Well 1:	50 mg/dl IgM	Well 2:	27 mg/dl IgM
Well 3:	6 mg/dl IgM	Well 4:	Serum control - 21 mg/dl IgM

Plate 4: Radial immunodiffusion of fractionated IgM from sucrose density gradient centrifugation of human serum. Wells 1-4 = IgM standards for standard curve. Wells 5-16 = IgM fractions.

C. Microneutralization Test

The microneutralization test was performed on the panel of sera (47) from the 24 children described in(B) of Materials and Methods, and on the IgM fractions from the 41 sera that were fractionated by sucrose gradients. The microneutralization titers and neutralizing IgM titers (of the sucrose fractions) of these sera are compared to ELISA results in Tables 17 and 18. The staining procedure for microtiter plates of the microneutralization test was a vital stain originally used by Hierholzer and Bingham (1978). An example of the stained microtiter plates for CBV microneutralization tests is illustrated in Plate 5.

PLATE 5

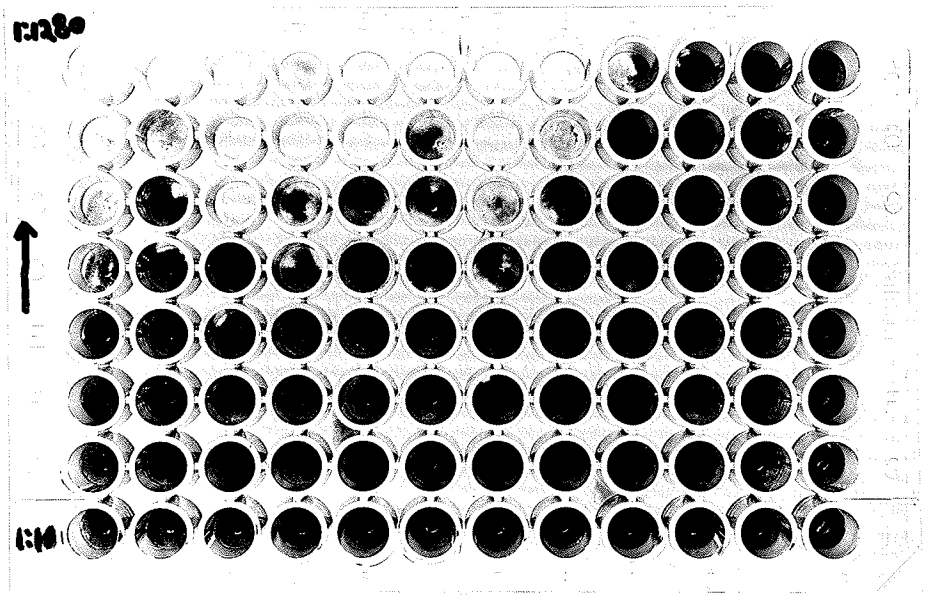


Plate 5: Crystal violet stained microneutralization test plate. (Dilution of human serum: 1:10 - 1:1280) Stained areas (dark areas) indicate intact (uninfected) cells, or negative cytopathic effect.

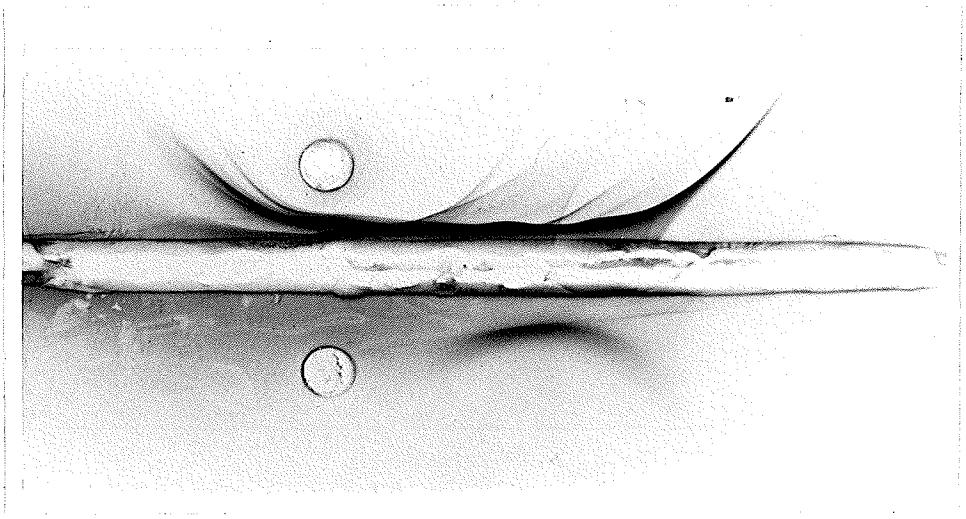
D. ELISA: Optimization Results

(i) Enzyme conjugates

The optimization of the three conjugates -- anti-horse IgG, anti-rabbit IgG, and anti-human IgM -- is illustrated in Tables 7, 8, and 9, respectively. The optimization curves of these conjugates are illustrated in Graphs 1, 2, and 3. Approximately 400 ug/ml of horse IgG was obtained from the purification process from 100 ml on normal horse serum. Plates 6a and 6b demonstrate the purity of the horse IgG and gamma globulin fraction by slide immunoelectrophoresis. Rabbit IgG was purified elsewhere (Greg James, M. Sc. Thesis, University of Manitoba, Winnipeg, Mb.). The optimal concentrations of the anti-horse and anti-rabbit conjugates were determined as a 1:6000 dilution, as this gave a high level of sensitivity, yet, only a low level of background activity.

The sensitivity of the anti-horse IgG conjugate was between 10 and 100 ng of horse IgG, and the sensitivity of the anti-rabbit IgG conjugate was between 0.1 and 1 ng of rabbit IgG. The anti-human IgM conjugate was optimized at a 1:6000 dilution also, with a sensitivity between 100-1000 ng of human IgM.

PLATE 6a



Contents of Wells

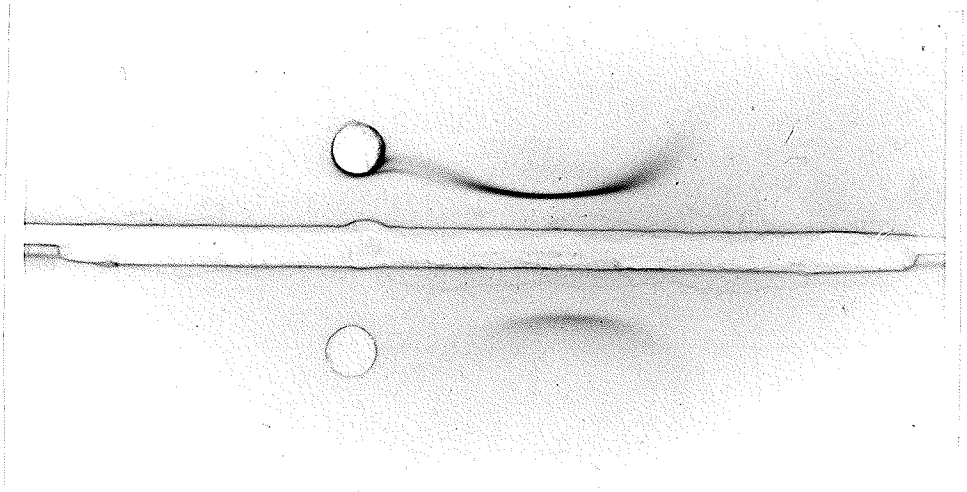
Upper well: Horse serum

Trough : Anti-horse whole serum

Lower well: Gamma-globulin fraction

Plate 6a: Slide immunoelectrophoresis of ammonium sulfate precipitated gamma globulin of horse serum.

PLATE 6b



Contents of Wells

Upper well: Gamma-globulin fraction

Trough : Anti-horse IgG

Lower well: Purified horse IgG

Plate 6b: Slide immunoelectrophoresis of purified horse IgG (from DEAE-cellulose ion-exchange chromatography).

TABLE 7

Optimization of Peroxidase Conjugated F(ab')2 Fragment
Goat Anti-Horse IgG

Conjugate Dilution	Horse IgG (ng) 1000	100	10	1	0.1	0.01	0.001	[2]Cut off
1:10,000	[1]0.869	0.257	0.053	0.060	0.046	0.056	0.058	0.090
1:8000	1.117	0.285	0.069	0.069	0.067	0.068	0.056	0.102
1:6000	1.381	0.381	0.092	0.083	0.083	0.080	0.073	0.140
1:4000	1.686	0.483	0.157	0.146	0.133	0.140	0.153	0.260

[1]Optical density (OD) at 492 nm. All values are the means of duplicate tests.

[2]Cut-off levels determined as 2X the OD of the background activity, i.e., non-specific activity of the conjugate.

GRAPH 1

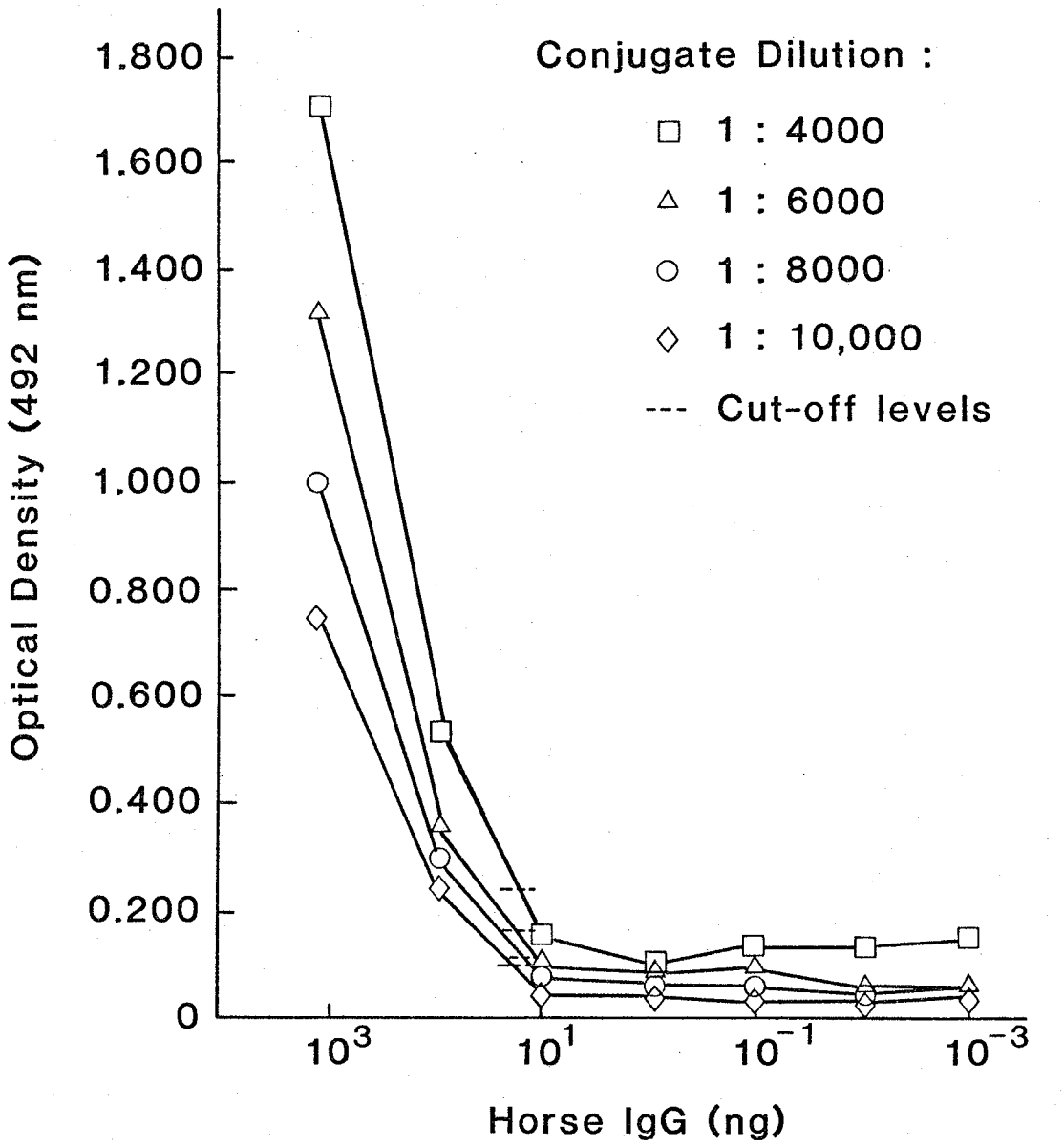

Optimization of Anti-Horse IgG Conjugate
Optical Density (492 nm)
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0
Conjugate Dilution :
□ 1 : 4000
△ 1 : 6000
○ 1 : 8000
◇ 1 : 10,000
--- Cut-off levels
10^3
10^1
10^{-1}
10^{-3}
Horse IgG (ng)

TABLE 8

Optimization of Peroxidase Conjugated F(ab')2 Fragment
Goat Anti-Rabbit IgG

Conjugate Dilution	Rabbit IgG (ng) 1000	100	10	1	0.1	0.01	0.001	[2]Cut off
1:10,000	[1]1.455	0.705	0.669	0.075	0.026	0.029	0.030	0.056
1:8000	1.500	0.962	0.534	0.080	0.040	0.041	0.040	0.058
1:6000	1.793	1.021	0.738	0.091	0.041	0.036	0.027	0.058
1:4000	1.883	1.562	0.956	0.098	0.057	0.051	0.043	0.068

[1]Optical density values at 492 nm. All values are the means of duplicate tests.

[2]Cut-off levels determined as 2X the OD of the background activity, i.e., non-specific activity of the conjugate.

GRAPH 2

Optimization of Anti-Rabbit IgG Conjugate

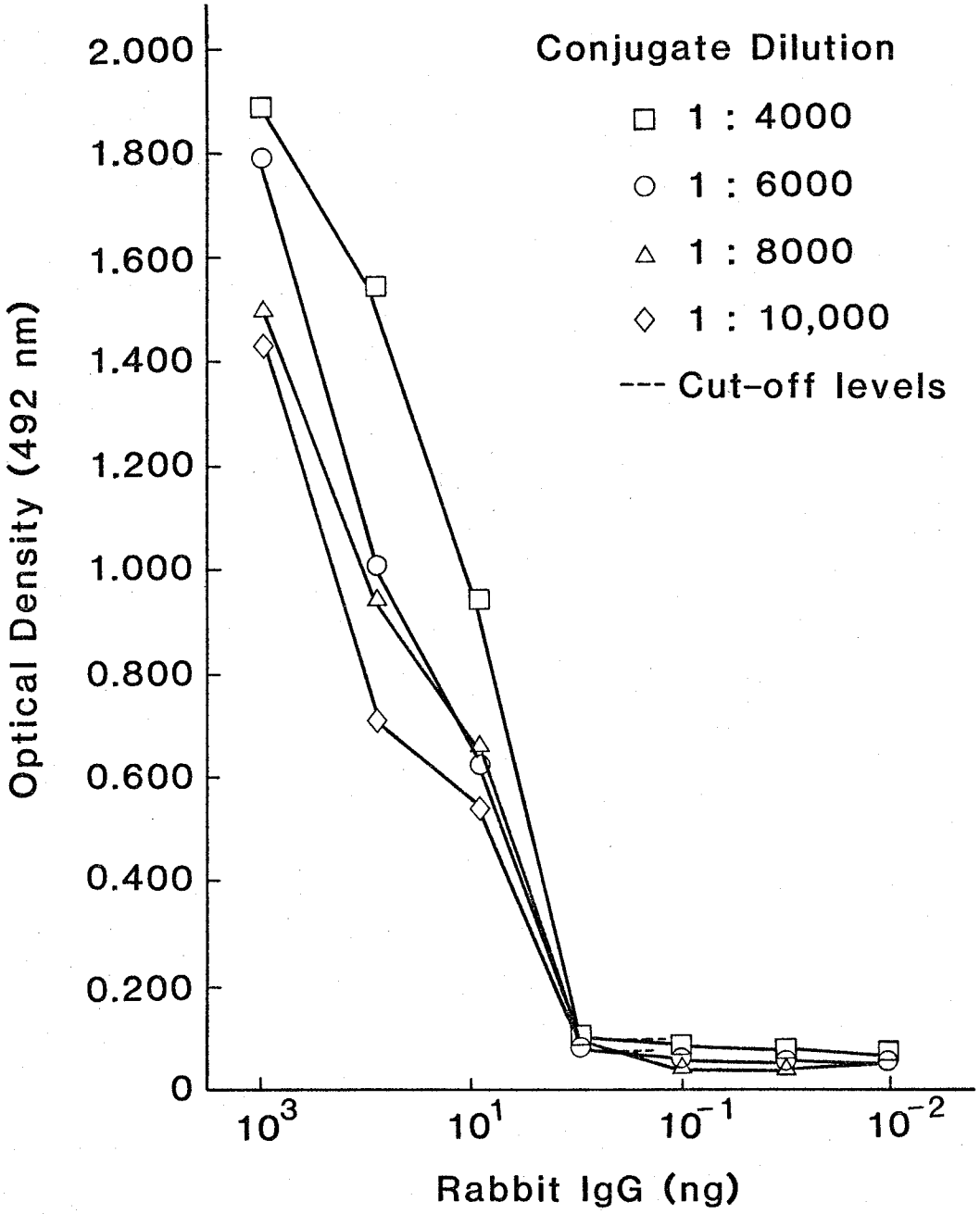


TABLE 9

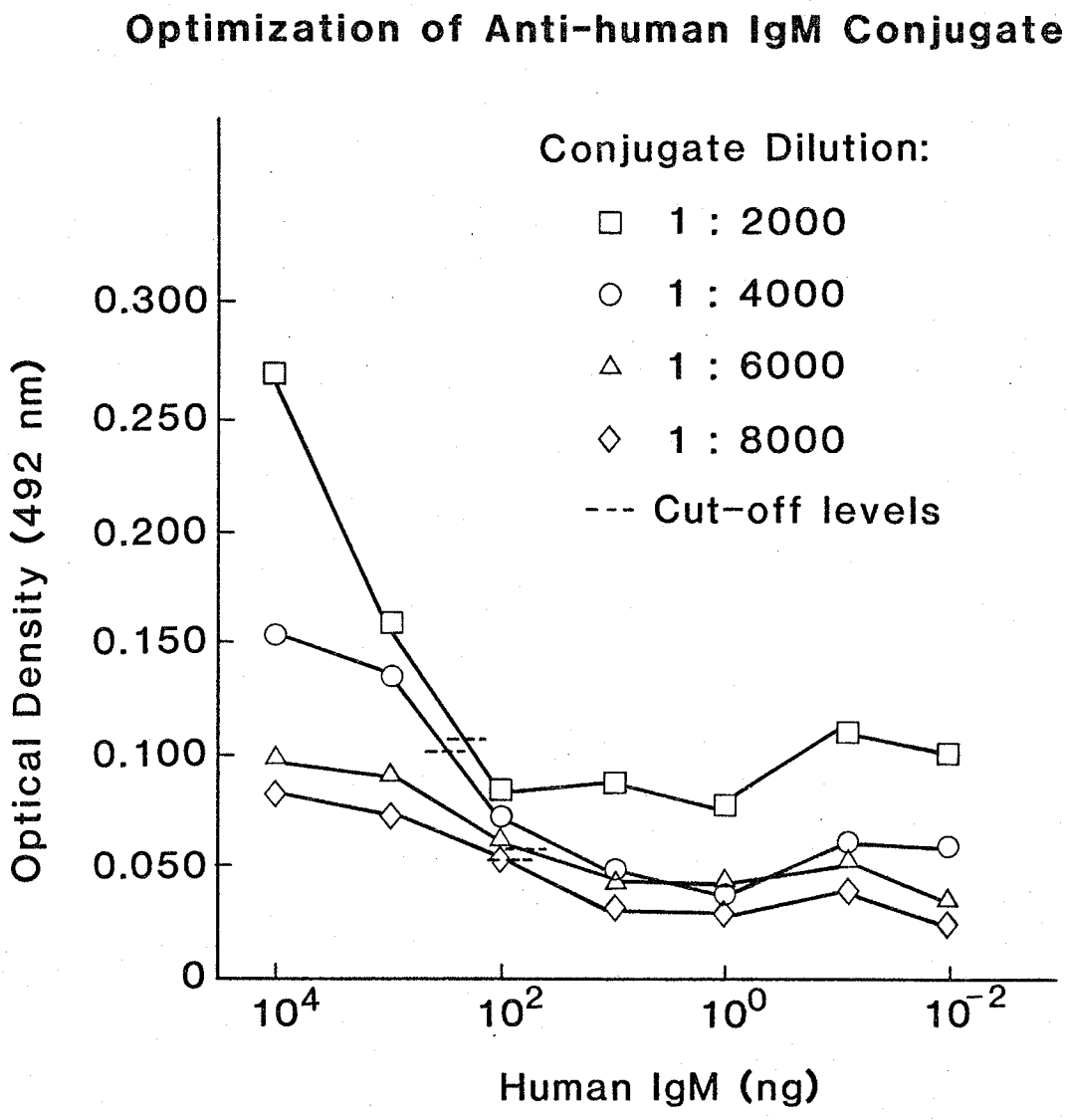
Optimization of Peroxidase Conjugated F(ab')2 Fragment
Goat Anti-Human IgM

Conjugate Dilution	Human IgM (ng) 10,000	1000	100	10	1	0.1	0.01	[2]Cut off
1:8000	[1]0.082	0.072	0.051	0.029	0.028	0.036	0.026	0.052
1:6000	0.098	0.088	0.062	0.045	0.038	0.050	0.033	0.064
1:4000	0.154	0.137	0.072	0.049	0.036	0.066	0.062	0.104
1:2000	0.273	0.160	0.086	0.090	0.074	0.113	0.098	0.114

[1]Optical density at 492 nm. All values are means of duplicate tests.

[2]Cut-off levels determined as 2X the background activity of the peroxidase conjugate.

GRAPH 3


Optimization of Anti-human IgM Conjugate
Conjugate Dilution:
1 : 2000
1 : 4000
1 : 6000
1 : 8000
--- Cut-off levels
Optical Density (492 nm)
0.300
0.250
0.200
0.150
0.100
0.050
0
10^4
10^2
10^0
10^{-2}
Human IgM (ng)

(ii) Capture Antibody

The sensitivity of three anti-human IgM antibodies is illustrated in Table 10 and Graph 4. From these results, the affinity purified goat anti-human IgM was chosen as the capture antibody as it demonstrated the greatest IgM binding ability of the three capture antibodies. This capture antibody was also utilized at a lower concentration of 1 ug/bead while still achieving a sensitivity of between 100 and 1000 ng of human IgM. Using concentrations greater than 1 ug/bead did not increase the sensitivity of the capture antibody.

When comparing the relative sensitivities of two affinity purified capture antibodies, the two capture antibodies were very similar in their ability to bind IgM (Table 11 and Graph 5). The capture antibody obtained from Cappel Laboratories, Cochranville, Pa., however, demonstrated a slightly higher capture ability than the antibody from Dakopatts Antibodies, Westbury, N.Y.

TABLE 10

Comparison of Sensitivities of Three
Anti-Human IgM Antibodies

[3]Anti-human IgM	Human IgM (ng) 10,000	1000	100	10	1	[2]Cut off
Standard	[1]0.301	0.116	0.071	0.075	0.079	0.160
Affinity Purified	0.329	0.191	0.091	0.111	0.084	0.160
Monoclonal	0.116	0.086	0.086	0.113	0.079	0.142

[1]OD at 492 nm. All values are means of duplicate tests. Peroxidase conjugated anti-human IgM utilized at 1:6000.

[2]Cut-off levels determined as 2X the background activity, i.e., non-specific activity of conjugate.

[3]Optimum concentrations of capture antibody:
Standard goat anti-human IgM -- 5 ug per bead
Affinity purified goat anti-human IgM -- 1 ug per bead
Mouse monoclonal anti-human IgM -- 1 ug per bead

GRAPH 4

Sensitivity of Three Anti-human IgM Antibodies

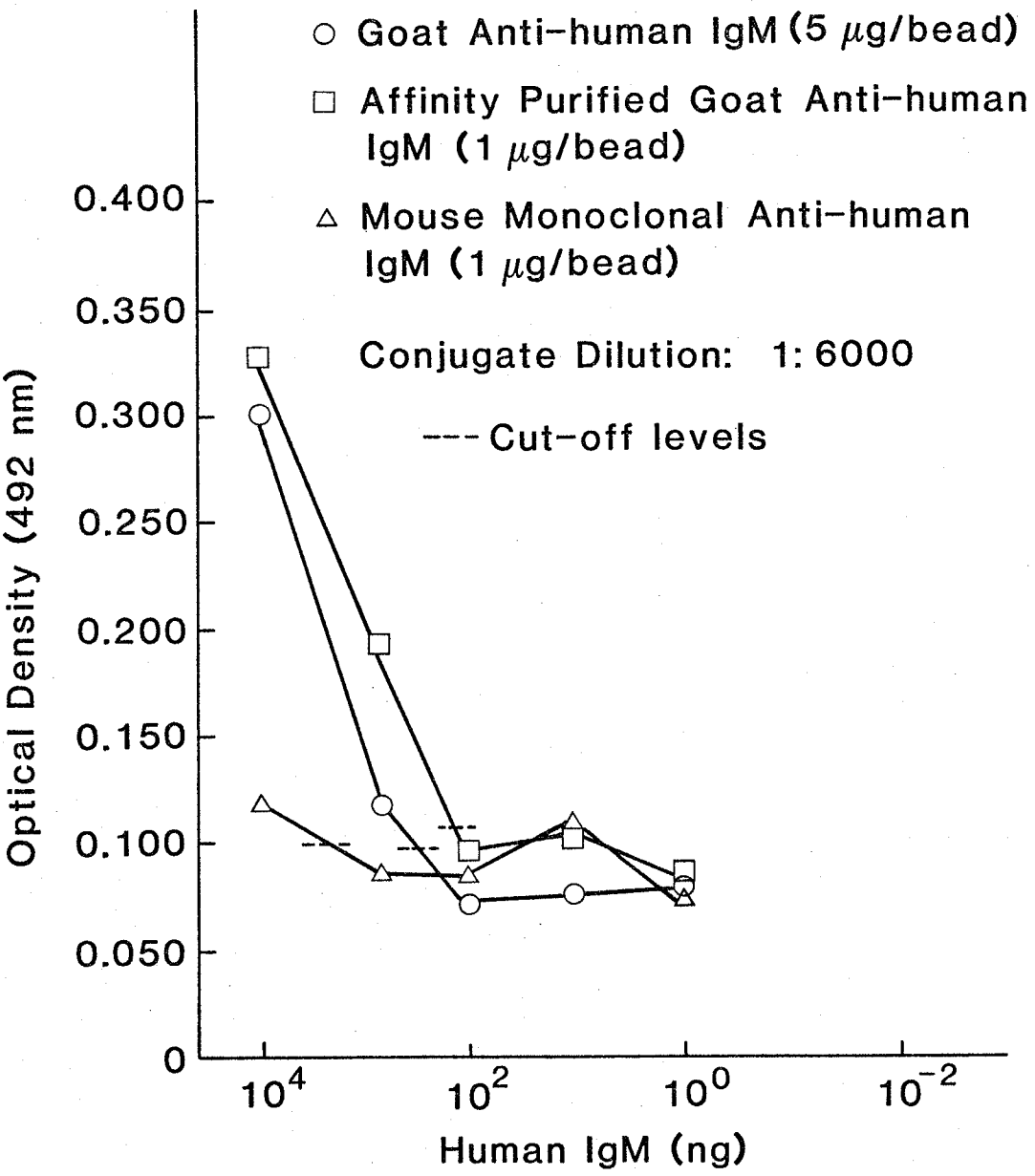


TABLE 11

Comparison of Sensitivities of Two Affinity Purified Anti-Human IgM Antibodies

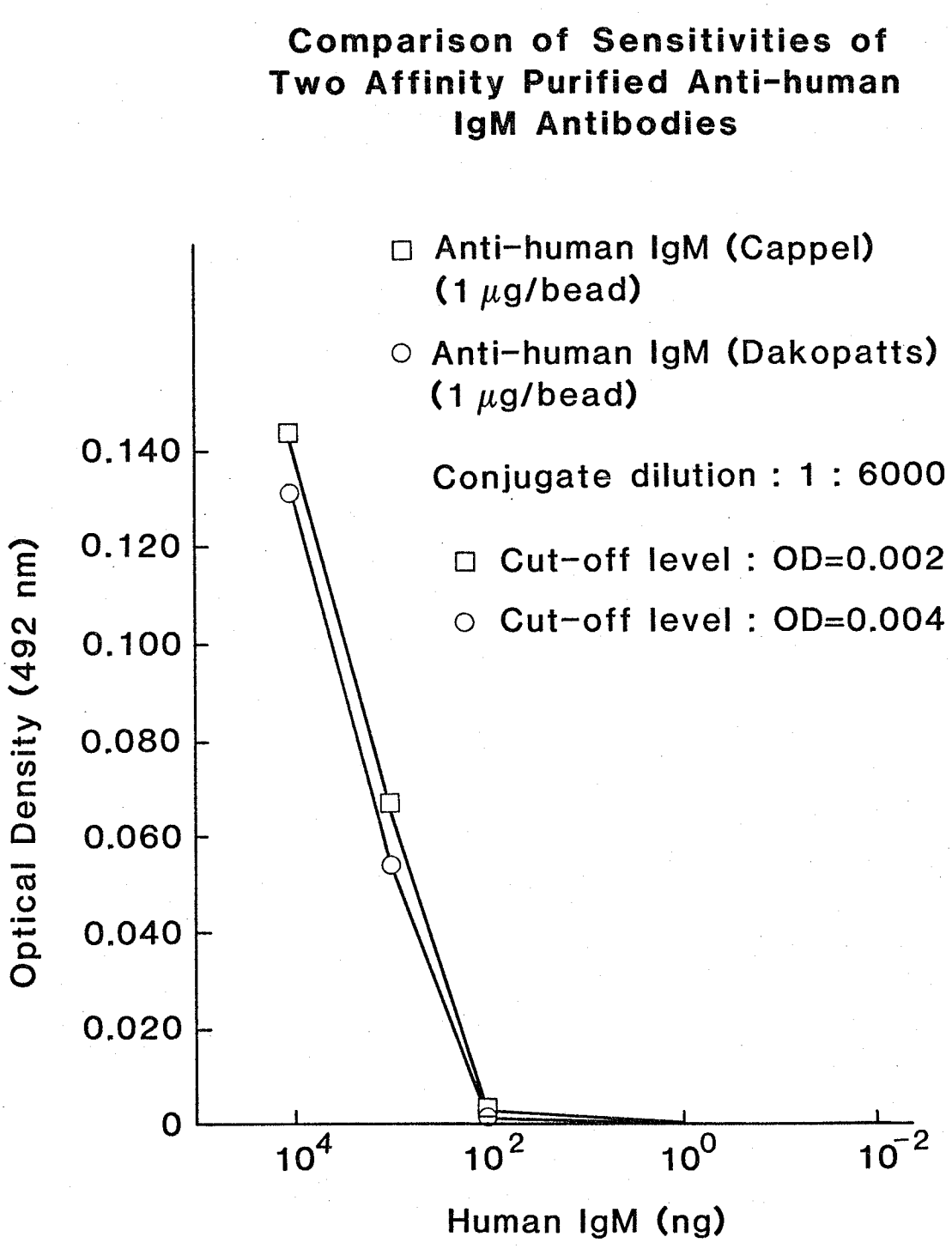
Anti-human IgM	Human IgM (ng) 10,000	1000	100	10	1	[2]Cut off
1	[1]0.143	0.068	0.003	0.000	0.000	0.002
2	0.131	0.055	0.002	0.000	0.000	0.004

[1]OD at 492 nm. All values are the means of duplicate trials.

[2]Cut-off levels determined as 2X the OD of the background activity, i.e., non-specific activity of the conjugate.

[3]Anti-human IgM 1: Affinity purified goat anti-human IgM (u-chain specific) - Cappel Lab., Cochranville, Pa.
Anti-human IgM 2: Affinity purified goat anti-human IgM (u-chain specific) - Dakopatts Antibodies, Westbury, NY

GRAPH 5


Comparison of Sensitivities of Two Affinity Purified Anti-human IgM Antibodies
□ Anti-human IgM (Cappel) (1 μg/bead)
○ Anti-human IgM (Dakopatts) (1 μg/bead)
Conjugate dilution : 1 : 6000
□ Cut-off level : OD=0.002
○ Cut-off level : OD=0.004
Optical Density (492 nm)
0.140
0.120
0.100
0.080
0.060
0.040
0.020
0
10^4
10^2
10^0
10^{-2}
Human IgM (ng)

(iii) Specificity of CBV Antisera

By neutralization tests, both the CBV horse and rabbit antisera were specific to the homolgous virus types with negligible neutralizing antibody titers to heterologous virus types. The heterologous horse antiserum titers were < 1:20 (Table 12), and the heterologous rabbit antiserum titers were < 1:10 (Table 13).

The effects of varying antiserum and antigen concentrations by indirect ELISA are illustrated in Table 14 and Graph 4. In these experiments, antigen was first adsorbed to the solid phase before reaction with the antisera and conjugate (see Figure 3). The results show that a decrease in the concentration of purified CBV antigen by one log of infectivity resulted in a dramatic decrease in reactivity, while saturating antibody levels occurred between 50-100 U of antibody for 10^6 - 10^7 $TCID_{50}$. The range between 10^4 - 10^5 $TCID_{50}$ was too low, however, to estimate the saturating antibody concentrations.

The results of the specificity of the horse CBV antisera by indirect ELISA are illustrated in Tables 15 and 16. All values represented in the tables are corrected OD readings whereby the readings are obtained after subtraction of the background activity due to each of the CBV 2-5 antisera and conjugate. The antisera demonstrated only a slightly greater specificity for the homologous virus type than heterologous types when utilizing a crude preparation of CBV antigen. Specificity was only demonstrable in one direction of the checkerboard titration (Table 15), with the highest reactions obtained for the homologous virus types using each antisera individually. From the results, it appears that the crude CBV 3, 4, and 5 preparations

have the most group specific determinants exposed as the OD readings indicate substantial reactivity with each of the CBV 2-5 antisera. Probably, the CBV 2 preparation was the best, although, there was a high level of activity with CBV 3 antiserum.

The antisera specificity, when utilizing purified virus preparations, was also only slightly greater for the homologous virus type than the heterologous types. The specificity was only apparent in one direction in the checkerboard titration. However, CBV 3 antisera and antigen demonstrated no specificity (Table 16), although a different purified CBV 3 antigen preparation had in previous experiments, demonstrated the greatest specificity of the four CBV types. Nevertheless, the homologous antiserum-antigen reactions of CBV 2, 4, and 5 were higher than the reactions obtained utilizing crude CBV preparations, but not significantly higher as major cross-reactions still occurred. The purified CBV 2 preparation was probably the worst as it had more exposed group specific determinants as seen with the substantial cross-reactions with CBV 3 and 4 antisera. The CBV 3 preparation was probably the best, but it was still not a strictly pure preparation of CBV 3 type-specific determinants. The CBV 4 preparation had substantial reactivity with the CBV 3 antiserum, but was relatively unreactive with CBV 2 and 5 antisera. Consequently, the specificity of the virus antisera may vary with different antigen preparations.

TABLE 12

Neutralization Titers of Horse
CBV 2-5 Antiserum

[1]Coxsackie B Antigen	Coxsackie B Antiserum B2	B3	B4	B5
B2	[2]1:20,480	< 1:20	< 1:20	< 1:20
B3	< 1:20	1:640	< 1:20	< 1:20
B4	< 1:20	< 1:20	1:1280	< 1:20
B5	< 1:20	< 1:20	< 1:20	1:10,240

[1] 100 $TCID_{50}$ per 0.025 ml

[2] Reciprocal titers per 0.025 ml

TABLE 13

Neutralization Titers of Rabbit
CBV 2-5 Antiserum

[1]Coxsackie B Antigen	Coxsackie B Antiserum			
	B2	B3	B4	B5
B2	[2]1:640	< 1:10	< 1:10	< 1:10
B3	< 1:10	1:1280	< 1:10	< 1:10
B4	< 1:10	< 1:10	1:320	< 1:10
B5	< 1:10	< 1:10	< 1:10	1:640

[1] 100 $TCID_{50}$ per 0.025 ml

[2] Reciprocal titers per 0.025 ml

TABLE 14

The Effect of Variation of Virus Antiserum and Purified Antigen Concentrations by ELISA

Coxsackie B4 Antigen (TCID50/0.025 ml)	[1]Coxsackie B4 Antiserum (U)				
	200	100	50	10	5
10^7	[2]1.075	0.995	0.824	0.286	0.166
10^6	0.216	0.229	0.175	0.112	0.062
10^5	0.064	0.021	0.041	0.034	0.007
10^4	0.031	0.036	0.031	0.034	0.000

[1]A Unit (U) of antibody is defined as the neutralization titer of the virus antiserum.

[2]OD readings at 492 nm corrected for background activity by subtraction of non-specific activity of the virus antiserum. All values are means of duplicate tests.

GRAPH 6

The Effect of Variation of Virus Antigen and Antiserum Concentrations by ELISA

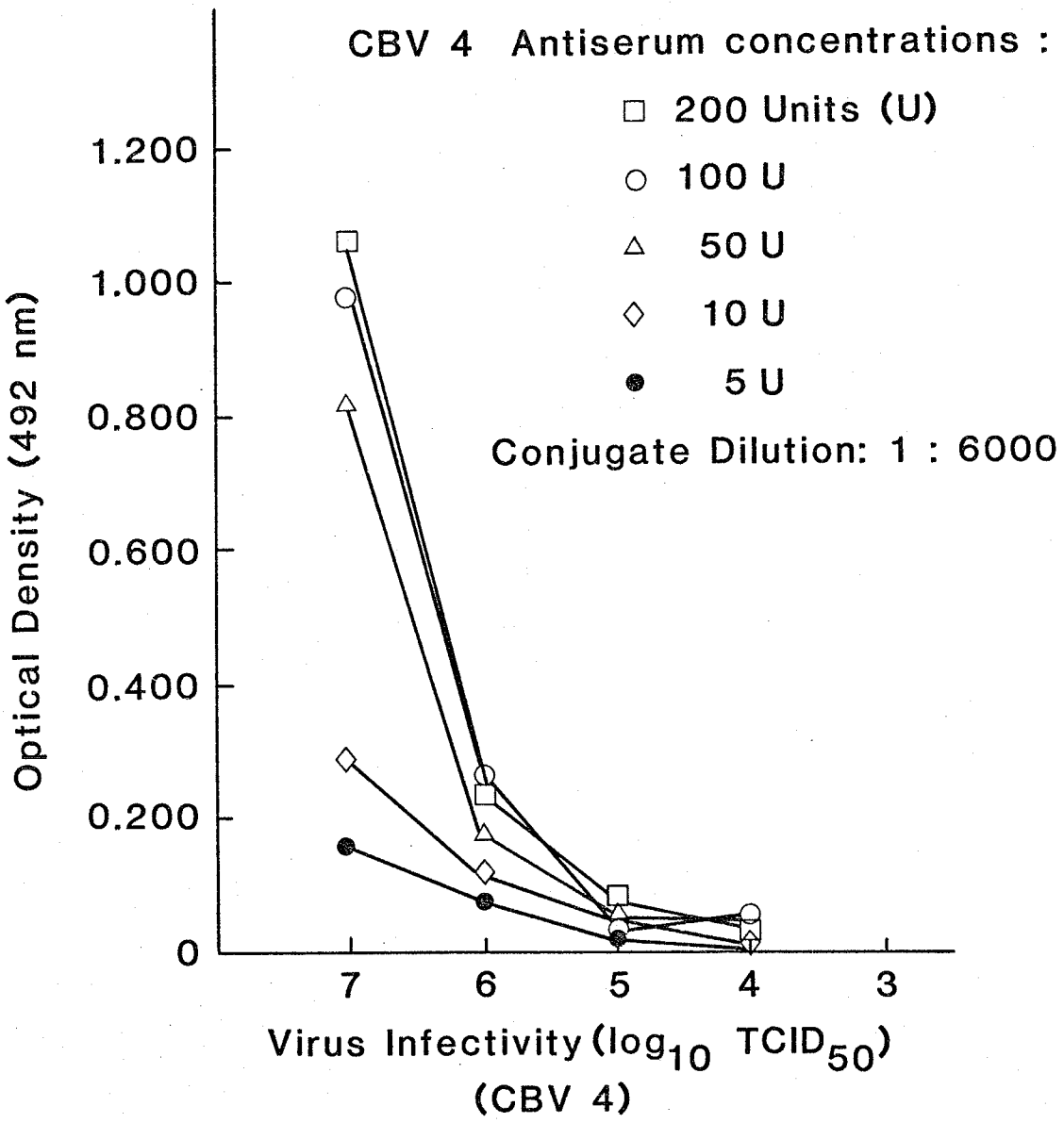


TABLE 15

Specificity of Crude CBV 2-5 Preparations and Horse CBV 2-5 Antisera by ELISA

CBV Antigen[3]	CBV Antiserum[2] B2	B3	B4	B5
B2	[1]0.101	0.250	0.133	0.097
B3	0.088	0.370	0.194	0.114
B4	0.082	0.351	0.247	0.131
B5	0.069	0.326	0.116	0.145

[1]OD at 492 nm. All values are means of duplicate tests.

[2]Antiserum concentrations = 50 Units/0.2 ml

[3]Antigen concentrations = 20 ug/bead

TABLE 16

Specificity of Purified CBV 2-5 Preparations and Horse CBV 2-5 Antisera by ELISA

CBV Antigen[3]	CBV Antiserum[2] B2	B3	B4	B5
B2	[1]0.066	0.240	0.145	0.033
B3	0.015	0.112	0.039	0.013
B4	0.013	0.371	0.746	0.027
B5	0.039	0.210	0.168	0.133

[1]OD at 492 nm. All values are means of duplicate trials.

[2]Antiserum concentrations = 50 Units/0.2 ml

[3]Antigen concentrations = 1 ug/bead

E. ELISA: Test Results

(i) ELISA Sensitivity and Specificity

The negative cut-off optical density, which was determined as the mean net OD plus three standard deviations of a 1:100 dilution of 42 cord bloods of newborn children known to be seronegative for antibody to CBV, was established as 0.080 at 492 nm. A net OD in the ELISA above 0.080, thus, was considered a positive result for CBV-specific IgM.

Experimental results demonstrating the sensitivity of the ELISA are illustrated in Table 19. With the stronger positive IgM serum, the ELISA detected IgM at a very high dilution of 1:800. The lower titered IgM serum was also positive by ELISA at a serum dilution of 1:200.

The results of a blocking assay using two human sera that demonstrated IgM antibody to four CBV types demonstrated that the homologous rabbit CBV 2-5 antisera blocked the detection of the IgM/CBV complex in ELISA by the horse CBV 2-5 antisera. Utilization of the rabbit CBV 4 antiserum to attempt to block all four horse CBV antisera types resulted in only blockage of the reaction of CBV 4 antiserum, and not the reaction of CBV 2, 3, or 5.

(ii) Test Results

The results obtained by ELISA were compared to results obtained by microneutralization tests of whole patient serum and and sucrose gradient IgM fractions in Table 17.

By microneutralization tests, one patient demonstrated a seroconversion (patient 6), i.e., a four-fold or greater rise in neutralizing antibody titer, to one CBV type (CBV 4). Another patient (patient 2) seroconverted to two CBV types, and one patient (patient 15) demonstrated a seroconversion to CBV 4 with high static neutralizing antibody titers to CBV 5.

Of the 19 patients who had CBV 4 isolated (Table 17), 16 demonstrated CBV-specific IgM by ELISA in either the acute or convalescent sera or both. In comparison, 13 of these 19 individuals demonstrated CBV-specific neutralizing IgM after sucrose density gradient fractionation of the serum and microneutralization. By ELISA, no CBV-specific IgM was detected in the acute or convalescent sera of patient 2, who had seroconverted to both CBV 2 and 4.

Of the 16 patients positive for virus-specific IgM by ELISA, 10 had a homotypic IgM response to CBV 4, 2 had an IgM response to three CBV types, and 4 patients had an IgM response to four CBV types. One patient demonstrated CBV-specific IgM to two CBV types in the acute serum and IgM to four CBV types in the convalescent serum.

In comparison to the IgM titers obtained by sucrose gradient fractionation and microneutralization, the ELISA detected IgM in all sera demonstrating CBV-specific neutralizing IgM except for five sera. Treatment of these five sera with 2-mercaptoethanol to remove 19S immunoglobulins (IgM) revealed that in only 2/5 sera was neutralizing antibody of the IgM class abolished. With 3/5 sera, there was still neutralizing antibody present.

Five sera from individuals who had no CBV isolated during the CBV

outbreak were also tested by the three different methods (Table 18). By microneutralization tests, 3/5 individuals had neutralizing antibody to one or two CBV types. No CBV-specific IgM, however, was detected either by ELISA or by microneutralization tests with the IgM sucrose gradient fractions.

Five IgM positive and two IgM negative sera were tested in duplicate on separate days to test day to day variability of the ELISA. Results of both days were in agreement (the five positives remained positive and the two negative sera remained negative). The low number of sera tested, however, precluded any statistical analysis.

TABLE 17

Comparison of Results from Microneutralization Tests,
Sucrose Gradient Fractionation, and ELISA

Patient	Serum	Coxsackie B Microneutralization Titers				Coxsackie B Neutralizing IgM Titers[1]				Coxsackie B IgM by ELISA			
		B2	B3	B4	B5	B2	B3	B4	B5	B2	B3	B4	B5
1	A	<1:10	<1:10	1:80	<1:10	ND	ND	ND	ND	-	-	+	-
	C	<1:10	<1:10	1:160	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	-	+	-
2	A	1:160	<1:10	1:80	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	-	-	-
	C	1:640	<1:10	1:640	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	-	-	-
3	A	<1:10	<1:10	1:160	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	-	+	-
	C	<1:10	<1:10	1:320	1:20	<1:2.5	<1:2.5	1:2.5	<1:2.5	-	-	+	-
4	A	<1:10	<1:10	1:160	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	+	+	+	+
	C	<1:10	<1:10	1:160	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	+	+	+	+
5	C	1:80	<1:10	1:80	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	+	+	+	+

Abbreviations: A = acute; C = convalescent; ND = not done (insufficient serum volume)

* All patients were positive for CBV 4 isolation.

[1] Microneutralization titers of the IgM fractions obtained by sucrose gradient fractionation.

Table 17 Cont'd

Patient	Serum	Coxsackie B Microneutralization Titers B2	B3	B4	B5	Coxsackie B Neutralizing IgM Titers[1] B2	B3	B4	B5	Coxsackie B IgM by ELISA B2	B3	B4	B5
6	A	<1:10	<1:10	<1:10	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	-	-	-
	C	<1:10	<1:10	1:320	<1:10	<1:2.5	<1:2.5	1:10	<1:2.5	-	-	+	-
7	A	<1:10	<1:10	1:640	<1:10	ND	ND	ND	ND	ND	ND	ND	ND
	C	<1:10	<1:10	1:320	<1:10	<1:2.5	<1:2.5	1:5	<1:2.5	-	-	+	-
8	A	<1:10	<1:10	1:640	<1:10	<1:2.5	<1:2.5	1:5	<1:2.5	-	-	+	-
	C	<1:10	<1:10	1:320	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	-	+	-
9	A	<1:10	<1:10	1:640	<1:10	<1:2.5	<1:2.5	1:5	<1:2.5	-	-	+	-
	C	<1:10	<1:10	1:320	<1:10	<1:2.5	<1:2.5	1:2.5	<1:2.5	-	-	+	-
10	A	<1:10	<1:10	1:320	<1:10	<1:2.5	<1:2.5	1:2.5	<1:2.5	-	-	+	-
	C	<1:10	<1:10	1:320	<1:10	<1:2.5	<1:2.5	1:2.5	<1:2.5	-	-	-	-

Abbreviations: A = acute; C = convalescent; ND = not done

* All patients were positive for CBV 4 isolation.

[1]Microneutralization titers of the IgM fractions obtained by sucrose gradient fractionation.

Table 17 Cont'd

Patient	Serum	Coxsackie B Microneutralization Titers B2	B3	B4	B5	Coxsackie B Neutralizing IgM Titers[1] B2	B3	B4	B5	Coxsackie B IgM by ELISA B2	B3	B4	B5
11	A	1:160	<1:10	1:160	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	+	+	+	+
	C	1:80	<1:10	1:160	<1:10	<1:2.5	<1:2.5	1:2.5	<1:2.5	+	+	+	+
12	A	<1:10	<1:10	1:640	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	+	+	+
	C	<1:10	<1:10	≥1:1280	<1:10	<1:2.5	<1:2.5	1:2.5	<1:2.5	-	+	+	+
13	A	1:20	<1:10	1:320	<1:10	<1:2.5	<1:2.5	1:2.5	<1:2.5	-	-	+	+
	C	1:40	<1:10	1:640	<1:10	<1:2.5	<1:2.5	1:10	<1:2.5	+	+	+	+
14	A	<1:10	<1:10	1:160	<1:10	<1:2.5	<1:2.5	1:2.5	<1:2.5	-	-	+	-
	C	<1:10	<1:10	1:160	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	-	-	-
15	A	<1:10	<1:10	1:160	≥1:1280	<1:2.5	<1:2.5	1:2.5	<1:2.5	-	-	-	-
	C	<1:10	<1:10	1:640	≥1:1280	<1:2.5	<1:2.5	1:5	1:2.5	-	+	+	+

Abbreviations: A = acute; C = convalescent

* All patients were positive for CBV 4 isolation.

[1]Microneutralization titers of the IgM fractions obtained by sucrose gradient fractionation.

Table 17 Cont'd

Patient	Serum	Coxsackie B Microneutralization Titers B2	B3	B4	B5	Coxsackie B Neutralizing IgM Titers[1] B2	B3	B4	B5	Coxsackie B IgM by ELISA B2	B3	B4	B5
16	A	<1:10	<1:10	1:320	<1:10	<1:2.5	<1:2.5	1:20	<1:2.5	-	-	+	-
	C	<1:10	<1:10	1:640	<1:10	<1:2.5	<1:2.5	1:20	<1:2.5	-	-	+	-
17	A	1:20	<1:10	1:160	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	-	-	-
	C	1:80	<1:10	1:320	<1:10	<1:2.5	<1:2.5	1:5	<1:2.5	-	-	+	-
18	A	<1:10	<1:10	1:320	<1:10	ND	ND	ND	ND	-	-	-	-
	C	<1:10	<1:10	1:640	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	-	-	-
19	A	<1:10	1:160	1:160	<1:10	ND	ND	ND	ND	-	-	-	-
	C	<1:10	1:320	1:160	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	-	-	-

Abbreviations: A = acute; C = convalescent; ND = not done

* All patients were positive for CBV 4 isolation.

[1]Microneutralization titers of the IgM fractions obtained by sucrose gradient fractionation.

TABLE 18

Comparison of Results from Microneutralization Tests, Sucrose Gradient Fractionation, and ELISA with Patients with Negative Virus Isolation

Patient	Serum	Coxsackie B Microneutralization Titers				Coxsackie B Neutralizing IgM Titers[1]				Coxsackie B IgM by ELISA			
		B2	B3	B4	B5	B2	B3	B4	B5	B2	B3	B4	B5
1	A	1:320	<1:10	1:160	<1:10	ND	ND	ND	ND	-	-	-	-
	C	1:320	<1:10	1:640	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	-	-	-
2	A	<1:10	<1:10	<1:10	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	-	-	-
	C	<1:10	<1:10	<1:10	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	-	-	-
3	A	<1:10	<1:10	1:40	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	-	-	-
	C	<1:10	<1:10	1:80	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	-	-	-
4	A	<1:10	<1:10	<1:10	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	-	-	-
	C	<1:10	<1:10	<1:10	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	-	-	-
5	A	<1:10	<1:10	1:320	<1:10	ND	ND	ND	ND	-	-	-	-
	C	<1:10	<1:10	1:320	<1:10	<1:2.5	<1:2.5	<1:2.5	<1:2.5	-	-	-	-

Abbreviations: A = acute; C = convalescent; ND = not done (insufficient serum volume)

[1]Microneutralization titers of the IgM fractions obtained by sucrose gradient fractionation.

TABLE 19

Sensitivity of ELISA for Detection of CBV-Specific IgM

Serum	Serum Dilution 1:100	1:200	1:400	1:800
1	[1]0.394	0.114	0.108	0.110
2	0.110	0.130	0.070	0.089

[1]O.D. readings at 492 nm. All values are means of duplicate trials. Negative cut-off level = 0.080.

[2]Serum #1 - IgM titer = 1:10
#2 - IgM titer = 1:2.5

IV. DISCUSSION

IV. DISCUSSION

A. Microneutralization Test

Since it is generally believed that the evaluation of CPE remains the best criterion for the presence and quantitation of virus, the neutralization test is a method which utilizes this characteristic in the evaluation of a virus infection. The basis of this test is that neutralizing antibody to a particular virus when present in the serum of an individual will bind to that virus in the test and prevent adsorption of the virus into the cells. Prevention of virus infection of the cells by neutralizing antibody will result in no CPE, whereas, absence of neutralizing antibody will allow the virus to infect the cells, eventually resulting in CPE. Consequently, the microtechnique for performance of the neutralization test has been an important development for diagnostic virus serology. The microneutralization test reduces time, materials, and some effort when compared to conventional tissue culture tube methods. There is also a reduction in serum requirements for the test making it particularly appropriate for neutralizing antibody studies of children or infants where only small volumes of serum are usually available. Results of micro-neutralization tests have also been shown to agree well with results obtained using the conventional techniques (Rosenbaum et al., 1963).

Antibody titers of the panel of sera in this thesis were only determined for CBV types 2-5 as it has been observed that CBV 1 and 6 isolates are not commonly detected by viral culture in Manitoba

(Annual Reports, Manitoba Health Services Commission, 1977-82).

The technical disadvantages of the microneutralization test became more apparent to this author as the tests were performed on the various patient sera. This test, although standardized in procedure, was subject to the day to day variations involved with use of biological materials such as cell culture and viruses. Consequently, due to this variation in the test, there was a requirement for retesting of some sera in order to obtain a more definitive result. Finally, this test was quite laborious and time consuming when compared to a technique such as ELISA.

Since the basis of a serologic diagnosis of infection with the microneutralization test is the demonstration of a seroconversion, this test was not very sensitive for recent CBV infections. From the data obtained with the sera from 19 individuals with a virologically confirmed CBV 4 infection, only 3/19 (16%) had a seroconversion to CBV 4. However, one of these individuals also seroconverted to CBV 2 besides CBV 4, while another individual seroconverted to CBV 4 with high static antibody titers to CBV 5. Consequently, the neutralization test is not totally reliable, as low seroconversion rates, anamnestic antibody responses, and high static antibody levels make diagnosis of a recent infection difficult. (Morgan-Capner and McSorley (1983) observed that an elevated neutralizing antibody titer was an unreliable indicator of a recent CBV infection since in their study, only 29% (5/17) of the sera with elevated CBV 4 neutralizing antibody titers contained detectable specific IgM.

B. Sucrose Density Gradient Fractionation

The techniqe of sucrose density gradient centrifugation involves the separation of serum macromolecular components by ultracentrifugation which exploits their differences in size and shape (Brakke, 1967; Vesikari and Vaheri, 1968). This method of IgM fractionation from human serum was chosen as the reference procedure since it has been used routinely for determination of IgM antibody to rubella in conjunction with other serological tests (Palmer et al., 1977; Meurman et al., 1977).

Sucrose density gradient fractionation in conjunction with neutralization tests has demonstrated to be more sensitive and specific than microneutralization tests in the data obtained in this thesis. Virus-specific IgM was detected with the combination of the two methods to the infecting CBV serotype (CBV 4) in 13/19 patients with a virologically confirmed CBV 4 infection. In contrast, by microneutralization tests, serodiagnosis could not be made in the majority of the individuals due to less then four-fold rises in neutralizing antibody titers, static antibody levels, and heterotypic antibody responses.

Although the technique of sucrose density gradient fractionation incorporated with the neutralization test was more sensitive and specific than the microneutralization test alone, this method was more time consuming and required specialized equipment (an ultracentrifuge).

C. ELISA

(i) Design and Development

In the development of an ELISA, the solid support phase is one of the more important considerations of the system. A number of solid phase supports have been used in the past, including test tubes, beads, filter papers, discs, resins, and microtitration plates. The fact that protein molecules can bind to a number of plastics by means of hydrogen bonds simplifies the process of attaching antibody to the solid phase. Several plastic polymers are commercially available and they include polystyrene, polyvinyl, polycarbonate, as well as aminoalkylsilyl glass and silicone rubber. Antibody or antigen can be passively adsorbed to such surfaces, with polystyrene and polyvinyl being the most commonly employed supports. Advantages of utilizing plastics as support surfaces are the simplicity and ease of handling, and the requirement for only small quantities of antibody. However, the disadvantages of utilizing plastic supports include variation of the quantity of antibody adsorbed from one plastic to another, only a small quantity of material is actually fixed, denaturation of adsorbed macromolecules may take place with time, and desorption of the immobilized substance can occur.

The type of solid phase utilized in the immunoassay will have an effect on the variation and accuracy of the assay. It has been observed that with adsorbed antigen, there was noticeable leakage during the assay from both polystyrene (30%) and nylon (60%) supports (Lehtonen and Viljanen, 1980). Another important factor to consider is the consistency of binding of material to the solid phase as this

will determine the reproducibility of the immunoassay. The level of background activity, i.e., the activity due to binding of nonspecific materials to the plastic support surfaces may also vary between support types.

In choosing the solid phase for the ELISA for CBV-specific IgM in this thesis, a surface was required that provided for good antigen or antibody adsorption, was easy to handle, and did not bind nonspecific reagents significantly. Polystyrene and polyvinyl plates have both been demonstrated to yield good results (Bidwell et al., 1977), with the polyvinyl surface demonstrating a slightly greater binding ability without significant nonspecific binding (Yolken, 1980). However, a disadvantage of the microtiter plates is that there is relatively small surface area available for binding. Consequently, polystyrene beads were chosen as the solid phase for the ELISA in this research project, as they provided a much greater surface area for binding and are relatively equal to polyvinyl in binding ability. The reactions in the ELISA were performed in 20-well polystyrene reaction trays which accommodated the polystyrene beads, and have been used successfully by the Abbott Company for hepatitis A and B serology. This system also had the advantage of ease of manipulation and the potential for semi-automation by the use of the Abbott Pentawash, a washer-aspirator device. The search for the ideal solid phase support, however, is still continuing.

Since the adsorption of proteins to plastic surfaces is due primarily to hydrophobic bonds, optimum conditions must be observed to promote maximum adsorption. These conditions are pH, ionic strength,

temperature, time, and protein concentration. Maximum hydrophobic interactions are promoted when the net charge of a molecule is zero. For adsorbing IgG, for example, this criterion is approached at alkaline pH's of 8.5 to 10.0. Concentration of salts are also important as these will neutralize localized charges on a protein, thus, promoting hydrophobic interactions. Time and temperature are factors that affect the probability of protein molecules coming into contact with the solid phase surface, and the extent of saturation of the surface. Increasing the temperature will increase the diffusion rate which increases the rate at which proteins contact the plastic surface. Room temperature is usually sufficient, but high temperatures can be used if the protein in question is not heat labile. The time required for saturation of the solid phase usually occurs within a matter of minutes to one or two hours. However, if a protein is stable, an overnight incubation is usually more convenient. Consequently, an overnight incubation was used for all adsorption steps in the IgM ELISA in this thesis. The protein concentration required to promote maximum adsorption will vary between different proteins, and also between different solid phase supports. With the polystyrene beads (Precision Plastic Ball Co., Chicago, Ill.), it is believed that approximately 1 ug of protein can be readily adsorbed. In fact, it was observed in this research project that adsorption of more than 1 ug of the capture antibody did not increase the sensitivity of the ELISA, indicating that saturating levels may be reached at 1 ug of protein.

Another method of attaching proteins to plastic solid phase supports is by covalent conjugation with cross-linking reagents, or

covalent linkage to nonplastic materials in hopes of increasing binding capabilities (Yolken, 1982). While there are theoretical advantages in establishing a covalent bond between the capture antibody and the solid phase support, for example, the process is much more complex than simple adsorption to plastic materials. Also, the limiting factor of the use of these nonplastic materials is that there is an increased nonspecific adsorption of immunoreactants to these surfaces, therefore, leading to a decrease in reaction specificity (Yolken, 1980). Consequently, most binding experiments only require placement of the serum or immunoglobulin in an appropriate buffer, i.e., 0.06M carbonate-bicarbonate buffer, pH 9.6, which will result in a large percentage of binding over a four hour period at 37° C or overnight at 4° C (Pesce et al., 1978). Thus, this was the system adopted for the ELISA in this research project as it was simple and convenient, and allowed a high level of sensitivity and specificity to be attained.

A major problem of these types of immunoassays is background activity due to nonspecific adherence. In IgM antibody assays, the background is usually higher than in IgG antibody assays since IgM is a more avid or "sticky" molecule than IgG. To eliminate or decrease this type of adherence, often dilution of immunoreagents is performed in buffers containing a nonionic detergent such as Tween 20 and an excess of inactive protein such as bovine serum albumin (BSA), fetal bovine serum, or gelatin. Preincubation of antibody-coated or even antigen-coated solid phases with BSA (Locarnini et al., 1977), animal serum (Meurman et al., 1977), or gelatin (Kangro et al., 1978) can

decrease background activity by blocking remaining free nonspecific protein binding sites. Background activity can also be reduced by a series of washing steps with an excess of detergent-containing buffer. Therefore, the diluent used in the IgM ELISA contained BSA and Tween 20, and all washing steps were performed with PBS containing 0.05% (v/v) Tween 20. Optimal concentrations of BSA (2% w/v) and Tween 20 (0.15% v/v) were used in the diluent for the IgM ELISA as suggested by Herrmann et al. (1979). Post-coating of the capture antibody beads with BSA was also observed to aid in decreasing the background activity in the IgM ELISA.

In solid phase anti-IgM assays, nonspecific binding has also been observed when low serum dilutions are used (Yolken and Leister, 1981). However, the sensitivity offered by these immunoassays allow higher serum dilutions (up to 1:10,000, Roggendorf et al., 1980; Mortimer et al., 1981b) to be utilized in the test so that nonspecific binding of serum immunoglobulins is no longer a major problem. The optimal dilution of serum determined for the IgM ELISA was 1:100 which provided a good level of sensitivity and economy, and low level of background activity.

Rheumatoid factor (RF), an anti-IgG antibody of the IgM class can cause false-positive IgM antibody results in immunoassays. In an IgM ELISA, the RF can bind to the anti-IgM on the solid phase that will in turn bind to other IgG antibodies and the labelled antibody. Since RF binds to the F_c portion of the IgG molecule, the problem of false-positives can be negated with the use of enzyme-labelled F(ab')2 fragments as the indicator antibody (Kato et al., 1979; Duermeyer et al., 1979). Consequently, all enzyme conjugated antibodies utilized

in this research project were F(ab')2 fragments.

When choosing the enzyme label for use in an ELISA, several criteria have to be met (Wisdom, 1976). The enzyme must be available inexpensively in high purity, must have high specific activity, must be stable under assay and storage conditions, must be soluble, must be absent from biological fluids, and must be capable of retaining activity while undergoing appropriate linkage reactions to antibody. Also, substrates, inhibitors, and disturbing factors must be absent from biological fluids, and the assay method for the enzyme reaction must be simple, sensitive, rapid, and inexpensive. The most widely used enzymes in EIA systems have been peroxidase and alkaline phosphatase. Peroxidase is the least expensive of the two, and it can be obtained easily in large quantities from various commercial sources. Peroxidase is glycoprotein with a carbohydrate content of 10-15% (Avrameas et al., 1971; Nakane and Kawaoi, 1974). Therefore, the theoretical advantage of linkage of the peroxidase enzyme to antibody via the carbohydrate portion of the enzyme molecule is that there is less interference with enzyme function after conjugation. The peroxidase substrate also produces an intense dark color that provides for definitive visual determinations. However, the disadvantages of the peroxidase system are that peroxidase conjugates can lose their activity when contaminated with microorganisms (Yolken, 1980). Also, peroxidase activity is very sensitive to the action of antibacterial agents such as methanol and sodium azide, so care must be taken to exclude these compounds from peroxidase conjugates (Schonbaum, 1973; Straus, 1971). Some of the peroxidase chromogens such as benzidine,

o-toluidine, and o-phenylenediamine have also been found to have carcinogenic or mutagenic effects (Scharpe et al., 1976). However, for the IgM ELISA in this thesis, a peroxidase conjugated F(ab')2 fragment indicator antibody was chosen as the enzyme label because it was readily available in a conjugated form to many antibody types, relatively inexpensive, stable, and had an easily measured enzyme product.

A factor which enables one to obtain the maximum sensitivity from an enzyme system is to optimize the conditions in which the enzyme functions. Bovaird et al. (1982) examined various aspects that may affect peroxidase activity including the effects of phosphate, pH, substrate and enzyme concentrations, and stopping reagents. Consequently, the substrate buffer utilized in the IgM ELISA was 0.01M citrate-phosphate buffer, pH 5.0, which was optimized under the conditions outlined by Bovaird et al. (1982) for horseradish peroxidase. The substrate utilized (H_2O_2 + OPD) was chosen as it was inexpensive and easily prepared. The peroxidase enzyme reaction was stopped using sulfuric acid (H_2SO_4). However, when stopping agents are used, the spectrum of the chromophore changes. For peroxidase systems, the absorbance is usually read at 435 nm, but upon addition of the stopping reagent, the chromophore spectrum shifts. Therefore, the absorbance maxima of the peroxidase enzyme products was measured at a wavelength of 492 nm (Bovaird et al., 1982).

In summary, the assay design was chosen to offer the maximum sensitivity and specificity, and to reduce nonspecific activity to a minimum. Therefore, the capture antibody principle was utilized for

the ELISA for detection of CBV-specific IgM in human serum. The advantages this system had over indirect ELISA methods included lack of competition between IgG and IgM for the antigen binding sites, a higher sensitivity, and decreased interference by rheumatoid factor. Since the ELISA was developed to detect IgM to four CBV serotypes, an extra step was added to the basic procedure outlined in Figure 3. This was done to circumvent the need for conjugating peroxidase enzyme to the four CBV antisera. Thus, only one enzyme conjugate directed against the species in which the virus antisera was produced was required for all four CBV antisera types.

(ii) Optimization of Reagents

In order to obtain the maximum sensitivity and specificity from the ELISA, all the reagents utilized in the immunoassay were optimized. A series of experiments were designed to optimize the various components of the ELISA before testing the complete system on clinical specimens. The optimum concentrations of each of the reagents were determined as that concentration which offered the best balance between sensitivity, specificity, economy, and non-specific background activity.

The capture antibody is one of the more important reagents of the IgM ELISA, as the IgM capture ability of the antibody will determine the sensitivity of the immunoassay. Thus, an affinity purified antibody was chosen as the capture since it demonstrated the highest avidity for IgM. The sensitivities determined by the optimization experiments in this thesis, however, may not be true representations of the capture

antibody's actual sensitivity as this is dictated in part by the sensitivity of the detector antibody, the peroxidase conjugate anti-human IgM, used in the optimization experiments. Since the concentration of reagents used in the ELISA is always in excess, an approximate sensitivity could be determined from these experiments which aided in choosing the capture antibody for the IgM ELISA. The true sensitivity of the capture antibody was more correctly assessed in the IgM ELISA by testing dilutions of IgM positive sera, and it was demonstrated that the ELISA capture antibody was very sensitive as virus-specific IgM was detected by the complete ELISA system at high serum dilutions. To more accurately assess the sensitivity of the capture antibody of the ELISA, though, the positive IgM serum should be diluted in human serum negative for CBV-specific IgM, and then tested by ELISA. In this manner, the ratio of nonspecific IgM to virus-specific IgM in the serum is increased, which would more accurately reflect a human serum with low titers of CBV-specific IgM.

In the ELISA procedure, the final step before addition of substrate involved the transfer of the beads to new plates. This was done to avoid the problem of increased background activity due to nonspecific adsorption of ELISA reagents, particularly the conjugate, to the surface of the wells of the polystyrene reaction trays. Therefore, only the reagents bound to the bead would be evaluated in the ELISA test.

The length of time of incubation involving the virus antigen was observed to be a less critical factor in the ELISA. The incubation period of either overnight at 4° C, or 1 hour at 37° C did not have any significant effects on the final IgM ELISA results with patient sera.

The quantitation of virus antigen for the ELISA was done by protein content. Infectivity titers do not accurately reflect the amount of antigenic material in a virus preparation as they only detect intact, infectious particles, and not non-infectious, defective, or incomplete virus particles. Therefore, the determination of the protein contents of the virus preparations was necessary to standardize the preparations of CBV 2-5 for the ELISA. In this manner, equal concentrations of antigenic material of each CBV type was presented in the IgM ELISA.

Both the horse and rabbit antisera demonstrated similar specificities by neutralization tests, but the horse antisera were chosen as the detector antibody since they demonstrated a lower background activity than the rabbit antisera in the IgM ELISA. Also, the neutralizing antibody titers of the horse antisera, with the exception of the CBV 3 antiserum, were much higher than the titers of the rabbit antiserum. Therefore, the horse antisera could be utilized at a low concentration for many more ELISA tests without the need for changing and re-evaluating antiserum lots.

The antisera concentrations chosen for the IgM ELISA varied between CBV 2 and 5, and CBV 3 and 4. Although the neutralization titers of the CBV 2 and 5 antisera were much higher than the CBV 3 and 4 antisera, a higher concentration of CBV 2 and 5 antisera (25 U) was utilized as it was observed that the avidity of these antibodies was much lower than those in the CBV 3 and 4 antisera. Therefore, it was necessary to reduce the concentrations of CBV 3 and 4 antisera, and increase the concentrations of CBV 2 and 5 antisera used in order

to obtain comparable optical density readings in the ELISA.

In order to develop a type-specific ELISA for detection of CBV-specific IgM, the virus antisera utilized as the detector antibody in the enzyme immunoassay also must be type-specific. The virus antisera were highly type-specific by neutralization tests; however, by indirect ELISA, the specificity of the antisera was difficult to ascertain. By indirect ELISA, a slightly greater type-specific reaction was observed with both crude and purified antigen. Substantial group-specific reactions were observed to occur indicating that group antigenic determinants were exposed in both types of antigen preparations. In actual IgM ELISA test conditions with human sera, the reaction of the antisera was very type-specific, as a blocking assay using rabbit CBV antisera to block the reaction of the horse CBV detector antisera demonstrated. Each rabbit CBV 2-5 antisera blocked the reaction of the homologous horse CBV 2-5 antisera indicating that the horse CBV antisera was type-specific. Attempts to block the reaction of all four horse CBV antisera with only rabbit CBV 4 antisera resulted in the blockage of only the horse CBV 4 antiserum.

An important factor observed to influence the sensitivity of the ELISA was the concentration of CBV antigen utilized in the assay. Checkerboard titrations with both purified and crude virus preparations of CBV antigen with CBV antisera indicated that the ELISA reaction was highly dependent on the concentration of antigen. Although the use of purified antigen in the ELISA may theoretically impart greater specificity, no obvious advantage was observed with the results. Therefore, it was not economical to utilize highly purified antigen

preparations for a routine diagnostic test. Hence, a crude preparation of antigen was utilized.

Katze and Crowell (1980) and Dörries and ter Meulen (1983) have indicated that perhaps a more purified antigen preparation would contribute to a greater type-specificity by ELISA. The indirect ELISA experiments with CBV antisera and crude and purified virus preparations in this thesis indicate that there was no significant increase in specificity by using more purified virus in the ELISA. Katze and Crowell (1980) have suggested that adsorption of virus onto solid phases may expose group antigenic determinants, thus, resulting in greater group-specific reactions by indirect ELISA. This still is speculation, however, as there is no evidence at present for changes in virus conformation due to solid phase adsorption. Nevertheless, there may similarities between CBV and other enteroviruses whereby exposure of group determinants (Schmidt et al., 1963), loss of VP 4 from the virus particle (Breindl, 1971; Maizel et al., 1967), or gross conformational rearrangements of the virus capsid proteins (Lonberg-Holm and Yin, 1973) can occur upon heating of the virus.

(iii) Test Results

The IgM ELISA utilized each patient serum to be tested as its own control as each serum was observed to vary in the degree of nonspecific activity in the ELISA. Consequently, each patient serum was tested in duplicate in wells containing virus antigen and in wells containing control antigen with the mean net optical density of the reaction used as the test result.

The CBV-IgM ELISA developed in this thesis had a positivity rate of 84% (16/19 patients) compared to the reference standard of CBV 4 isolation. Five individuals with negative virus isolation and with or without preexisting neutralizing antibody to one or more CBV types were negative for CBV-specific IgM by ELISA and also by IgM neutralization tests after sucrose density gradient fractionation. Although the number of these sera with negative virus isolation was small, indications are that the IgM ELISA demonstrated 100% specificity for absence of a recent infection. In comparison to the neutralization test, the IgM ELISA was much more sensitive as only 16% (3/19) of the individuals demonstrated a seroconversion by microneutralization tests. The IgM ELISA was also more sensitive than microneutralization tests after sucrose density gradient fractionation of the IgM which only detected IgM in 13/19 patients. The increased sensitivity of the ELISA was further demonstrated as the ELISA detected virus-specific IgM which was undetected in sucrose gradient IgM fractions at a titer of 1:2.5, the lowest dilution used in the microneutralization test. Thus, the ELISA for detection of CBV-specific IgM in a single serum specimen could be employed as a sensitive diagnostic test for detection of recent CBV infections.

The ELISA for detection of CBV-specific IgM developed by El-Hagrassy et al. (1980) had a sensitivity of 83% (10/12 patients) which is very similar to results obtained in this thesis. His ELISA also utilized a crude antigen preparation, but differed from the ELISA in this thesis in that a pool of CBV antigens was used. Banatvala et al. (1983) developed an ELISA for detection of CBV IgM

in children with insulin-dependent diabetes mellitus. Examination of sera from children with virologically confirmed CBV infection (either by CBV isolation and/or four-fold rise in neutralizing antibody titer) revealed that 81% (29/36) of children aged 6 months to 4 years had a homotypic IgM response, whereas 77% (44/57) of persons aged 15 years had a homotypic response. Of the sera from institutionalized children with virologically confirmed CBV 4 infection (by CBV isolation) in this thesis, 68% (13/19) of children aged 3 to 9 years demonstrated a homotypic IgM response by ELISA with the remaining 32% (6/19) exhibiting a heterotypic IgM response.

Morgan-Capner and McSorley (1983) remarked that if the IgM response in CBV infections is not type-specific, it may be impossible to obtain a type-specific IgM assay. In fact, the data obtained in this thesis strongly indicated that the cross-reactivity of the patient's IgM may be a problem in establishing a type-specific reaction by ELISA. The heterologous IgM reactions observed with the results in this thesis may be non-neutralizing IgM directed against a group determinant on the CBV virion as well as virus-specific neutralizing IgM directed against the currently infecting serotype. Conversely, in some of the sera found to be IgM negative at a dilution of 1:2.5, virus-specific IgM as detected by ELISA may possibly be non-neutralizing type-specific IgM directed against the currently infecting serotype.

The reasons why some individuals will manifest a heterotypic IgM response while others demonstrate only homotypic IgM antibody to the infecting serotype (even though they have IgG neutralizing antibody to more than one serotype) are not clear at present. However, it may be

related to the number of CBV types or other enteroviruses to which the individual has been exposed. Many of the children studied in this thesis exhibited high neutralizing antibody titers to more than one CBV type indicating a probable anamnestic IgG antibody response induced either by the currently infecting CBV serotype or a recent infection with another CBV serotype. The presence of neutralizing antibody was detected in 5 patients with negative virus isolation. Although these individuals exhibited CBV neutralizing antibody, both ELISA and neutralization tests after IgM extraction by sucrose density gradient fractionation revealed that these individuals did not have virus-specific IgM, strongly suggesting that they did not have a detectable current CBV infection.

Perhaps the most perplexing observation derived from the experimental results was the presence of group-specific IgM antibody in some individuals as detected by ELISA, but where the same individuals exhibited only neutralizing antibody to the currently infecting CBV serotype. These experimental results may be due to two possibilities. Since the antigen used in the IgM ELISA was not strictly type-specific, i.e., the antigen preparations contained both type- and group-specific determinants, group reactions may be expected if group-specific IgM was present in the patient's serum. However, the implications of this observation are that heterologous IgM antibody may not be directed against the neutralizing determinants of the virion. The latter possibility reflects the observation that in one individual, virus-specific IgM was detected by ELISA to CBV 3, 4, and 5, yet, the patient demonstrated only neutralizing IgM antibody to CBV 4. Another

individual demonstrated virus-specific IgM to CBV 4 and 5 in the acute serum, and IgM to all four CBV types in the convalescent serum, yet, only had neutralizing IgM antibody to CBV 4. The patient population studied in this thesis were clinically institutionalized children and presumably, the CBV 4 infection was the first CBV infection encountered by these children. This may be related to the presentation of novel virus group determinants which may be exposed in the infected person, resulting in the formation of group-reactive IgM antibodies. The nature of the host immune response to the individual virus polypeptides requires further investigation. Obtaining more type-specific antisera for the assay would not aid in developing a more type-specific assay as this would be irrelevant if virus cross-reactivity of the patient's IgM is the natural immune response. The only advantage of utilizing more specific antisera might be to increase the sensitivity of the ELISA by decreasing the background test activity. Preadsorption of the virus antigen with heterologous rabbit antisera before the addition of the homologous horse antiserum in the IgM ELISA may help in obtaining a more type-specific reaction with the detector antisera. If group reactivity of the patient's IgM is the difficulty, a type-specific IgM ELISA could only be developed if a more type-specific antigen is obtained. Consequently, a purified type-specific determinant (either a type-specific virion protein or peptide), if it could be obtained, would definitely aid in the development of a more type-specific IgM ELISA.

With reference to future evaluation of the IgM ELISA, other sera from individuals demonstrating seroconversions by neutralization tests

with CBV will be evaluated. As well, sera from individuals showing neutralizing antibody to more than one CBV type and persons with high static neutralizing antibody titers will be tested. To investigate the extent of cross-reactivity of virus-specific IgM in CBV infections to other enteroviruses, serum from individuals with a confirmed coxsackie A virus or echovirus infection could be analyzed. It may also be interesting to obtain serum samples from individuals who have recently been inoculated with poliovirus vaccine to examine the extent of the group IgM response. Evaluation of serum from persons with hepatitis A virus infections may also provide valuable information regarding the specificity of the CBV-IgM ELISA test.

In conclusion, the IgM ELISA results demonstrate that the assay can be utilized as a diagnostic test for CBV infections. Because of the rapidity and sensitivity of the test, it can potentially be used in place of the conventional techniques such as the neutralization test. However, as the results have indicated, it may be very difficult to obtain a type-specific ELISA due to the cross-reactivity of the patient's IgM response. Nevertheless, a test for detection of group-specific IgM to CBV is important for diagnosis of a recent infection.

V. SUMMARY

V. SUMMARY

An enzyme-linked immunosorbent assay (ELISA) utilizing the capture antibody principle was developed for the detection of coxsackie B virus-specific IgM in human serum. The detection of virus-specific IgM was used as an indicator of a recent infection with a particular virus, and avoided some of the difficulties associated with conventional techniques such as the microneutralization test. The immunoassay utilized an affinity purified anti-human IgM antibody adsorbed to polystyrene beads as the capture antibody. The ELISA involved addition of CBV antigen, CBV antisera, and then a peroxidase conjugated F(ab')2 fragment anti-horse IgG conjugate. A positive reaction in the ELISA thus depended on the presence of virus-specific IgM in the serum which is separated from other serum components by binding to the capture antibody followed by binding of the remaining reagents in the ELISA. The negative cut-off optical density of the ELISA was established as the mean optical density plus three standard deviations of cord blood obtained from 42 newborn infants known to be seronegative for antibody to CBV. Acute and convalescent sera from 19 children with a virologically confirmed CBV 4 infection were tested by ELISA, microneutralization, and by microneutralization after extraction of IgM by sucrose density gradient fractionation of the sera. The ELISA could detect CBV-specific IgM which was undetected in sucrose gradient IgM fractions at a titer of 1:2.5 (the lowest dilution used in the microneutralization test). In

a serum with a CBV IgM titer of 1:2.5 and a serum with a CBV IgM titer of 1:10, the ELISA could also detect virus-specific IgM at serum dilutions of 1:200 and 1:800, respectively. The sensitivity of the ELISA was 84% (16/19 patients) in comparison to the reference standard of virus isolation. The ELISA was more sensitive than the microneutralization test which demonstrated a 16% (3/19 patients) positivity rate, and more sensitive than neutralization tests based on sucrose density gradient fractionation of IgM from serum which detected virus-specific IgM in 13/19 patients. Five sera from children with negative CBV isolation and with or without preexisting neutralizing antibody to CBV were also tested. With the small number of these sera tested, the ELISA indicated 100% specificity for absence of a recent CBV infection as no CBV-specific IgM was detected either by ELISA or by neutralization tests of sucrose gradient IgM fractions. Of the 19 individuals examined with a confirmed virus infection (CBV 4 isolation), 68% (13/19) exhibited a homotypic IgM response to the currently infecting CBV serotype, whereas 32% (6/19) exhibited a heterotypic IgM response. In conclusion, the capture antibody ELISA for detection of CBV-specific IgM antibody has proven that it can be employed as a more rapid and sensitive method than conventional techniques for diagnosis of a recent CBV infection. However, it probably detects group as well as type-specific IgM antibodies, both of which may be produced naturally following a CBV infection.

VI. APPENDIX

APPENDIX

ELISA Buffers and Reagents

(1) Coating Buffer -- 0.06M Carbonate-Bicarbonate Buffer, pH 9.6

1.59 g Na_2CO_3

2.93 g $NaHCO_3$

Make up to volume of 1 liter with distilled water and store at 4° C. Discard buffer after 2 weeks.

(2) Washing Buffer -- Phosphate-Buffered Saline-Tween 20 (0.05% v/v), pH 7.2 (PBST)

1.096 g Na_2HPO_4 (anhydrous)

0.315 g $NaH_2PO_4 \cdot H_2O$

8.5 g NaCl

0.05 ml Tween 20

Make up to volume of 1000 ml with distilled water.

(3) Diluent Buffer -- Phosphate-Buffered Saline-BSA (2% w/v)-Tween 20 (0.15% v/v), pH 7.3 (PBS-BSAT)

0.639 g $NaH_2PO_4 \cdot H_2O$

2.186 g Na_2HPO_4 (anhydrous)

8.5 g NaCl

Make up to volume of 1000 ml with distilled water. Dissolve 2 g of bovine serum albumin (BSA) in PBS and make up to volume of 100 ml with more PBS. Add 0.15 ml Tween 20 to the buffer and mix. Store at 4° C.

APPENDIX CONT'D

(4) Substrate Buffer -- 0.01M Citrate-Phosphate Buffer, pH 5.0

0.510 g citric acid

0.7296 g Na_2HPO_4 (anhydrous)

Make up to volume of 1000 ml and store at 4° C.

(5) Substrate Solution

40 mg o-phenylenediamine (OPD) (Sigma Chemical Co., St. Louis, Mo.)

0.040 ml 30% H_2O_2 (J.T. Baker Chemical Co., Philipsburg, N.J.)

100 ml 0.01M citrate-phosphate buffer, pH 5.0

Make up substrate solution immediately before use.

VII. LITERATURE CITED

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