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IMMUNE CROSS-REACTIVITY BETWEEN INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS AND HUMAN CYTOMEGALOVIRUS

by

Kristin Marie Abraham

A Thesis Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE
WITH A MAJOR IN MICROBIOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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ACKNOWLEDGMENTS

The author is indebted to Dr. Randall F. Pritchett for his guidance and time during this study.

The author would also like to thank Dr. Wayne R. Ferris for his assistance with the electron microscopy, and Dr. B. Sue Criswell for her advice and expertise.

Finally, the author wishes to express her appreciation to her parents, Mr. and Mrs. Donald Olson, and her husband, Mr. John Abraham, for their continued encouragement and support.

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ABSTRACT

Antigen specific lymphocyte blastogenesis assays can be used to indicate in vivo immune reactivity to viral antigens after immunization. This study investigated the immune cross-reactivity of two herpesviruses, human Cytomegalovirus and Infectious Bovine Rhinotracheitis Virus, as demonstrated by blastogenic assay of rabbit peripheral blood lymphocytes.

In all cases, statistically significant lymphocyte responses to homologous antigens were observed, as well as significant responses to cross-reacting viral antigens. Cellular control antigen preparations were stimulatory only in the case of lymphocytes taken from animals immunized with CMV antigen preparations when stimulated by antigens derived from the host cell used to propagate CMV. The ability of lymphocytes committed to one virus to react with or be stimulated by a similar but different herpesvirus may lead to clinical applications of this cross-reactivity.

INTRODUCTION

Human Cytomegalovirus (CMV) and Infectious Bovine Rhinotracheitis Virus (IBRV; also referred to as Bovine Herpesvirus Type 1), two viruses of the herpesvirus group, are ubiquitous important pathogens. Herpesviruses range in size from 180 nm to 200 nm in diameter. They consists of an innermost section or "core" which is 76 nm in diameter that contains the double stranded DNA genome. The molecular weight of the genome among individuals of the group ranges from 82 x 10^6 daltons to 150 x 10^6 daltons. The core is enclosed in an icosahedral capsid 95-105 nm in diameter which is made up of 162 capsomers. The capsid and enclosed core are referred to as the nucleocapsid. The nucleocapsid is surrounded by a granular zone, or tegument, which in turn is enclosed by an envelope from which small spikes project. During morphogenesis nucleocapsids assemble in the nucleus and acquire envelopes via budding through the inner nuclear membrane. Host membrane glycoproteins may be incorporated into the envelope during the budding process. The typical herpesvirus virion consists of approximately 70% protein, 7% DNA, 22% lipid (phospholipid - cellular in origin) and 2% carbohydrate (1).

CMV is individually characterized by its ability to cause enlargement of cells or "cytomegaly", by the extraordinarily high molecular weight of its genome (150 x 10^6 daltons), and by its narrow host range. CMV infections are also characterized by the production of aberrant forms of the virus referred to as dense bodies.

Dense bodies are spherical forms containing homogeneous electron-dense material which is enclosed within membranes. These forms contain 22 of the 23 polypeptides found in the complete virus, but do not contain any DNA. The 22 structural polypeptides, including 8 glycoproteins, range in molecular weight from 24,000 to 171,000 daltons (2). Dense bodies which are synthesized in large amounts in infected cells possibly serve as immunogens in the naturally infected host when little extracellular virus is produced (3).

IBRV, a bovine pathogen, has not been reported to cause human disease. However, it does replicate in human cells. IBRV has a wide host range, and with respect to CMV, a short replication cycle. Unlike CMV, dense body forms are not seen in cells infected with IBRV. Electrophoretic analysis of purified IBRV revealed the presence of 25 to 33 structural polypeptides, eleven of which were found to be glycoproteins (4).

Herpesviruses are characterized by their ability to produce latent infections, with reactivations possible after variable periods of quiescence. CMV is thought to become latent in human peripheral blood lymphocytes, as evidenced by IgM antibody levels to CMV developing in seronegative patients who receive transfusions of blood from seropositive healthy donors. The form of CMV in these cells is unclear, as the virus is neither found complete, free in the plasma, nor whole within the lymphocytes (5,6). Latent herpesvirus infections typically occur after acute primary infections. In the case of CMV, primary infection often occurs without overt disease and the virus remains in the host as an endogenous latent infection.

Primary CMV infections and reactivations of latent endogenous CMV result in significant and often severe clinical manifestations in the human population. Currently, prenatal and neonatal CMV infection constitutes a greater danger to children than does rubella (7). From 0.5% to 1.0% of all children born excrete CMV in their urine, or have antibodies against CMV which persist longer than maternal antibodies (8). Cytomegalic inclusion disease (CMID), is the best characterized and most severe form of CMV caused disease contracted in utero. The clinical manifestations of CMID include microencephaly, cerebral calcifications, chorioretinitis, hepatosplenomegaly, thrombocytopenic purpura, deafness and hemolytic anemia (9). Other congenital infections may be inapparent in the newborn, but are activated in later life.

Most exogenous childhood and adult infections are thought to be spread via the respiratory route. Transmission via urine is also possible, as those children with apparent and inapparent viral infections contracted in utero may have protracted periods of viral shedding in the urine (10).

While most adults who become infected with the virus elicit little or no symptomatology, in children younger than 4 months of age the disease can progress and lead to hepatic and renal insufficiency, pneumonia, neurological symptoms and eventual death (11). The most likely mode of transmission in these cases appears to be via mothers' milk during breast feeding (12). In children over 4 months of age, the symptomatology is generally less severe, and the CNS is spared, although hepatitis or pneumonitis may result (13). Heterophile

negative mononucleosis is an exogenous infection caused by CMV, which is observed in CMV seronegative people who are recipients of multiple blood transfusions from latently infected donors (14).

Active infections with symptomatology in adults are generally due to the presence of a latent, endogenous virus contracted at an earlier age, which is reactivated due to allogeneic stimulation or immunosuppressive therapy. CMV infection is the most common and most important infection that occurs after allogeneic marrow transplantation. The most significant result of CMV disease after marrow transplantation is disseminated infection with pneumonia, although arthritis, hepatitis and leukopenia may result (15).

In patients receiving renal allografts, CMV and Herpes Simplex Virus (HSV) reactivations commonly occur (16). CMV infections following renal allografts have been reported in association with several syndromes including pneumonitis (17), fever and hepatitis (18), a mononucleosis-type syndrome, and hemolytic anemia accompanied by "transplant lung" syndrome and hepatic dysfunction (19). Humoral immunity appears to be unimpaired in infected transplant patients, as their neutralizing antibody titers are not significantly different from controls. Thus, it is likely that impairment of cell-mediated immune mechanisms are responsible for this complication of renal allograft surgery (20).

In the case of viral infections it has been shown that circulating antibody may be highly effective in protecting against infection and in decreasing the possibility of development of systemic

disease through limiting viremia. However, certain virus infections remain unresponsive to antibodies and may thrive in the face of high titers (21). Through the study of patients with T lymphocyte defects, it has been possible to elucidate the importance of the T cell in recovery from viral infection. This recovery is thought to be dependent on the elimination or restriction of virus infected cells (22).

In several cases of herpesvirus infections, the cellular response may be nonspecifically or specifically depressed in its level of activity in vivo (23). The latter is found in striking clarity in the case of cytomegalovirus infections, in which the reactivity of peripheral lymphocytes in infected patients is specifically suppressed to the CMV antigen (24). Thus, either through effects which result in elimination of the virus, or through suppressive effects brought about as a result of virus infection, the response of greatest consequence to the outcome of virus infection and production of disease is the cell-mediated response.

Treatment and preventative measures against CMV infection have been of great interest for the last several years. The use of antiviral chemotherapeutic drugs such as adenine arabinoside have not shown much promise (25), and the use of interferon during therapy has also been fairly ineffective as in most cases both substances caused only a transient decrease in urinary virus excretion (26).

The development of viral vaccines utilizing different laboratory strains of CMV began with the use of the AD-169 strain, which was originally isolated from a young girl undergoing adenoidectomy (27). Secondary efforts utilizing the Towne strain, isolated from an infant

with the signs of cytomegalic inclusion disease have also been undertaken (28). Both vaccines have undergone limited trials, with some success in the development of an antibody response and some indications of a cell-mediated response being elicited to the vaccine (29). The largest drawback to the use of these attenuated strains is their apparent ability to infect, and initiate latency in the vaccinated individual (30). As CMV, along with IBRV and other herpesviruses are suspected oncogenic agents (31,32), the long range suitability of live, whole virus vaccines is under some doubt (33). Trials with subunit vaccines have generally been disappointing, as vaccination resulted in poor protection when tested in an animal model system (34), and did not prevent the appearance of viremia in these animals.

An alternative approach to treatment of CMV infections involves the use of an antigenically related virus or preparation to cross-stimulate cell-mediated immune (CMI) responses. IBRV has been reported to cross-react immunologically with human CMV. Using serological methods such as immunofluorescence it was shown that CMV immune sera will react with IBRV infected cells (35). A preliminary report has suggested that oral administration of bovine transfer factor prepared from lymphocytes of an IBRV sensitized cow was able to stimulate a CMI response to CMV in a human patient with CMV disease (36). Also, IBRV has recently been shown in isolated cases to stimulate a blastogenic response in lymphocytes of donors with a history of infection with human herpesviruses (37). The possibility of using this related virus in some capacity to augment recovery from CMV

caused diseases has stressed the importance for documentation of its immune cross-reactivity with CMV.

This study was designed to establish whether antigenic components of CMV and IBRV are capable of cross-stimulating a CMI response. The experimental approach was first to immunize individual rabbits with either CMV or IBRV. The in vitro blastogenic responses of peripheral blood lymphocytes to the homologous and heterologous viruses were then assayed and the results compared.

MATERIALS AND METHODS

Animals

Adult male New Zealand White rabbits each weighing approximately 2 kilograms were maintained with rabbit pellets and tap water ad libitum throughout the experimental procedure.

Cell Cultures

Human foreskin fibroblast (HFF) cell cultures were prepared from human foreskins in stationary flasks. Subsequently the HFF cells were cultivated in 260 mm glass roller bottles (Bellco Glass, Inc., Vineland, N.J.). Cells were grown in Eagle's minimal essential medium (EMEM, Flow Laboratories, Rockville, Md.) supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.), 2 mM L-glutamine (GIBCO), 0.01 M Tricine, 0.075% $NaHCO_3$, and antibiotics (100 U Penicillin per ml and 100 µg of Streptomycin per ml). Cells were subcultured after reaching confluency at a split ratio of 1:3 once per week. Cells of passage levels 5 through 16 were used for virus propagation.

The Madin-Darby Bovine Kidney (MDBK) infinite cell line was obtained from Dr. John Maré (University of Arizona, Tucson, Az.) and cultivated in 32 oz. glass prescription bottles. The MDBK cells were grown in the same medium as HFF cell cultures and were subcultured after reaching confluency at a split ratio of 1:4 twice per week.

Virus Propagation and Assay

The Towne strain of human CMV originally isolated from the urine of an infant with cytomegalic inclusion disease was obtained from Dr. M. F. Stinski (University of Iowa, Iowa City, Ia.). Virus was adsorbed onto roller bottle cultures of HFF cells at a concentration of 0.1 plaque forming units (PFU) per cell. After an adsorption period of 90 minutes at 37C, cultures were overlayed with 80 ml of EMEM supplemented with 2% fetal calf serum, 2 mM L-glutamine, 0.01 M Tricine, 0.075% $NaHCO_3$, and antibiotics. Infected cell cultures were incubated at 37C and rolled at 0.25 rpm. At 4 days post-infection, the culture medium was replaced and L-arginine was added to a final concentration of 1.2 mM. Virus was harvested at 9 days post-infection from the extracellular culture fluid.

A stock preparation of IBRV was obtained from Dr. John Maré. Virus was adsorbed onto 32 oz. prescription bottle cultures of MDBK cells at a concentration of approximately 20 PFU per cell. After an adsorption period of 60 minutes at 37C, cultures were overlaid with 15 ml of EMEM supplemented with 2% fetal calf serum, 2 mM L-glutamine, 0.01 M Tricine, 0.075% $NaHCO_3$, and antibiotics. At 24 hours post-infection, virus was harvested from the extracellular culture fluid.

Infectivity titers of CMV in HFF cells and IBRV in MDBK cells were determined by the plaque technique under methyl cellulose overlays (38). Plaques of CMV were counted at 9 days post-infection and those of IBRV were counted at 4 days post-infection.

Radioisotope Labeling of Virus

To monitor virus purification, the DNA of IBRV was labeled by the addition of 15 µCi/ml of ^{3}H-thymidine (71 Ci/mmol, Amersham Corp., Arlington Heights, Ill.) to MDBK cell cultures immediately after virus adsorption.

Purification of Virus

All steps of the purification procedure were conducted at 4C or on ice. Pooled extracellular culture fluid (200 ml containing 2 x 10^{8} PFU per ml of IBRV or 1 x 10^{7} PFU per ml of CMV) was centrifuged in a Sorvall GSA rotor (DuPont Instruments, Newtown, Conn.) at 1500 x g for 15 minutes to pellet cells and debris. The virus was subsequently pelleted from the supernatant by centrifugation in a Sorvall SS-34 rotor at 27,000 x g for 90 minutes at 4C.

Virus pellets were resuspended in 3 ml of virus resuspending buffer (0.5 mM $NaPO_4$, pH 7.1) and homogenized with 20-30 strokes of a Dounce homogenizer. Suspensions were then clarified by centrifugation at 1100 x g in a SS-34 rotor for 15 minutes and layered onto a 10% to 60% sucrose (wt/wt, in virus buffer: 0.15 M NaCl-50 mM Tris-hydrochloride, pH 7.2) gradient. Gradients were centrifuged at 64,000 x g for 1 hour in a Beckman SW 25.1 rotor (Beckman Instruments, Inc., Palo Alto, Ca.). In the case of CMV gradients, two light scattering bands were observed in the gradients at the end of centrifugation: an upper distinct band consisting of complete virions and a lower diffuse band containing dense bodies. Both fractions were collected and diluted in virus buffer. In the case of IBRV gradients, only one diffuse light

scattering band was observed, collected and diluted in virus buffer. Virus preparations were subsequently pelleted by centrifugation in a Beckman SW 25.1 rotor at 83,000 x g for 90 minutes. Virus pellets were stored at -70C.

Virus pellets were resuspended in sterile deionized water and the virus further purified by centrifugation through a preformed cesium chloride gradient of density range 1.16 g/cm^3 - 1.37 g/cm^3 in a Sorvall AH627 rotor, at 100,000 x g for 4 hours at 20C. Gradients were subsequently fractionated into 0.5 ml fractions and the elution profile of the ^{3}H-thymidine labeled IBRV was obtained. CMV fractions corresponding to densities of 1.263 g/cm^3 and 1.219 g/cm^3 (38), and IBRV fractions corresponding to densities of 1.329 g/cm^3 and 1.258 g/cm^3 were pooled together independently, diluted with virus buffer and pelleted by centrifugation at 110,000 x g at 4C for 2 hours in a Sorvall AH627 rotor. Virus pellets were stored at -70C.

Preparation of Antigens

Virus pellets were resuspended in sterile deionized water and treated with a 1:4000 dilution of 37% formaldehyde at 37C for three hours. Preparations were then held at 4C for 18 hours and subsequently pelleted by centrifugation in a Sorvall SS-34 rotor at 27,000 x g for 60 minutes at 4C. Loss of infectivity of formalin-treated virus was assured by the inability of an undiluted portion to produce cytopathology in appropriate cell cultures. The formalin-inactivated purified preparations of CMV and IBRV served as virus antigens.

Freeze-thaw lysates of cells which were clarified by centrifugation at 1100 x g for 15 minutes in a Sorvall SS-34 rotor served as cellular control antigens.

Ultraviolet Sterilization of Antigens

Preparations to be used as antigens for animal injections and lymphocyte stimulation assays were sterilized by exposure to ultraviolet light (UA-3GE 360W) for 5 minutes at a distance of 35 cm. Sterility was assured by absence of growth after inoculation of preparations onto blood agar plates (5% sheep red blood cell; GIBCO).

Protein Concentration Determination of Antigens

Protein concentrations were determined for viral antigen and cellular control antigen suspensions using the Bio-Rad colorimetric technique (Bio-Rad Laboratories, Richmond, Ca.). Suspensions were then adjusted to 150 µg of protein per ml of sterile deionized water.

Electron Microscopy of Virus Preparations

Virus preparations were spread on Formvar treated, carbon coated copper electron microscopy grids, and negatively stained with a 2% phosphotungstate solution. Virus preparations were examined and photographs taken at various magnifications using a Phillips EM 200 electron microscope.

Sensitization of Animals

Rabbits were sensitized by injecting a total of 450 µg of purified CMV or IBRV antigen. Each of three injections was given subcutaneously and prepared as an equal volume of antigen to Freunds incomplete adjuvant (Difco Laboratories, Detroit, Mi.), with a total volume per injection of 2 ml. The three injections were given within 15 days, each 5 days apart.

Mitogens

Phytohaemeagglutinin (PHA-P, GIBCO) was prepared as a 10 mg/ml stock solution in sterile deionized water. Concanavalin A (Con A, Sigma Chemical Co., St. Louis, Mo.) was prepared as a 1 mg/ml stock solution in sterile deionized water. The stock solutions were filter sterilized through .45 µm filters and then diluted to appropriate concentrations in Roswell Park Memorial Institute 1640 medium (RPMI-1640, GIBCO).

Lymphocyte Culture Medium

RPMI-1640 was buffered with 10 mM HEPES and supplemented with 10 mM Gentamycin Sulfate and 250 mM Fungizone (GIBCO). Pooled normal rabbit serum which was heat inactivated at 56C for 30 minutes was added to a final concentration of 5%. Complete medium was stored at -20C.

Preparation of Peripheral Blood Lymphocytes for Assay

Approximately 18 ml of blood was collected via cardiac puncture into syringes containing 2 ml of 100 IU of heparin per ml. The heparinized blood was diluted 1:2 with sterile RPMI-1640, layered over Ficoll-Paque (Pharmacia, Piscataway, N.J.) and centrifuged at 400 x g for 30 minutes at room temperature. Peripheral blood lymphocytes were collected from the interface, washed in RPMI-1640 and pelleted by centrifugation at 100 x g for 20 minutes at 4C. Cell pellets were resuspended in RPMI-1640, viability assessed by trypan blue dye exclusion, and the concentration adjusted to 1.0×10^6 viable cells per ml in complete media.

Lymphocyte Stimulation by Antigens and Mitogens

Cells were dispensed in 0.2 ml volumes into wells of a 96 well flat bottom microtiter plate (Costar, Cambridge, Mass.). Ten, 15 or 20 µl amounts of test antigen or mitogen were added to appropriate wells and plates were incubated in a humidified atmosphere of 5% CO_2 at 37C. After 48 hours of incubation (unless otherwise indicated) in mitogen wells, and 5 days of incubation in antigen wells, 1 µCi of ^{3}H-thymidine (5.0 Ci/mmol ^{3}H-6-thymidine; Amersham Corp.) was added. After a pulse labeling period of 24 hours, cells were harvested onto glass fiber filters (Microbiological Associates, Walkersville, Md.) using a Mini-MASH multiple automated sample harvester (Microbiological Associates), and washed with 0.15 M NaCl. Filter pads were dried

under a heat lamp and transferred into plastic disposable scintillation vials (New England Nuclear, Boston, Mass.). Scintillation cocktail (Betamax; Westchem Products, San Diego, Ca.) was added to each vial in 5 ml amounts, and radioactivity in the vials was counted in a Tri-Carb liquid scintillation spectrophotometer (Model 332, Packard Instrument Co., LaGrange, Ill.) at a gain of 6.5 and window settings of 50 and 1000.

Statistical Analysis of Data

Data were analyzed using a Student's paired-t test on absolute counts per minute recorded on the Tri-Carb liquid scintillation spectrophotometer.

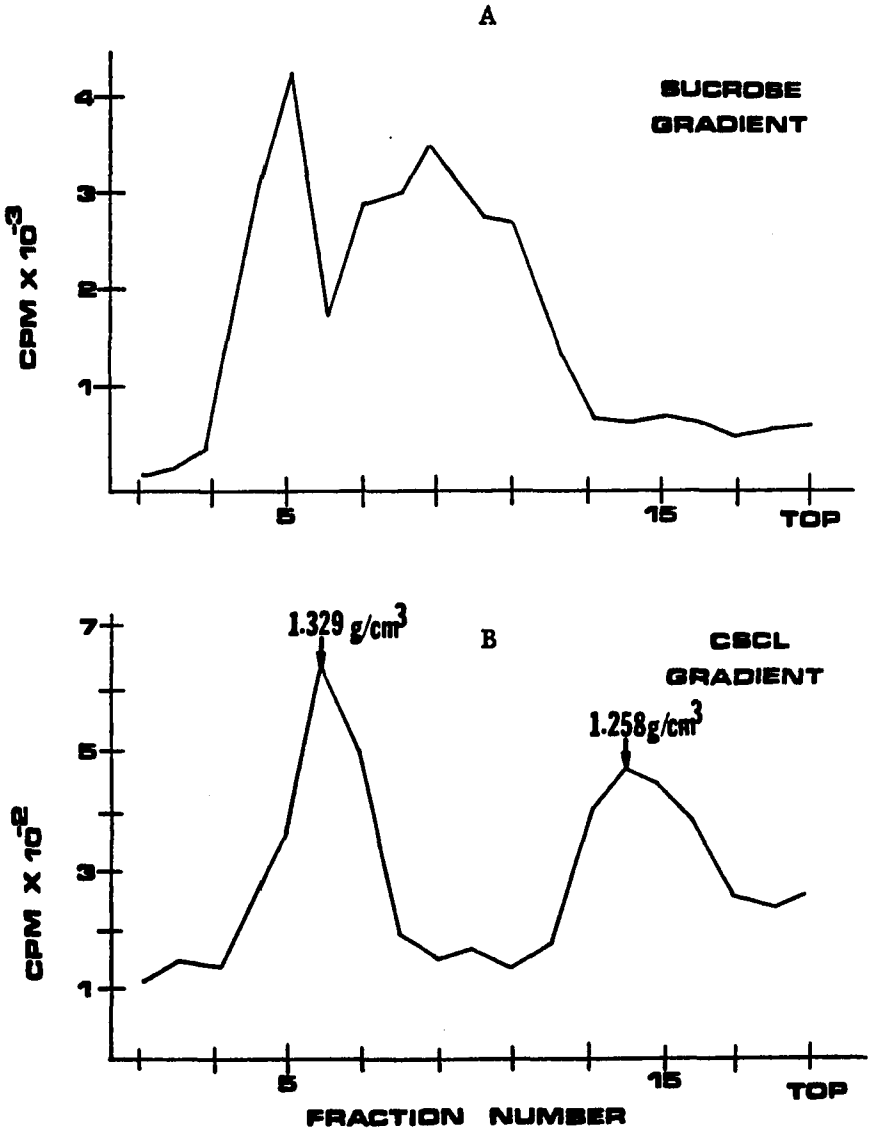

Figure 1 (A&B). ^{3}H-thymidine distribution in 10%-60% sucrose gradients (A) and preformed CsCl gradients (B) after centrifugation of labeled IBRV. Values represent corresponding densities in fractions 6 (nucleocapsids) and 14 (enveloped virions) of the CsCl gradient.

for CMV are 1.263 g/cm^3 for naked nucleocapsids and 1.219 g/cm^3 for enveloped virions and dense bodies (39).

Electron Microscopic Examination of Purified Virus

Viral preparations to be used as antigens were examined by electron microscopy. This provided for a description of the virions and subvirion particles present, and also for an approximation of the amount of extraneous debris present. Electron microscopic examination of purified CMV revealed the presence of enveloped virions and dense bodies (Fig. 2). IBRV preparations were found to contain enveloped virions and naked nucleocapsids (Fig. 3). Both the CMV and IBRV preparations were relatively free of extraneous membranous debris.

Kinetics of the Mitogenic Response to Con A and PHA

Preliminary experiments were conducted to assess the optimum dose requirements, and the time of maximum response in vitro to mitogenic stimulation. Results are expressed as stimulation indices (SI) which are the mean cpm of cells treated with mitogen divided by the mean cpm of cells incubated without mitogen (controls). The in vitro mitogenic response of rabbit peripheral blood lymphocytes was determined on day 2, 3, and 5 of incubation with 50 µg/ml of Con A (Fig. 4A). In all animals except rabbit #3, peak stimulations to mitogen were seen at 2 days, with decreased responses at 3 days and the lowest responses at 5 days. Rabbit #3 had a peak stimulation response

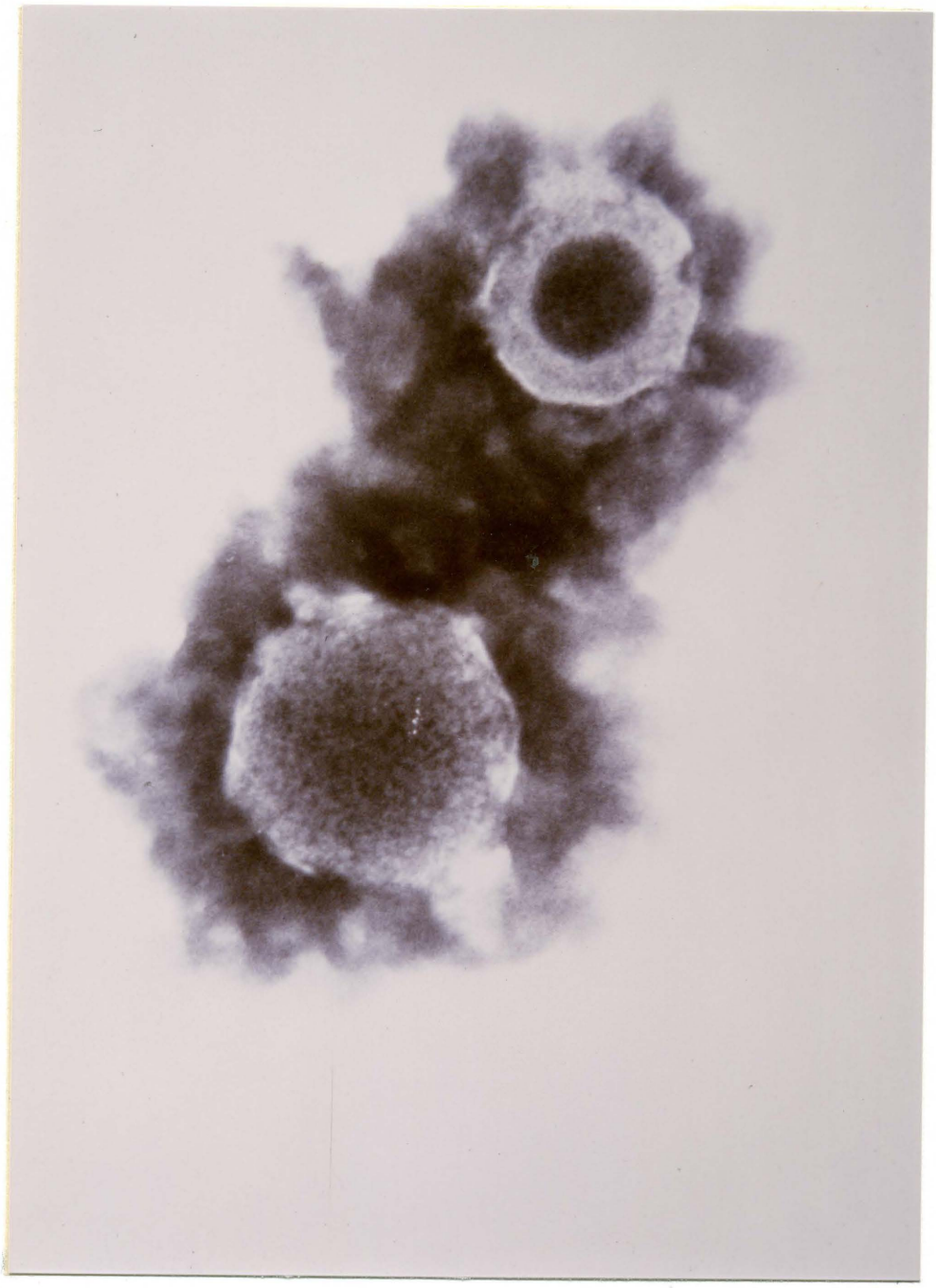

Figure 2. Electronmicrograph of the CMV virus preparation. (150,000 X)

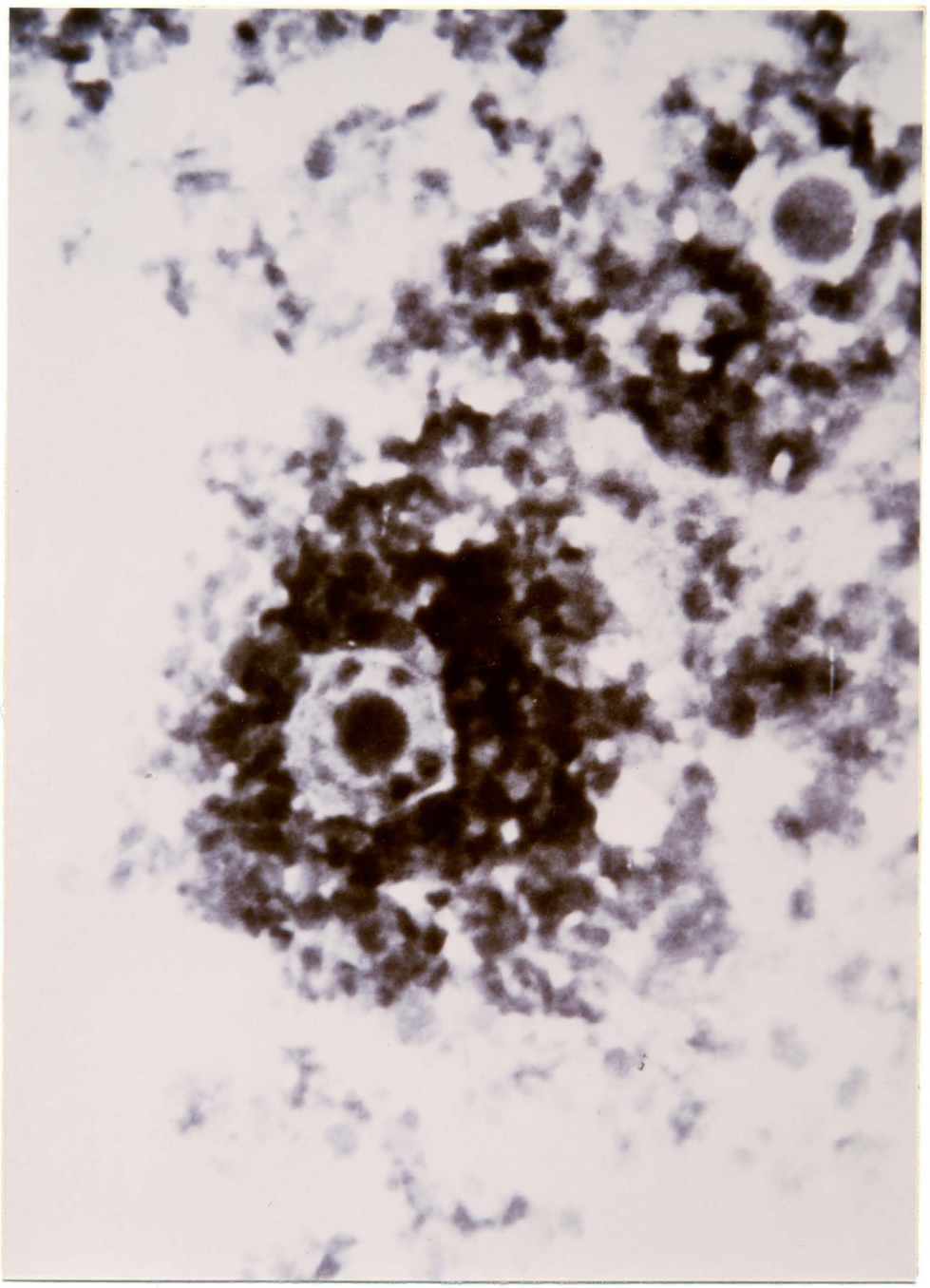

Figure 3. Electronmicrograph of the IBRV virus preparation. (150,000 X)

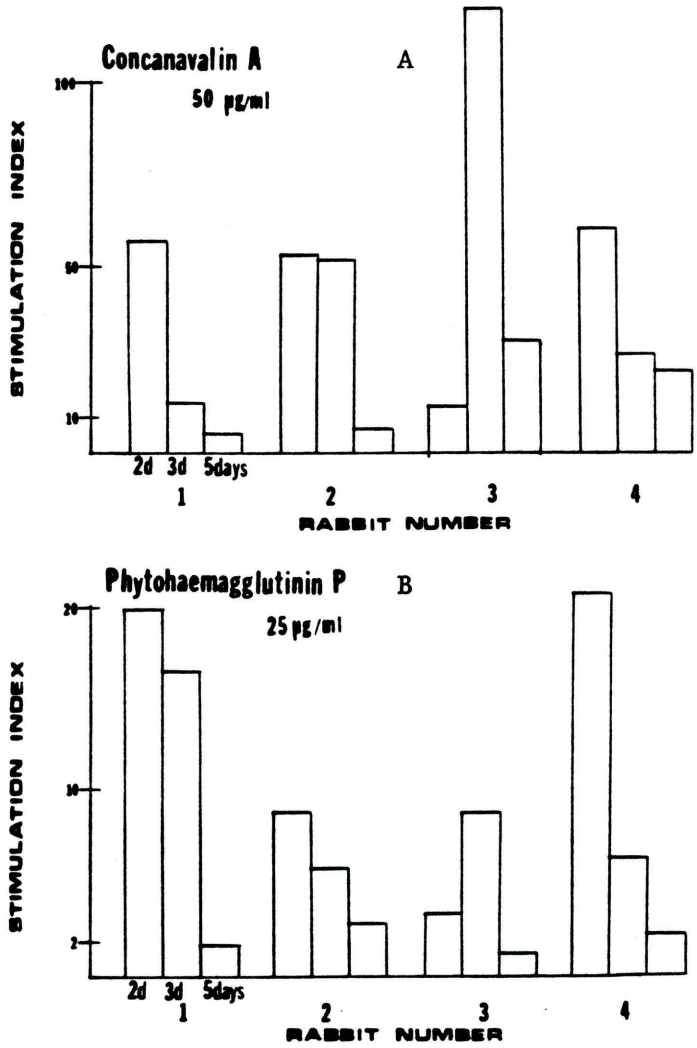

Figure 4 (A&B). Mitogenic response of rabbit lymphocytes to Concanavalin A and Phytohaemeagglutinin as a function of in vitro culture time for four test rabbits. Stimulation index = cpm in mitogen stimulated cultures ÷ cpm in control cultures.

to Con A at 3 days, with lower responsiveness at 2 days and 5 days. The mitogenic responses to PHA (25 µg/ml) on day 2, 3, and 5 of incubation are shown in Fig. 4B. For all rabbits except #3, peak responses were seen at 2 days, with lower responses at 3 days and 5 days. Lymphocytes of rabbit #3 had a peak response at 3 days, with lower responsiveness at 2 days and 5 days. In subsequent experiments, mitogen stimulated cultures were pulsed with ^{3}H-thymidine at 2 days of incubation.

The stimulation of peripheral lymphocytes as a function of Con A concentration is illustrated in Fig. 5A. Although the lymphocytes of rabbits #2 and #3 had quantitatively lower responses to the mitogen than those of rabbits #1 and #4, the peak stimulation in lymphocytes of all 4 rabbits occurred at a concentration of 25 µg/ml of culture. Shown in Fig. 5B are the responses of the peripheral blood lymphocytes as a function of concentration of PHA. The optimum response of rabbits #3 and #4 was at a dose of 12.5 µg/ml, while rabbit #1 responded best at 25 µg/ml and rabbit #2 at 62.5 µg/ml. Rabbits #1 and #2 had a quantitatively lower response than rabbits #3 and #4 to the PHA mitogen.

Lymphocyte Blastogenic Response of Rabbits Immunized with IBRV and CMV

To determine whether a CMI response would detect antigenic relatedness between IBRV and CMV, the in vitro lymphocyte blastogenic responses of four rabbits (two immunized with IBRV and two immunized with CMV) to homolgous and heterologous antigens were studied. At

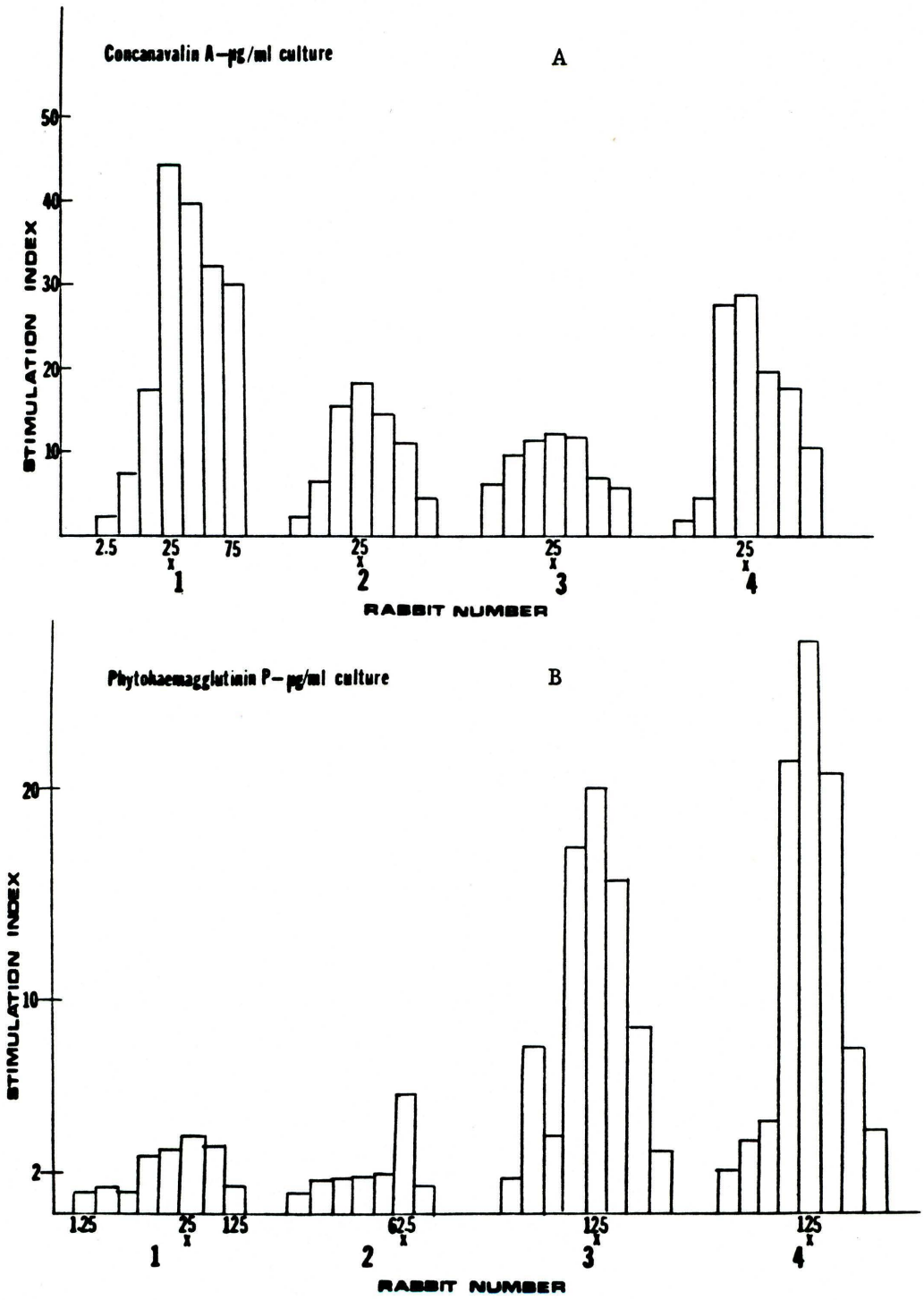

Figure 5 (A&B). Changes in mitogenic response of rabbit lymphocytes to Concanavalin A and Phytohaemeagglutinin as a function of concentration of mitogen. "X" denotes concentration optimum for each of four test rabbits.

every weekly bleeding period, six concentrations of both CMV and IBRV antigens were incubated with peripheral blood lymphocytes in culture. The mean cpm of triplicate samples was calculated, as well as standard deviations for each concentration. The response of peripheral lymphocytes of each rabbit at 1 week post-immunization is shown in Table 1. In the case of rabbit #1, optimal response to CMV was at a dilution of 1:2 of the original 150 µg/ml injection preparation, when 10 µl was added to the culture. Response to IBRV was optimal at 1:2 (10µl). Rabbit #2 had lymphocytes which responded best in culture to a concentration of 1:10 (10µl) of CMV and 1:2 (20µl) of IBRV. The amount of antigen found to give the greatest stimulatory response in lymphocytes of rabbits #3 and #4 was 20 µl of a 1:2 dilution of the 150 µg/ml injection preparation for both the CMV and IBRV antigens. The responses at the above antigen concentrations were plotted as a function of time (Figs. 6 and 7).

The mitogenic responses of rabbit peripheral lymphocytes were used to assess the in vitro cultivation technique. This technique was to be employed in subsequent assays to determine reactivity of the lymphocytes to viral antigens. The results of studies to determine optimum mitogen concentrations and proper timing of pulse-labeling were utilized in a standard procedure at each weekly bleeding to assess the culturing technique.

The in vitro lymphocyte blastogenic responses to IBRV and CMV antigens for two rabbits immunized with IBRV are shown in Figs. 6A (rabbit #1) and 6B (rabbit #2). The data plotted are mean cpm of

Table 1. ^{3}H-Thymidine uptake of peripheral lymphocytes at one week post-immunization as a function of virus antigen concentration.

Antigen Concentration		IBRV Immunized		CMV Immunized	
		Rabbit #1	Rabbit #2	Rabbit #3	Rabbit #4
CMV Antigen 1:2[a]	20 µl	1683(367)[b]	363(147)	3729(365)	5324(1246)
	10 µl	3293(527)	492(192)	3311(1099)	3390(1663)
CMV Antigen 1:10	20 µl	1466(333)	398(73)	3392(364)	4779(1960)
	10 µl	2690(176)	560(65)	2629(315)	1185(1379)
CMV Antigen 1:100	20 µl	990(249)	234(121)	1163(143)	262(33)
	10 µl	2290(576)	278(43)	1927(1121)	212(9)
IBRV Antigen 1:2	20 µl	5067(990)	990(14)	6020(396)	3242(506)
	10 µl	6723(724)	925(91)	3780(5282)	1162(211)
IBRV Antigen 1:100	20 µl	3119(1002)	751(47)	1652(253)	307(8)
	10 µl	4009(850)	578(108)	1768(784)	364(94)
IBRV Antigen 1:100	20 µl	1611(321)	295(47)	1016(309)	245(20)
	10 µl	2815(140)	369(156)	995(716)	308(29)
CONTROLS		3778(142)	582(111)	2355(414)	263(46)

[a]Dilution of stock 150 µg/ml antigen solution

[b]Arithmetic mean of cpm of triplicate samples (standard deviation)

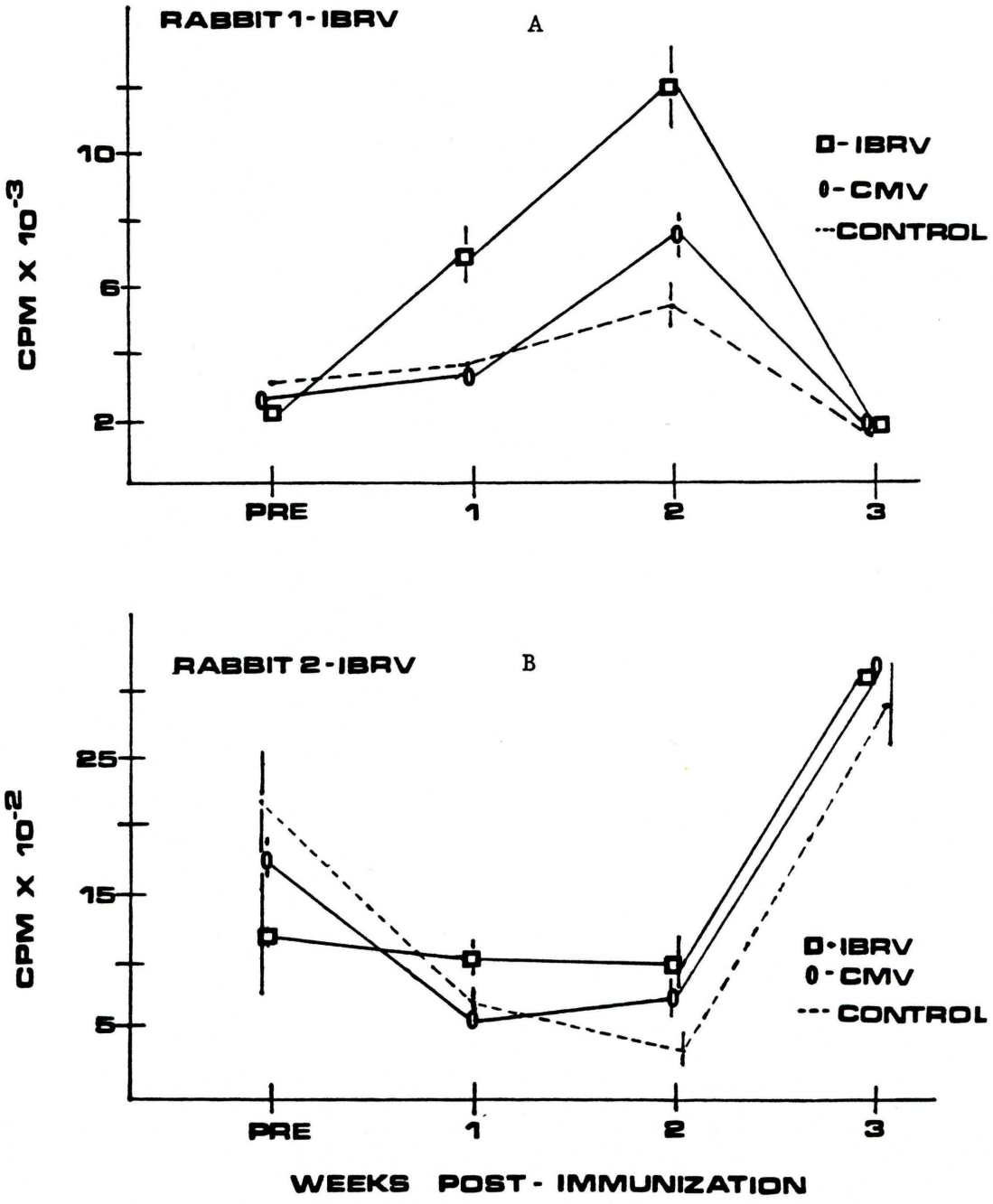

Figure 6 (A&B). Response of lymphocytes from rabbits immunized with IBRV to homologous and heterologous antigenic stimulation. Vertical lines represent (+/-) one standard deviation.

triplicate samples +/- one standard deviation. Lymphocytes of rabbit #1 at pre-immunization showed little response to antigenic stimulation, (i.e., cpm values in test wells were not significantly different from those in control wells). After 1 week post-immunization with IBRV antigen, a significant response was observed to the homologous antigen, IBRV, but the response to the heterologous antigen, CMV, was not significantly different from controls. At 2 weeks post-immunization, the homologous antigen response peaked, and a concomitant rise in response to the heterologous antigen was observed. By 3 weeks, response levels to both antigens had returned to near control levels. The response of lymphocytes from a second IBRV immunized rabbit (#2) to antigenic stimulation is shown in Fig. 6B. As with the first rabbit, pre-immunization stimulation responses to antigens were not significantly different from controls. One week after immunization with IBRV, the incorporation of ^{3}H-thymidine in controls dropped precipitously, while there was evidence of stimulation occurring to the homologous antigens. At 2 weeks post-immunization, the control stimulation dropped again, while stimulation with the homologous antigen, IBRV, again remained fairly constant, and an increase in response to the heterologous CMV antigen was observed. By the third week, all levels of response to antigen had returned to near control levels, and more closely resembled the responses observed at pre-immunization.

The in vitro lymphocyte blastogenic responses of two rabbits immunized with CMV, to IBRV and CMV antigens are shown in Figs. 7A

(rabbit #3) and 7B (rabbit #4). Figure 7A illustrates the lymphocyte blastogenic responses of rabbit #3, immunized with CMV, to antigenic stimulation. During the pre-immunization period, there was no significant response observed in the lymphocytes to test antigens as compared to controls. At the first week after immunization with CMV, there was an increase in the response of peripheral lymphocytes to both the homologous antigen, CMV, and the heterologous antigen, IBRV. By 2 weeks, the responses to both antigens were not significantly different from controls. The lymphocyte responses of rabbit #4, the second immunized with CMV, to antigenic stimulation are shown in Fig. 7B. At pre-immunization, test antigen and control antigen stimulation values of lymphocytes were not significantly different. At 1 week post-immunization, a substantial increase was seen in both the response to the homologous antigen (CMV), and the heterologous antigen (IBRV). Both responses were significantly different from controls. By the second week after immunization, the stimulation of lymphocytes in response to all antigenic stimulation was once again indistinguishable from controls.

Cellular control antigens were used in the blastogenic assays to determine if any sensitization to contaminating cellular debris had occurred during the immunization process. The ^{3}H-thymidine uptake induced in each of the four rabbits' lymphocytes during each week of the blastogenic assay procedure is shown in Table 2. Rabbits #1 and #2, both immunized with IBRV, show negligible responsiveness to either the MDBK antigen (corresponding to cells in which the IBRV

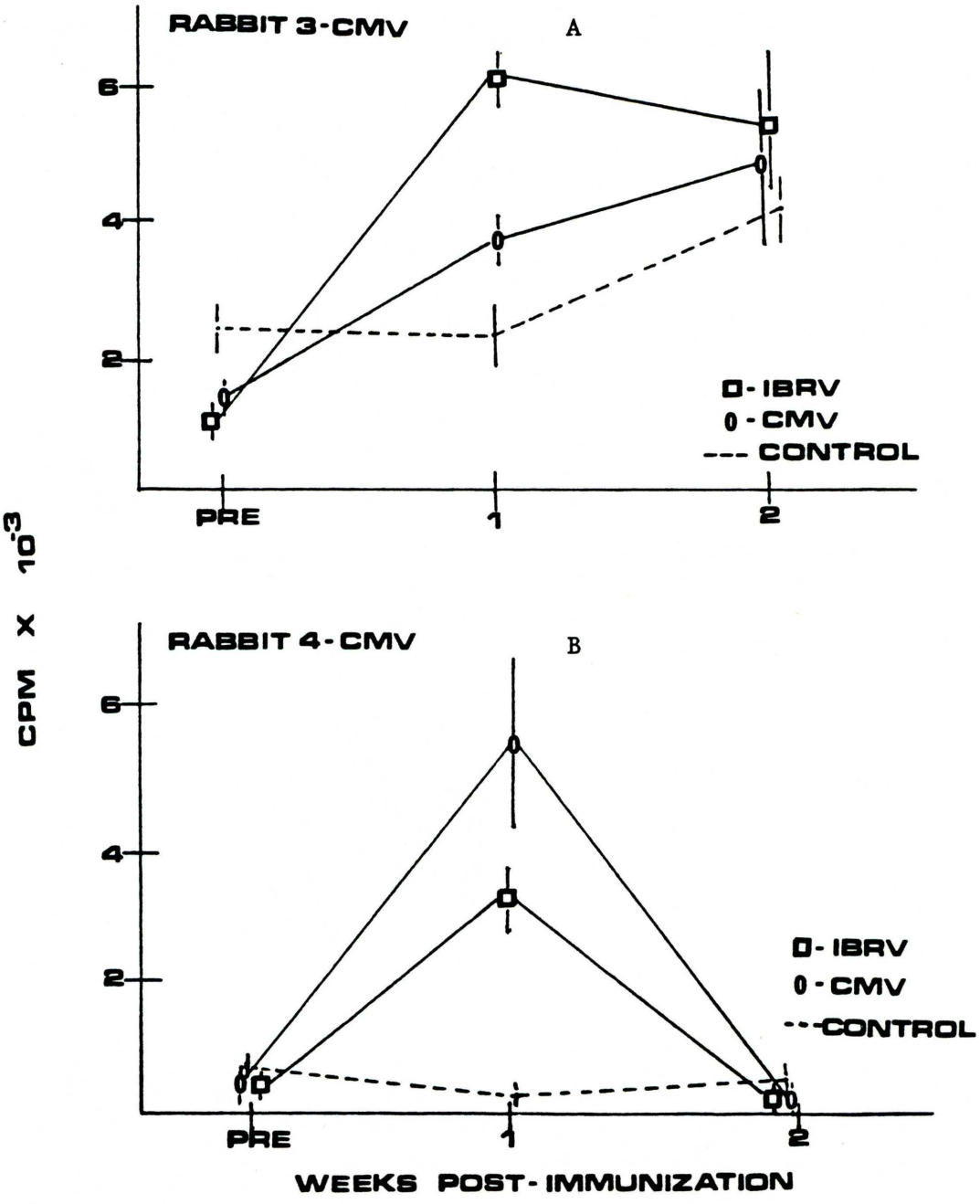

Figure 7 (A&B). Response of lymphocytes from rabbits immunized with CMV to homologous and heterologous antigenic stimulation. Vertical lines represent (+/-) one standard deviation.

Table 2. ^{3}H-Thymidine uptake of peripheral lymphocytes in response to cellular control antigens.

		PREBLEED	Week 1	Week 2	Week 3
Rabbit #1	HFF[a]	3837(882)[c]	2475(314)	5712(516)	2328(128)
	MDBK[b]	2092(70)	1171(143)	2616(303)	1992(615)
	Controls	3158(220)	3778(142)	5022(554)	2378(1052)
Rabbit #2	HFF	2015(321)	250(105)	475(198)	2328(128)
	MDBK	1884(360)	217(84)	453(101)	1992(615)
	Controls	2090(379)	582(111)	302(37)	2303(365)
Rabbit #3	HFF	2425(200)	3278(405)	3721(639)	
	MDBK	1325(65)	1198(1272)	2462(515)	
	Controls	2402(307)	2355(414)	4174(631)	
Rabbit #4	HFF	646(202)	3163(589)	134(25)	
	MDBK	404(43)	441(179)	182(67)	
	Controls	500(62)	263(46)	311(72)	

[a]Human Foreskin Fibroblast cellular control antigen, added as 20 µl of a 1:2 dilution of a 150 µg/ml stock solution.

[b]Madin-Darby Bovine Kidney cellular control antigen, added as 20 µl of a 1:2 dilution of a 150 µg/ml stock solution.

[c]Arithmetic mean of cpm of triplicate samples (standard deviation).

was propagated), or the HFF antigen (derived from the cells used to propagate CMV). The response seen against antigens in these rabbits can be said to be virus-specific, and not reflective of sensitization against contaminating cellular antigenic determinants. The lymphocytes from rabbits immunized with CMV (#3 and #4), show no responsiveness to MDBK antigens. However, at week 1, significant responses to the HFF cellular antigen control were observed.

A summary of statistical evaluations of the data is shown in Table 3. As evaluated by a paired-t test, all rabbits gave statistically significant reactions to the homologous antigen (IBRV: rabbits #1 and #2, and CMV: rabbits #3 and #4). Lymphocytes of all rabbits reacted significantly to the heterologous, or cross-reacting antigen in vitro (CMV: rabbits #1 and #2, and IBRV: rabbits #3 and #4). Rabbits #1 and #2 showed no statistically significant reactivity to cellular control antigens in vitro. Rabbits #3 and #4 showed significant reactivity to the HFF celluar control antigen, but not to the MDBK cellular control antigen.

Table 3. Significance of test cpm vs control cpm at optimum response as determined by a paired-t test.

RABBIT NO.	IBRV	HCMV	MDBK	HFF
1	99.0%[a]	99.5%[b]	NS	NS
2	99.5%	99.0%	NS	NS
3	99.5%	99.0%	NS	95.0%[c]
4	99.5%	99.0%	NS	99.0%

[a]Corresponding alpha value 0.01

[b]Corresponding alpha value 0.005

[c]Corresponding alpha value 0.05

DISCUSSION

As a means of obtaining virus preparations which were relatively free of contaminating cellular debris, a double gradient technique was utilized during purification. First, virus and subviral components were separated from cellular debris on the basis of size by velocity sedimentation through sucrose gradients. This technique also separated naked nucleocapsids, enveloped virions, and dense bodies. Second, the isopycnic banding of virus preparations in CsCl separated virus from cellular material on the basis of density, and enabled the estimation of densities of naked nucleocapsids and enveloped virions of IBRV.

Electron microscopic examination of virus preparations revealed the presence of virions with typical herpesvirus morphology in both the CMV and IBRV preparations. The virus suspensions appeared by visual inspection to be relatively free of cellular membraneous materials. The majority of material present in the preparations was viral in nature.

The response of lymphocytes to mitogenic stimulation by Con A and PHA was used to assess the in vitro cultivation technique. This basic technique was used in subsequent assays to determine the response of lymphocytes to antigenic stimulation. Examination of stimulation at three time intervals revealed in most cases, optimum response to both Con A and PHA occurred after 2 days of in vitro

culture. The optimum concentration of mitogen was also assessed. The concentrations giving optimum stimulation were used throughout all antigenic stimulation assays to assess the reactivity of cells in culture. Con A and PHA have been shown to stimulate separate subpopulations of T cells in the murine system (40), and it is thought that through their joint use the majority of T cells present in peripheral blood can be monitored. Throughout the procedure, all animals maintained acceptable mitogenic stimulation levels (SI of greater than 3), indicating the culturing technique was maintained satisfactorily, and reactivity of rabbit lymphocytes was near levels found at pre-immunization. In the case of rabbit #2, mitogenic stimulation dropped at week 1, and returned to near normal levels at week 3. This trend was very similar to the pattern found in this rabbit with antigenic stimulation.

As a means of determining the concentration of antigen which would give the maximum response in sensitized lymphocytes, responses to several concentrations of antigen were determined throughout the procedure. These concentrations appeared to be fairly individual, as most of the rabbits' lymphocytes reacted at different concentration optimums.

Rabbit #1 (immunized with IBRV), gave a rapid response to antigenic stimulation with IBRV which was significant at week 1. The response to CMV, the heterologous antigen, lagged a week behind and did not become significant until week 2, when the response to IBRV peaked. The lymphocytes of this rabbit injected with IBRV, could be stimulated by both the homologous antigen (IBRV) and the heterologous

antigen (CMV). This response indicates that lymphocytes sensitized against IBRV recognized certain similar or cross-reacting antigens of CMV.

Rabbit #2, also immunized with IBRV, had lymphocytes which gave a significant response to both IBRV and CMV at 2 weeks post-immunization. The basal reactivity rate of this rabbit's lymphocytes, along with their mitogenic reactivity, decreased dramatically at one week post-immunization.

A lymphocytic response to both the CMV and IBRV antigens was also detected in rabbits immunized with CMV (#3 and #4). Although significant responses to both the homologous (CMV) and heterologous (IBRV) antigens were detected in rabbit #3, the response to the heterologous antigen was statistically higher than the response to the homologous antigen. This could possibly be attributed to the time of stimulation of the cells in vitro. All antigenic lymphocyte responses were pulsed after 5 days of incubation with the antigen. The previous mitogen response experiments had revealed that lymphocytes of rabbit #3 had a different optimum time of response than the other 3 rabbits. Therefore, it is possible that the optimum response time of rabbit #3's lymphocytes in vitro to stimulation by antigen could also be different from the other animals.

The response in rabbit #4 was very brisk, peaking at one week post-immunization to both CMV and IBRV antigens. This response indicates that after immunization with the CMV antigen preparation, cells of the rabbit were sensitized against CMV, and were reactive against certain antigens found in the IBRV preparation.

Through the purification technique, an IBRV antigen was prepared which appeared to be relatively free of extraneous debris from the MDBK cells in which it was propagated, as lymphocytes from rabbits injected with IBRV gave no significant reaction to MDBK cellular antigens. The CMV preparation on the other hand, appeared to have a lesser degree of purity with respect to HFF cellular contamination, since the lymphocytes of the 2 rabbits immunized with CMV did give significant responses to HFF cellular control antigens.

All rabbits in the study showed responsiveness to the homologous antigen with which they were injected, indicating sensitization had occurred during immunization. Each rabbit also had lymphocytes which would react in vitro to the heterologous antigen, indicating that the two viruses, IBRV and CMV, share some similar or identical antigenic determinants. The reactivity of lymphocytes in the case of IBRV immunized animals can be said to be virus specific, as no reactivity to cellular antigens was demonstrated. The stimulation of CMV immunized rabbits' lymphocytes being virus specific is highly likely, but can be disputed as there was some reactivity elicited to host cellular antigens as well as viral antigens. Although the immunizing antigen preparation appears to be contaminated with HFF cellular material, it cannot account for the significant cross-reactivity observed between CMV and IBRV antigens. This is because the lymphocytes of CMV immunized rabbits were not stimulated by MDBK cellular antigens.

SUMMARY

Cytomegalovirus (CMV) is a human herpesvirus of current importance as an ubiquitous human pathogen. The need for therapeutic methods of control of cytomegaloviral diseases has brought to light the use of Infectious Bovine Rhinotracheitis Virus (IBRV), a bovine pathogen, to facilitate or augment the stimulation of an immune response against CMV. The immune cross-reactivity of these two viruses within the humoral immune system has been previously documented using immunofluorescent techniques, and recent reports also indicate that this cross-reactivity may be detected by cell-mediated immune responses as well.

In an effort to document or illustrate this cross-reactivity within the cell-mediated immune system, rabbits were injected with either CMV or IBRV. Blastogenic assays were used to assess the reactivity of the rabbit peripheral blood lymphocytes to both homologous and heterologous antigens.

Initial experiments characterizing the basic methodology necessary for in vitro cultivation of rabbit peripheral blood lymphocytes were accomplished using mitogenic stimulation. These basic conditions were maintained for antigenic blastogenesis assays. All animals were found, as a result of antigenic stimulation in the blastogenesis assays, to have lymphocytes which could be stimulated by the viral antigen which was used for immunization. The heterologous

antigen was also stimulatory in all cases, indicating that antigens of the two viruses which are recognized by cell-mediated immune mechanisms were cross-reactive.

This study highlighted trends which had been previously observed relating to the immune cross-reactivity of CMV and IBRV, and indicated such cross-reactivity can be determined and defined in the blastogenesis assay. The rabbit proved to be an appropriate host with which to study this phenomenon. The study encourages the further characterization of this cross-reactivity, and enhances the feasibility for the use of IBRV antigen, in some form, as a protective or therapeutic agent in CMV infections.

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