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PRODUCTION, CHARACTERIZATION AND APPLICATION OF MONOCLONAL ANTIBODIES AGAINST RETICULOENDOTHELIOSIS VIRUSES

By

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ABSTRACT

PRODUCTION, CHARACTERIZATION AND APPLICATION OF MONOCLONAL ANTIBODIES AGAINST RETICULOENDOTHELIOSIS VIRUS

By

Zhizhong Cui

Reticuloendotheliosis virus (REV) infections have been reported throughout the world, but its economic role is still not clear. More than 30 isolates were obtained from different avian species with various symptoms. They can not be differentiated antigenically. Some molecular studing is done for REV viral proteins recognized by polyclonal sera, but nothing is known about the relationship between antigenic components and biological functions. The monoclonal antibodies are expected to help us to further understand the problems.

In this study, a panel of MCAs against REV strain T are generated and characterized. MCAs 11C100 and 11F667 are strain T specific and recognized a 54-72K dalton glycoprotein. They are useful for subtyping REV isolates. Others are crossreactive with other members of REV and recognized 64K or both 64K and 21K dalton glycoproteins. MCAs 11A25 and 11B118 are strongly reactive with all the REV isolates tested.

To determine epitope-specificities of MCAs, a synergistic ELISA (sELISA) is developed. It is quite coincident with the classical competitive ELISA (cELISA) in the results of identification of epitope-specificities of MCAs. However, it is much simpler than the cELISA and could use culture supernatants instead of ascitic fluid for testing. These two advantages over cELISA should make sELISA very helpful in testing a large number of hybridoma samples and thus stimulate more interests in topological analysis of various antigen molecules. Several independent epitopes on REV glycoproteins are differentiated with both cELISA and sELISA. The neutralizing activity of some MCAs is also tested showing that the neutralization activity is related to only some epitopes on virions.

By using the combination of MCAs, which are REV group-common and recognizing glycoproteins on the surface of virions but different epitopes, a MCA-mediated ELISA is developed for direct detection of REV antigens from plasma, tissue suspensions, egg albumen, cloacal swabs, and semen. The sensitivity limit of the ELISA is 8-16 ng purified REV protein in 100 ul. It is 40-80 times more sensitive than complement fixation test (CF) the standard assay currently used. More importantly, The ELISA could directly detect REV antigens from bird samples, but CF could not do so without amplification of viruses in cultures.

DEDICATION

To my mother, Wang Meidi, my wife Miao Xiue,

my daughters Xiaoxia and Xiaoping,

my son Yuefeng

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ABBREVIATIONS

AGP: Agar gel precipitate test.

ALV: Avian lympho-leukosis viruses.

BMC: Bone marrow cell line.

BSA: Bovine serum albumin.

CBA: Competitive binding assay.

CEF: Chicken embryo fibroblast.

cELISA: Competitive ELISA.

CF: Complement fixation test.

CS-CEF: CSV infected CEF.

CSV: chicken syncytial virus.

DEF: Duck embryo fibroblast.

DIAV: Duck infectious anemia virus.

ELISA: Enzyme-linked immunosorbent assay.

EM: Electron microscopy.

FA: Fluorescent antibody test.

FFU: Fluorescent antibody focus-forming unit.

HVT: Herpes virus of turkey.

IFA: Indirect fluorescent antibody test.

MCA: Monoclonal antibody.

MD: Marek's disease.

MDV: Marek's disease virus.

MuLV: Murine leukemia viruses.

nd-REV: Non-defective REVs.

PBS: Phosphate buffered saline.

QEF: Quail embryo fibroblast.

RE: Reticuloendotheliosis.REV: Reticuloendotheliosis viruses.

REV-A: Reticuloendotheliosis-associated viruses.

REV-CEF: REV infected CEF.

RIA: Radioimmunoassay.

sELISA: Synergistic ELISA.

SNV: Spleen necrosis virus.

T-CEF: REV strain T infected CEF.

TEF: Turkey embryo fibroblast.

TEM: Transmission electron microscope.

VIF: Virus immunofluorescent antibody test.

INTRODUCTION

Reticuloendotheliosis viruses (REV) comprise a group of avian retroviruses serologically related to each other but distinct from avian leukosis viruses (ALV). Representatives of this group include strain T originally isolated from turkey (Robinson, 1974), chick syncytial (CS) virus (Cook, 1969), spleen necrosis (SN) virus (Trager, 1959), and duck infectious anemia (DIA) virus (Ludford, 1972). REV isolates were obtained from turkeys (Paul, 1976, 1977; Sarma, 1975; Soloman, 1976; McDougall, 1978; Witter, 1982, 1984a), Pheasants (Dren, 1983), chickens (Witter, 1982) and ducks (Grimes, 1973; Li, 1984) in many parts of the world. REVs were reported to cause various pathogenicities such as neoplasm, immunodepression, and a runting disease syndrome in a number of avian species (Purchase, 1973; Witter, 1984b). Little is known about the difference in antigenicity among REV strains or the relationship between antigenic structure and pathogenecity. Since all members of REV group are indistinguishable by an indirect fluorescent antibody (IFA) test with the convalescent serum (Purchase, 1973; Soloman, 1976; Dren, 1983), and since REV strains cross-react and exhibit only minor strain differences in virus neutralization tests (Witter,1970; Purchase, 1973; Paul, 1977), differential REV diagnosis and strain identification with conventional serum are often difficult.

Recent epidemiological surveys (Witter, 1982, 1985) showed that REV infection in commercial chicken and turkey flocks was more common than

previously recognized. Little is known of the natural incidence of REV infection, due mainly to the lack of a simple and sensitive test to identify REV infection. Even in cell culture, REV infections are not easy to be identified because they are not constantly cytopathic in chick cells (Soloman, 1976), although Cho (1983, 1984) reported focus formation of REV in a quail fibroblast cell line. Smith (1977) developed a specific REV micro-complement fixation (CF) procedure comparable in sensitivity to IFA for detecting REV infection in cell culture. But as astandard procedure for routine use, both CF and IFA require REV replication and amplification in cell culture, and consequently are cumbersome for mass screening of REV-infected samples from flocks.

An ELISA for detection of antibody against REV has been in use for serologicl survey of REV infection in commercial chicken and turkey flocks (Smith, 1983). However, persistent REV viremia exists in some tolerant chickens which do not show antibody activity in the serum (Bagust, 1979, 1981). These tolerant viremic chickens may transmit REV infection horizontally. It is necessary therefore to detect both REV antigen and antibody positive individuals in flocks for epidemiological surveys and eradication programs.

Although an ALV-ELISA (Smith, 1979) based on the group-specific antigen, p27, is in routine use for detecting antigen from different kinds of samples of ALV-infected chickens, there is need to develop a similar ELISA to directly detect REV antigen in various samples from REV-infected birds so that we can run the assays for both REV and ALV using the same samples.

Several different REV proteins have been identified using anti-REV

rabbit serum. The 29 Kd protein is probably the major virus structural core protein responsible for the REV group-specific antigenicity (Maldonado, 1975, 1976; Mosser, 1975; Tsai, 1985; Wong, 1980). Two of the remaining glycoproteins, 73 Kd and 19 Kd, were also found in strain CS- and DIA- infected cells (Maldonado, 1975, 1976). Since the polyclonal anti-REV rabbit serum and chichen convalescent serum do not distinguish between different strains well, it is not conclusive that the 29 Kd protein is the major structural protein responsible for the REV group-specific antigenicity. Monoclonal antibodies (MCAs) are useful for analysis of virus protein structure in detail at the level of antigenic epitope instead of the level of virus protien itself. Lutz (1983) developed three different MCAs against the major core protein (p27) of feline leukemia virus(FLV), a retrovirus, with each MCA directed against a different epitope of FLV-p27. These MCAs could readily be adapted to an ELISA for the specific study of FLV-p27. In the case of REV, also as a retrovirus, weexpect to find similar results.

The competition binding assays in both radioimmunoassay and ELISA were developed for determinating epitope-specificity of MCAs (Stone and Nowinsk, 1980; Roehrig et al, 1982). Since then, a great deal of work has been done to make epitopic analysis of antigenic determinants and topological mapping on virus antigens of different viruses (Lutz et al, 1983; Schlesinger et al, 1984; Bruck et al, 1982; Henchal et al, 1987). These assays are also used to relate the epitope(s) of virus proteins to their biological functions such as neutralization and hemmagglutination (Yewdell and Gerhard, 1982; Roehrig et al, 1983; Heinz et al, 1983). But no matter what kinds of alternatives were used

for CBA, immunoglobulin of each MCA has to be purified from asitic fluid with high titers and conjugated to enzymes or labeled with ^{125}-iodine befor samples could be tested for their epitope-specificity. It is labor-intensive and expensive, and identification of epitope-specificity could not be done until each hybridoma is rescreened and injected into mice for ascitic fluid. A simple assay is clearly needed, which does not involve labeling or conjugating each hybridoma sample and can be used to directly identify epitope-specificity of MCA in the hybridoma culture supernatant.

Although REV structural polypeptides were recognized by polyclonal antisera, nothing is known about relationships between antigenic structure and biological functions. Identification of epitopes with MCAs would probably help us further relate some antigenic epitopes on REV to their biological functions such as neutralizing activity. Objectives of this study are as followed.

1. To generate monoclonal antibodies (MCAs), both strain-specific and common group-specific against REV;
2. To identify antigenic differences among different strains of REV using strain-specific MCAs when they are available;
3. To analyse virus proteins and epitopes recognized by MCAs and determine proteins or epitopes responsible for strain-specific or common group-specific antigenicity; To establish the relationship between antigenic epitopes and their biological functions;
4. To locate the epitopes recognized by MCAs on the virions by electronic microscopy (EM). The MCAs recognizing antigenic epitopes on the surface of virions will be useful for detection of REV particles;
5. To develop a direct ELISA or sandwich ELISA to directly detect REV

antigen in various samples by using a combination of common group-specific MCAs against different epitopes on the surface of virions. The ELISA should be much simpler and more sensitive than the current standard procedures, CF and IFA, both of which could detect antigen indirectly only;

6. To develop an assay simpler and more practical than the classical CBA for determining MCA epitope-specificity.

LITERATURE RIVIEW

Reticuloendotheliosis and reticuloendotheliosis viruses

Reticuloendotheliosis (RE) designates a group of pathologic syndromes including acute reticulum cell neoplasia, chronic neoplasia of lymphoid or other tissues, runting disease syndromes and immunodepression in various avian species, such as turkeys, chickens, ducks, geese, and quail. The diseases were caused by a group of retroviruses collectively called reticuloendotheliosis virus (REV) and which was antigenically different from another group of avian retroviruses, avian lympho-leukosis viruses (ALV).

Epidemiology and pathology of REV

The initial REV isolate, Strain T, was obtained in 1958 from a moribund turkey with lymphomas in liver and spleen (Robinson and Twiehaus, 1974). During the subsequent 16 passages, in which liver cellular inocula were used, in both chicks and turkey poults, the infection agent increased in virulence, causing nearly 100% mortality. The most common gross lesions in both chicks and turkey poults consisted of enlarged livers and spleens with subcapsular white foci (Robinson and Twiehaus, 1974; Sevoian, 1964). The virus which produced

reticuloendotheliosis in experimentally infected 1 to 14 day old chicks, Japanese quail and turkey poults appeared to be antigenically unrelated to several known ALVs and differ morphologically from ALV (Theilen et al, 1966).

Trager(1959) reported a new virus which produced a rapidly fatal disease in ducks with enlargement and necrosis of the spleen, and severe anemia as a companion of Plasmodium lophurae. The virus could also kill chicks and cause white tumor-like lesions in spleens and enlarged livers when inoculated at 2-days of age. The virus was then named as spleen necrosis virus (SNV).

Cook (1969) repeatedly isolated a viril agent producing a syncytial-type cytopathic effect in chick embryo fibroblast was isolated repeatedly from the CAL-1 strain of Marek's disease tumor (Bankowski, 1969). The filtrates of the tissue culture grown virus induced a disease resembling Marek's disease when inoculated into chicks; the virus was named chicken syncytial virus (CSV).

Ludford et al (1972) isolated another virus associated wuth Plasmodium lophurae from ducks with anemia and named it duck infectious anemia virus (DIAV). The virus was highly contagious and could spread from infected ducks to susceptible ducks housed in the same cages. Purchase (1973) studied the serological relationship of strain T, SNV, DIAV and CSV, indicating that all four viruses were indistiguishable by indirect fluorescence antibody test (IFA) in virus-infected chicken embryo fibroblast (CEF) or duck embryo fibroblast (DEF) cultures, and concluded that they formed a new group of oncogenic viruses for which reticuloendotheliosis virus (REV) group was designated. REV strain T, SNV, DIAV, and CSV were recognized as representatives of the REV group.

Since then a series of pathogenicity studies have been conducted in experimental infection of different strains of REV. Sevoian et al (1963) demonstrated that cellular preparation of REV strain T was highly lethal for various genetic lines of chickens causing 100% mortality of the inoculated chicks by the acute lymphomatosis within the first eight days postinoculation. All the inoculated chicks manifested greatly enlarged livers and spleens, with subcapsular grayish-white focal lesins irregular in shape and ranging up to 1 cm. Extensive tumor nodules were seen also in the gonades, heart, kidney and other visceral organs. When the virus was diluted, the pathological response in chicks was graded and delayed, but the gross lesions were similar. They also compared the host response to cellular versus cell-free preparations of REV strain T, and indicated that mortality was less and the incubation was longer in chicks receiving cell-free virus preparations than in birds receiving comparable dilution of cellular inocula. Lorose and Sevoian (1965) further conducted comparative titration of the REV strain T in chicks of 6 age groups from 1 day to 10 weeks old, utilizing cellular virus preparation and found that the day-old group was more susceptible than the older groups. In their experiments no mortality or morbility occurred in the contact control birds, but antibodies to strain T virus were found in a low percentage of birds within the 12-week experimental period indicating a low-grade horizontal transmission.

An acute runting syndrome was induced in young chicks by intra-abdominal inoculation of hepatic and splenic materials prepared from chicks previously infected with REV strain T (Mussman and Twiehaus, 1970). Experimentally infected young chicks were emaciated,

lethargic, anemic and retarded in growth: the gross lesions were marked hepatosplenomegaly with a consistant decrease in size of the thymus and bursa of Fabricius. Histologically, the lesions were composed of proliferating histocytoid cells of the reticuloendothelia system.

The histopathologic and hematologic changes during morbid stages of chicks inoculated with strain T-infected liver suspension were studied (Olson, 1967). There were marked proliferation of reticuloendothelia cells around the vessels of the liver, spleen, pericardium, and mesentery in the lymph follicles of the gastro-intestinal tract, bursa of Fabricius and thymus. The hematologic changes consisted of a reduction in packed cell volume and total leukocyte count with increased sedimentation rate and clotting time.

REV strain T displayed a wide spectrum of infectivity (Taylor and Olson, 1971). Two-day-old chickens, quail, ducklings, goslings, turkeys, pheasants, and guinea keets were found susceptible to the virus by intraperitoneal injection of chicken liver virus preparations. The lesions observed in the various species of birds were similar; and all the birds that died of virus infection had grossly enlarged livers and spleens. Irregular white foci, 1 to 3 mm in diameters, were scattered over the surface of the liver and spleen. The Bursa was atrophied. Microscopically, there was a proliferation of an undifferentiated mesenchymal cell in affected organs.

Witter et al (1970) described gross lymphoproliferative lesions similar to Marek's disease in the peripheral nerves of chickens inoculated with REV strain T infected CEF or DEF culture. The infected birds did not die early and lacked visceral reticuloendotheliosis tumors when killed at 6 weeks. However, grossly enlarged peripheral

nerves were found. The enlargenent was due to accumulation of lymphocytes and plasma cells between the nerve fibers, a lesion similar to Marek's disease. Most peripheral nerves in affected chickens developed lesions, but the most noticeable enlargement was in the cervical portion of the vagus nerve.

Franklin et al (1974) isolated and cloned REV-transformed cell lines from bone marrow of strain T infected chickens. The culture fluids from cultures of the transformed chicken bone marrow cell line (BMC) could be used as a souce of oncogenic REV strain T.

When CEF cultures were infected with viruses of the REV group, virus production with the development of a cytopathic effect occured after a brief latent period (Temin and Kassner, 1974). Following the acute phase, the cytopathic effect disappeared but the culture still produced virus persistently (Temin and Kassner, 1975). Hoelzer et al (1979) developed a focus assay for quantitating in vitro transformation by oncogenic REV in Japanese quail embryo fibroblast (QEF) and demonstrated that oncogenic stock of REV strian T was composed of a mixture of tranforming and nontransforming viruses. The transforming virus could cause acute RE in birds and transform QEF and CEF, but it was replication defective in CEF and QEF and no free virus was released from the fransformed cell line culture. In contrast with it, the nontransforming virus referred to as reticuloendotheliosis-associated virus (REV-A) could be released from the persistently infected cultures and is non-defective in replication in CEF or QEF, but could not transform either CEF or QEF cultures. When REV-A was injected into 1-day-old chickens, they failed to develop the hepato-spleenomegaly characteristic of the prototype of REV strain T, instead, the latent

period became protracted and the birds developed an acute runting disease accompanied by paralysis. Breitman et al (1980) also studied attenuation of oncogenic REV strain T stock during serial passage in fibroblast culture. According to the electrophoretic analysis of virus RNA, they suggested that oncogenic REV was retained during serial passages in chick bone marrow cells because virus infection selected for a population of stably growing REV-transformed cells but viral attenuation might occur in fibroblast culture because REV-transformed fibroblasts did not have a significant growth advantage over REV-A infected or uninfected fibroblasts. As a consequence, transforming REV would be diluted out during serial passage. It was found that other members of REV, such as DIAV, SNV and CSV, also failed to transform fibroblast culture or induce neoplastic disease in experimentally infected birds (Hoelzer et at,1979).

Witter et at (1981) reported that chickens inoculated as embryos with non-defective REVs (nd-REV) REV-A and CSV generally developed a "tolerant" infecton characterized by lack of immunofluorescent antibody indicatiing immunodepression, and by a viremia that persisted through 93 weeks. Chickens inoclulated at hatching generally developed a "non-tolerant" infection characterized by antibody development that gradually waned and by the presence of a transient or intermittent viremia. After a long latent period (17 to 93 weeks), nd REV-infected chickens developed lymphomas involving the Bursa and other visceral organs at high frequency and developed sarcomas, carcinomas, and inflammatory nerve lesions at a lower frequency.

In addition to the above REV members, more strains were isolated from naturally outbreaks of the disease in differrent bird species. Sarma et

al (1975) and Paul et al (1976) reported natural outbreaks of RE in turkeys in Minnesota. It involved a flock of 30,000 Nicholas white turkeys, of which 14% died at the farm and another 2% were condemned due to lymphoproliferative disease at the time of slaughtering, and another flock of 11,000 turkeys, of which approximately 5% died. Infected birds had grossly demonstrable lesions at postmortem examination in the liver, spleen, kidney, heart, lungs and other visceral organs. Histologically, affected tissues were focally or diffusely infiltrated by proliferative lymphoreticular cells. Two virus isolates were obtained from two flocks respectively. The virus isolates had typical C-type RNA virus structure and were antigenically identical to REV strain T according to the neutralizing test. In the further study (Paul et al, 1977a) the two virus isolates were designated as REV strain MN81 and MN67 respectively. They could replicate in CEF, DEF amd Turkey embryo fibroblast (TEF) cultures and produced syncytial cytopathic effects in DEF and TEF cultures. Paul et al (1977b) also demonstrated that when inoculated in 1-day-old turkey poults, TEF-culture-propagated viruses of strains MN81 and MN67 were pathogenic and caused about 22-33% mortality with incubation of 8-11 weeks. The macroscopic RE lesions were observed in the livers, spleens, intestines, pancreas and kidneys. Peripheral nerve enlargement was also seen occasionally. Microscopically, the lesions were composed of neoplastic lymphoreticular cells.

Solomon et al (1976) studied 25 normal turkey flocks and found tumors from 8 sources. REV was isolated from tumors of two flocks and from one normal flock. The REV isolates were antigenically similar to the prototype REV strain T but were of low pathogenecity. Because there was

no evidence of infection with ALV or MDV in any of the turkeys tested, the etiological role of REV in some though not all forms of turkey neoplastic disease was suggested.

Witter and Glass (1984) reported a natural RE outbreak with 2% mortality due to lymphoid tumors mainly between 20 and 30 weeks of age in a flock of 6,750 Nicholas turkey breeder hens in Texas. Of 10 turkeys submitted for diagnosis, all had gross lymphoproliferative lesions. Diffuse and focal tumors were seen regularly in the liver, spleen and kidney. Nodular tumors were seen in the intestine. The virus isolate, designated REV strain 339, produced an acute neoplastic disease when inoculated into young chickens.

Another REV isolate, strain RU-1, was isolated from leukotic liver tissue of a white Pekin duck with large nodular lymphoid tumors in liver and spleen. The duck was part of an experiment on Plasmodium sp. infection, and other ducks in the group had died apparantly as the results of similar neoplasms (Li et al, 1983). When inoculated into ducks, it caused mortality of 80-100% during 4- or 6-month experimental period regardless of age at infection, and route of exposure. Most deaths were from non-neoplastic conditions (stunting, bacterial infection), but 17 of 69 (25%) infected ducks developed a variety of neoplasms.

In Australia, REV was first isolated from an adult duck with lymphoreticular cell tumors of various organs by Grimes and Purchase (1973). Experimental infection of 1- to 7-day old ducklings with the virus resulted in low mortality, tumors of visceral tissues, within 5-9 weeks after inoculation. The virus was designated as REV/Q/1/73 by Bagust and Dennett (1977) who produced feathering defects in 2- to

4-week old chickens inoculated at 1-day-old with the virus. The serum from the infected chicks was positive to REV strain T in IFA. Attention was subsequently drawn to the pathogenic potential of REV in chickens when widespread losses occured among Australian poultry flocks (Jackson et al, 1977) following the use of a commercial Marek's disease vaccine which was subsequently proven to be contaminated with REV (Bagust et al, 1979). Grimes et al (1979) showed that infection of young chickens with an Australian isolate of REV resulted in feathering defects and poor growth rate. Other findings in birds that died or were culled during 40-week experimental period included mild anemia, leucopenia, hypoplasia of organs of the immune system and inflammation in visceral organs and nervous system. Lymphoreticular-cell tumors of the liver, kidney or spleen were found in two birds aged 22 to 24 weeks, suggesting that REV may also caused tumors in adult fowls. Ratnamohan et al (1980) reported another field case of REV infection. Histiocytic lymphosarcomas of the intestine, liver, spleen and sciatic nerve were found in a 36-week-old laying hen that was culled from a flock of 1,800 birds in which 148 hens were sick or dead between 27-51 weeks of age. Type C virus particles were observed in ultrathin sections of liver and spleen, and REV antigen was found in the sera of the hen.

In England, McDougall et all (1978) reported outbreaks of turkey leukosis in 1975. In the seven affected flocks visited, including three adult breeding flocks and four commercial rearing flocks, mortality had increased during the 8th to 12th week of life and had continued at 1 to 2% per week throughout the life of the affected flocks resulting in overall mortalities in excess of 20%. The initial rise in mortality was associated with persistent diarrhea and leg

weakness. All birds had enteritis affecting particularly the small intestine. Pericarditis and peritonitis were also present in the majority of the birds. In affected turkeys the liver was usually diffusely enlarged up to three times normal size. In some birds, discrete tumors were present throughout the liver, spleen and some other visceral organs. Virus was isolated from ailing culled turkeys and from cell culture prepared from embryonated eggs produced by a flock with the disease. The isolates were antigenically related to REV strain T or CSV by direct FA or AGP. Therefore it was a member of the REV group and has been designated REV-HPRS-1. McDougall et all (1980) further studied the pathogenicity of REV-HPRS-1 in turkeys infected at 1-day of age, four weeks of age and by contact exposure from 1 day of age. Only the 1-day-old inoculated group appeared clinically sick with mild diarrhea three weeks post inoculation. Lymphoid tumors and mortality from RE occurred in all three groups, but at a lower level and later in life in turkeys inoculated at four weeks of age.

A naturally occuring lymphoproliferative was found in three flocks of Japanese quail in Mexico. The tumor-like lesions were detected mainly in livers and spleens. MDV was not isolated from 74 quail tested, nor were antibodies to MDV detected in 84 sera. Antibodies to REV (3/24) and ALV subgroup A (2/20) were present. It suggested that REV involvement in the disease appeared more likely (Schat et all, 1975).

An outbreak of a lymphoproliferative disease in pen-raised pheasants was described in Hungary (Dren et al, 1983). In the affected flock of about 3,000 pheasants during the period of 6-12 month of age, 120 pheasants died or were culled of which 19 had gross and/or microscopic tumors. Nodular or diffuse tumors were found on the head and in various

internal organs. The lesions consisted of undifferentiated lymphoreticular cells. A typical C-type virus, similar to REV, was isolated in tissue culture. The isolate was antigenically related to REV strain T in IFA. Antibodies to REV were also demonstrated in serum samples from naturally diseased and experimentally infected birds. All phesant chicks inoculated with cell suspension prepared from the skin tumor and the spleen of a naturally diseased pheasant died with tumor between 16-34 days after inoculation. Infection of chickens with spleen cells from a field case with tumor also caused feathering defect syndromes, some gross lesion of tumor in the heart, and liver, and high mortality, but inoculation with supernatant of infected cell culture caused only feathering defect in some infected chickens.

RE outbreaks induced by REV-contaminated MDV vaccine were also reported in other part of the world. Yuasa et al (1976) described a disease with delayed growth, anemia, abnormal feathers, and leg paralysis as main symptoms which broke out in flocks of chickens inoculated with MD vaccine produced by certain manufacturers in various part of Japan over a period from Spring to Fall in 1974. In the flocks examined, many chicks were affected with the disease at about 30 days of age. The culling rate exceeded 50% in some flocks. Histological examination revealed swelling and multiplication of Schwann's cells and mild cell infiltration accompanied with edema in peripheral nerves. The infiltrating cells were plasmacytes and lymphoid cells. A virus was isolated from affected birds in the field and the same lot of MD vaccine as inoculated into these birds. The virus had a common antigenicity to REV strain T. When chicks were inoculated with it, they presented essentially the same symptoms as the birds affected in the

field. Kawamura et al (1976) and Tagayanagi (1976) showed the same reports. In Australia, Jackson et al (1977) reported a low mortality at 10 days of age followed by a low incidence of nervous symptoms and a feathering abnormality in MD-vaccinated broiler breeders between two and three weeks of age. Reports received from all states in Australia indicated that the disease might be associated with the use of particular batches of commercial HVT vaccine contaminated by REV. Bagust et al (1979) isolated in cell culture REV from commercial MD vaccine (HVT) and re-isolated the virus from the organs of vaccinated chickens. Runting and feathering abnormalities were produced when 1-day-old SPF chickens were inoculated with the REV. Serological responses to REV were also detected by IFA in chickens directly inoculated with the contaminated vaccine, and spread of REV infection to in-contact chickens was demonstrated by histopathological and serological investigations.

In addition to the symptoms and lesions mentioned above, REV also caused immuno-depression in infected birds. Bulow (1977) first reported that even minor contaminations with REV markedly reduced the efficacy of MD vaccine and decrease the antibody titers against HVT in chickens inoculated with MDV vaccine, especially in young chicks. Witter et al (1979) further investigated in detail the effect of infection with low-virulence, tissue culture-propagated strains of REV on protective vaccinal immunity against MD lymphomas and found that MD-vacinated chickens inoculated at hatching with more than 10^4 focus-forming units of REV and challenged with MDV were poorly protected against MD lesion development. Furthermore, the response of blood lymphocytes to mitogen stimulation and the antibody response to sheep erythrocytes and

Brucella abortus were less in REV-inoculated chickens than in controls. The REV-induced depression of immune response was generally transient. Depression of cell-associated immunity by REV infection was studied in chickens. Carpenter et al (1977) and Rup et al (1979) demonstrated that infection of chickens with REV-A resulted in suppression of PHA-induced responses of spleen cells from infected birds. This inhibition of T-lymphocyte proliferation was mediated by a host-derived population of suppressor cells that were activated or induced during REV infection. The immunosuppression induced by REV-A was transient, because PHA-induced lymphocyte responses of infected birds returned to normal by 5 weeks after infection. Other non-transforming members of REV group, CSV, DIAV and SNV, also induced immunosuppression in chickens within six days after infection. Depression of immunity against other viral or bacterial diseases was also reported. After inoculation of lentogenic B1 strain or mesogenic strain TCND from the live vaccine of Newcasle disease virus (NDV), the antibody response was suppressed and duration of NDV recovery prolonged in REV-infected chicks compared to controls (Yoshida, 1976, 1980, 1981). Motha (1982a, 1982b) studied the interaction between infectious larygotrachitis virus (ILTV) and REV , and found that the resistance to ILTV in chickens infected with REV was much lower than that in control chickens and there was a significantly higher proportion of ILT vaccination "takes" in the group inoculated with REV than that in controls. Motha and Egerton (1983) also reported that the mortality due to S.typhimurium in chickens inoculated with REV was markedly higher than in control chickens.

Morphological and molecular structure of REV

Most isolates of REV group have been observed under the transmission electron microscope (TEM). The virus varions were typical C-type virus with diameter of 80-110 nm according to different investigators. Zeigel et al (1966) studied prototype REV strain T in spleens of infected turkey or Japaness quail and in infected CEF or QEF using the TEM and indicated that the mature extracellular viral particles ranged from 85-110 nm. REV particles budding from cells showed double-layered inner crescent and a outer layer which was continuous with cell plasma membrane. Some REV particles from cell culture fluid showed pleomorphic "tail-like" extension of their outer coat. The patterns of budding (viral proliferation) were studied in detail by Zeigel et al (1966). During the budding process, the material that ultimately participated in forming the virus nucleoid appeared to originate as a crescent in the 2-dimensional micrographs and , presumably, as a dome or cup-shaped accumulation in 3-dimensional beneath the plasmalemma of the cell. There was an apparent separation of a single nucleoid component or the presence of two distinct nucleoid components, the outermost representing the intermediate ring. This particular configuration differed distinctly from the pattern of budding observed in the immature form of ALV and resembled the immature form of a murine leukemia virus, e.g. Rauscher virus. Baxter-Gabbard et al (1977) also demonstrated the enveloped REV strain T in diameter of approximately 100 nm.

Cook (1969) described the morphology of CSV with diameter of 100 nm similar to C-type virus particle. It was resistant to 5-Iododeoxyuridine so that it bolonged to RNA virus group.

Purchase et al (1973) compared all four representatives of REV group in the ultrathin sections of infected DEF or CEF, and found that all isolates of REV strain T, CSV, DIAV and SNV were identical but could be distinguished from ALV under EM. Kang et al (1975) made a further comparative ultrastructural study of the four representatives of REV group. The virions were spherical with a diameter of approximately 110 nm. The uranyl acetate negative staining showed that the viral envelopes were covered with apparently hollow peplomers approximately 10±1.5nm in diameter at the tip by 6±1nm long. The peplomers appeared to be tapered so that at the viral membrane, the peplomers were only 4±1.5 nm in diameter. The center to center distance of surface projection was about 14 nm. There were about 100 of those peplomers per virion even though Zeigle et al (1966) did not find the "hole" of projection or knobs in REV particle by other different staining methods. The budding virions contained crescent-shaped electron-dense cores 73 nm in diameter with electron-lucent centers. After release of the virions the cores became condensed to 67 nm in diameter. The distribution of budding REV on cells appeared random over the cell surface, and occasionally aberrant multiple forms of budding virions were observed. They also indicated that the virions of REV appeared to resemble mammalian leukemia and sarcoma viruses more closely than ALV.

The morphology of other REV isolates also was studied by Sarma et al (1975), Paul et al (1977), Koyama et al (1976), Yuasa et al (1976), McDougall et al (1978), Grimes et al (1979), indicating the same C-type virion structure. An ultrastructural comparison of REVs to ALV and MuLV (murine leukemia viruses) was conducted by Moelling et al (1975), showing that the immature particles of REV could be morphologically

distinguished from both MuLV and ALV. The mature REV particles were very like that of MuLV and quite different from that of ALV. They indicated that a number of 1,000-2,000 budding and extracellular REV particles per cell was estimated, much less than that of ALV and MuLV. They also found that about two of ten REV budding or extracellular particles were immature structurally, i.e., exhibited an electron-lucent core, which incidence was two- to five-fold higher than for ALV or MuLV.

The intact virions of the REVs had a denstity of 1.15-1.18g/ml in sucrose gradient (Campbell et al, 1971; Sarma et al, 1975; Baxter-Gabbard et al, 1971; Paul et al, 1976; Koyama et al,1976). Campbell et al (1971) also demonstrated ethyl-ether-treated REV strain T virions lost their outer envelopes. In the majority of virions after ether-treatment, the material which usually surrounded the cores was partially disrupted or absent. It was concluded that this material also contained ether-soluble lipids. When the ether-treated virus suspension was subjected to sucrose density-gradient centrifugation, 2 distinct bands were detected. The bottom band with density of 1.25-1.26g/ml, when examined with EM, was observed to consist of naked cores, i.e., the outer membranes were absent and the intermediate layers were partially disrupted or absent.

Further studies (Baxter-Gabbard et al, 1971) indicated that the genomes of REV contained a single-strand RNA under EM. Maldonado and Bose (1973, 1975) found two RNA species with approximate sedimentation values of 64S and 4S after sucrose gradient centrifugation of RNA extracted from REV strains T, CSV and DIAV grown in CEF. Beemon et al (1976) showned that RNA of REV strain T propagated in CEF had a

complexities of 3.9 x 10^6 of 60-70S. Because REV was found to have an unique sequence genomic complexity near the molecular weight of a single 30-40 S viral RNA subunit, they also concluded that the genome of REV is at least largely polyploid.

However, genomes of non-defectve REVs and replication-defective strain T differed. As mentioned previously, the original REV was a mixture of transforming and non-transforming viruses, the former was defective in replication in CEF culturue, the later, called REV-A, was non-defective. An analysis of the RNA monomers from particles released from virus-producing REV-transformed clones on denaturing methylmercuric hydroxide gel indicated that two distinct RNA species were present (Hoelzer et al,1980). The larger RNA species of REV-A had a molecular length of 8.7kb and the smaller RNA monomer of transforming REV had a molecualr length of 5.9 kb. Therefore, the defectiveness of REV was due to deletion of sequence essential for replicaton. The same phenomenon was reported by Brietman et al (1980). Hu et al (1981) indicated that replication-defective Strain T had a deletion of 3.69 kb in the gag-pol region, confirming the genetic defectiveness of the virus. In addition, REV strain T lacked the sequence corresponding to the env gene but contained, instead, a contiguous stretch (1.6 to 1.9 kb) of the specific sequences presumably related to viral oncogenicity. Cohen et al (1981) also reported two regions of REV-A sequences which were deleted in the defective REV strain T genome. The first region encompasses 3 kb of sequences in the 5'-half of the genome, presumably corresponding to the gag-pol genes. The second region represents 1.5 kb of the env sequences. They have also shown that approximately 3% of the genomic sequences of transforming REV strain T are unrelated to the

non-oncogenic nd REVs.

By using cDNA specific for REV, Wong and Lai (1981) have shown that transforming REV strain T contains a new class of transforming-specific sequences (ret) which were present in normal uninfected vertebrate and most related to or probably derived from normal turkey DNA. In contrast, the sequences related to REV-A could not be detected in any normal vertebrate cells. The ret sequences were not contained in other REVs. Chen and Temin (1982), Rice et al (1982) further experimentally analyzed and compared the genomic RNA structure of transforming replication-defective REV strain T and other REVs by molecular biology techniques to study the rel-gene and its location. Rice et al (1982) also showed that transforming REV strain T specific segment (rel-gene) was derived from avian DNA, because a cloned fragment of the transfoming REV was able to hybridize with the DNA from an uninfected chicken. So, the transforming REV appeared to be the product of recombination between a replication-competent virus and host DNA. The highly oncogenic replication-defective REV strain T contained the oncogene v-rel. There was a large c-rel locus in the turkey genome which contained all of the sequences homologous to v-rel (Wilhelmsen et al, 1984a). It was thought that REV strains T arose when a virus similar to REV-A, the helper virus of REV strain T, infected a turkey and recombined with c-rel from that turkey. Wilhelmen et al (1984b) sequenced v-rel and its flanking sequences, and found that each of the regions of the c-rel locus from turkey was homologous to v-rel and their flanking sequences, and the coding sequence for env and part of pol of REV-A. Comparison of REV-A, transforming REV-T, and c-rel indicated that the v-rel sequences might have been transduced from the

c-rel (turkey) locus by a novel mechanism. Rice et al (1982) assumed a gene order of 5′-gag-pol-env-3′ for REV-A or other nd-REVs similarly to those of other C-type viruses. Most or all of REV-A pol gene was deleted and its env gene was also partially deleted in defective REV T. The 1.9 kb oncogene rel segment which is unrelated to REV-A and supposed to be oncogene rel follows the env gene.

DNA polymerase activity of REV primed with synthetic template was first found (Baxter-Gabbard et al, 1971) on purified REV strain T grown in CEF culture. Peterson et al (1972) further studied the DNA polymerase activity of REV and found that a large amount of REV protein was required to demonstrate the in vitro polymerase reaction. There were tremendous numbers of virus particles present, yet the activity of DNA polymerase expressed was surprisingly low, suggesting that the virus RNA polymerase was involved in the infection nature of the virions. The RNA-directed DNA polymerase was demonstrated from REV virions, but its molecular size was reportedly different. Mizutani and Temin (1974) isolated the DNA polymerase from the REV strain T and showed that the molecular weight of the enzyme was approximately 70-75K. They (1975) also isolated the DNA polymerase from SNV indicating its molecular weight of 68K. Kieras and Faras (1975) reported the presence in REV of a virion-associated DNA polymerase by employing exogenous synthetic homopolymers as tempelate primer, although no endogenous RNA-directed DNA polymerase could be detected. But Kang (1975) has shown that there was an endogenous RNA-directed DNA polymerase activity in disrupted virions of REVs grown in CEF if manganous ions were added to the reaction. Enzyme activity could be inhibited by pre-treatment with RNase and the DNA product of the endogenous DNA polymerase reaction was

hybridized to REV RNA, but not to avian leukosis virus RNA. Moelling (1977) reported a purified RNA-dependent DNA polymerase with a molecular weight of 84K from REV. An RNA polymerase activity was also found in the core of purified REV virions (Mizutani and Temin, 1976).

Structural polypeptide composition of REV virions was also studied in detail. Halpern et al (1973) and Maldonado and Bose (1973) separately reported that five polypeptides of REV-T grown in CEF were resolved by polyacrylamide gel electrophoresis (PAGE). Among them, two were glycosylated. The major non-glycosylated polypeptide did not comigrate with those of ALVs. Halpern et al (1973) indicated two surface proteins were detected and corresponded to the two viral glycoproteins by lactoperoxidase catalyzed iodination. Maldonado and Bose (1975) compared the polypeptide composition of different members of REV group. They found that two glycosylated polypeptides of gp 73K and 19K and four non-glycosylated polypeptides of p29K, 22K, 15K, 13K existed in all three strains T, CSV and DIAV of the REV group tested, but strain T grown in CEF had an additional non-glycosylated polypeptide p37 which was abscent in CSV and DIAV. The nonglycosylated polypeptide p29 was the major internal non-glycosylated polypeptide in the virion (Maldonado and Bose, 1976). Mosser et al (1975) studied the polypeptide composition of SNV and determined ten polypeptides by PAGE. Two glycosylated proteins, gp71 and gp22, were located on the outer surface of the lipid envelope, as demonstrated by lactoperoxidase-catalyzed iodination and by bronelain digestion. The non-glycosylated polypeptides were p77, p62, p50, p36, p30, p26, p14, p12. The p30 was the major polypeptide which consisted of 38.8% of total counts/min. recovered in ^{14}C-labeled amino-acids. The results also suggested that

two of the minor polypeptides, p36 and p26 were also located on the outer surface of the virions.

Wong et al (1980) studied the assembly of REV. They have demonstrated that a virus-specific ribonucleoprotein complex was present in the cytoplasma of REV-transformed chicken bone marrow cells. The complex contained viral reverse transcriptase activity and could represent a precursor to the budding virions. The major viral polypeptide associated with the complex was a polypeptide of 63K. This protein exhibited a precursor-product relationship with the major REV structural core protein p29. The core polypeptides were not associated with the intracellular ribonucleoprotein complex. Thus, p29 was incorporated into the virions in the form of its precursor pr63. The cleavage of pr63 in the complex was accomplished either during the budding process or shortly after the release from the cell.

Tsai et al (1985) have described five gag-gene -encoded structural proteins which were purified from REV and designated p12, pp18, pp20, p30 and p10. Based on amino-acid composition and NH_2- and COOH-terminal sequence analysis, p12, pp18, p30, and p10 were distinct from one another, whereas pp20 was likely identical to pp18 in primary structure. Sequence comparisons among the retrovirus family showed that pp18/pp20 and p10 were homologs of phosphoproteins and nucleic acid-binding proteins respectively. The REV-A gag-gene-encoded precursor polyproteins, pr60 was identified and the organization of pr60, viz., NH2-p12-pp18-p30-p10-OH was established.

Serological relationships among the members of REV and with other retroviruses

Although different members of REV were isolated from various avian species and caused quite different lesions and syndromes, they were antigenically closely related. Antigenic relationships between neurotrophic and viscerotrophic REV stocks were studied by cross-neutralization test (Witter, 1970). In most cases homologous and heterologous neutralization titers of sera were similar both in vitro and in vivo. Also, it was indicated that the fluorescence in CSV-infected cells stained with REV T antiserum was indistiguishable from that in REV T-infected cells. Purchase et al (1973) compared the antigenic relationships of all four representatives of the REV group. All REV isolates tested were serologically indistiguishable. The antisera to one of REV-T and one of DIAV gave an approximately equal staining intensity in FA to cells infected with all nine tested REV isolates including three REV-T isolates, 4 DIAV isolates, SNV and CSV. Cook (1969) first indicated a possible antigenic relationship between REV-T and CSV in FA.

As mentioned earlier, the nonglycosylated protein p29 was the major internal polypeptide in the virion. Maldonado and Bose (1976) demonstrated a crossreactivity of antiserum against p29 purified from REV-T infected CEF culture with CSV, SNV and DIAV in both AGP and CF tests, indicating that the p29 was a group-specific antigen shared by the viruses of REV group. Bulow (1977) also failed to find antigenic differences between CSV and three isolates of REV-T by FA and AGP.

Some new REV isolates in U.S., such as REV strain SC and VA (Soloman et al, 1976), MN81 and MN67 (Paul et al, 1977) from turkeys, and REV

strain RU-1 (Li, 1983) from duck, were also found to be antigenically related to the REV group. Recently a comparison of antigenic relationships among all 26 REV isolates obtained in U.S. was made by cross neutralization test with chicken sera (Chen et al, 1987). The results indicated that the isolates were all strongly related by neutralization assays and probably constitute a single serotype.

Other REV strains isolated from Japan (Yuasa, 1976; Koyama, 1976), Australia (Grimes and Purchaxe, 1973; Bagust and Grimes, 1979a), Britain (McDougall et al,1978), Hungary (Dren et al, 1983) were also shown to be antigenically related to REV strain T by AGP or FA assays.

Although all strains of REV group were indistingushible serologically, some minor differences between isolates were noticed by some workers. By using cross-neutralization test. Purchase et al (1973) compared REV strain T, CSV, DIAV and SNV, and Paul et al (1977) compared their strains MN81 and MN67 with all four representatives of REV. Their results showed that neutralization titers were higher in the homologous than in the heterologous strains. It would be helpful if we could find some way to differentiate different REV strains of origins or pathogenicity.

ALV is another group of retroviruses which could cause tumors in chickens and some other avian species. REVs were quite different antigenically from ALV (Theilen, 1966; Aulisio and Shelokov, 1969; Purchase et al, 1973; Paul et al, 1977). It was also indicated that the group-specific antigen of ALV was not detected in concentrated and purified REV (Maldonado and Bose, 1971), and antiserum against ALV-group specific antigen did not react with four representatives of REV group in AGP (Maldonado and Bose, 1976). But the cross-reactiveness

of ALV with some REV isolates was accidently noticed. Purchase et al (1973) reported the presence of ALV gs antigen detected by COFAL test in CEF culture infected by one origin of REV-T and one origin of DIAV. Baxter-Gabbard (1973) found that purified REV-T even after SDS-treatment induced immunity not only against REV but also against Rous sarcoma virus, indicating the possible antigenic relationship of REV to some members of ALV. The mutual cross-protective immunity and cross-neutralization activity between REV strain T and some subgroup of ALV were also experimentally demonstrated (Baxter-Gabbard, 1980). Apparantly the serological cross-reactivity between REV and ALV could cause confusion in diagnosis of two similar diseases or in distinguishing two groups of retroviruses.

There was no REV antigenic cross-reactiveness with other avian viruses tested, such as Marek's disease virus, newcastle disease virus, infectious bronchitis virus, haemorrhagic enteritis viruses (Ianconescu, 1977). Interestingly, REV showed some serological relationships with mammalian C-type oncogenic retroviruses. Charman et al (1979) found that REV p30 shares cross-reactive determinants and a common NH2-terminal tripeptide with mammalian C-Type viral p30s. By using a double-antibody radioimmunoprecipitation, Barbacid et al (1980) demonstrated a close antigenic relatedness in the major structural proteins between REV-A and a mammalian retrovirus OMC-1 isolated from an owl monkey, a new world species of the Aotus genus. Tsai et al (1985) compared the antigenic relationship between REV-A and other retroviruses by the electroblotting-immunoautoradiography technique and found that antisera to REV-A gag-gene-encoded major internal structural protein p30 could cross-react with the similar p30 proteins of various

mammalian type-C retroviruses, such as two mouse retroviruses of subgroup I (R-MuLV and M-MuLV), a feline endogenous virus RD-114, a baboon endogenous virus of subgroup II, two macaque endogenous viruses of subgroup IV, a gibbon ape virus of subgroup IV, and 3 type-D viruses such as MPMV (Mason-Pfizer monkey virus), SMRV (the sole new world type-D virus) and PO-1Lu (an old world type-D virus found in the spectacled langur).

Economic and biological significances of REV

Even though naturally sporadic outbreaks of RE have been continuously reported in various avian species from different parts of the world since the 1960's, its economic significance was still not clear. Probably, one of the reasons was that the diversity of symptoms and lesions caused by REV from nonspecific runting-symdromes and immunodepression to tumors in different organs and tissues, made it easy to confuse REV infection with other similar diseases such as infectious bursa disease, MD, and lympholucosis, and made it difficult to diagnose the disease. However, the serological and epidemiological surveys indicated that the REV infection might spread more widely than thought according to earlier field diagnosis reports. By using FA test, Aulisio and Shelokov (1969) made a serological survey of chicken eggs from 12 states in U.S.A. for antibody against REV and shown that 147 of 905 egg samples tested from 41 of 92 flocks tested were antibody positive, and that the positive samples were distributed through 9 of 12 states surveyed. Purchase et al (1973) made another serological survey of serum antibodies against REV in different avian species. Two of 65 turkey flocks within seven states, five of 43 duck flocks within

17 states, three of nine goose flocks within eight states were found positive. A recent serological survey within different parts of U.S.A. by Witter et al (1982) has documented probable infection with REV in 21.0% of 101 layer flocks, 23.5% of 85 broiler and broiler-breeder flocks, 2.3% of 43 backyard chicken flocks, and 4.8% of 125 turkey production and breeder flocks according to the FA test with sera. The infected flocks mainly were located in southern states, such as Florida, Georgia, Mississippi, North Calolina, but also in some northern states, such as Illinois, Indiana, Michgan and Pennsylvania. Witter and Johnson (1985) further studied epidemiology of REV in broiler-breeder flocks. Six broiler breeder flocks from two companies in Mississippi were tested at intervals for REV infection. Virus was isolated and antibody was demonstrated in all six flocks. Infection was first detected at ages ranging from 13-47 weeks. The REV isolated from these flocks were immunosuppresive and oncogenic when inoculated into day-old chicks. A moderate (3-16%) incidence of neoplasms was induced by contact exposure to these field isolates in the laboratory. Recently, natural outbreaks of REV infection with significant economic losses were reported in some turkey-breeder flocks with high percentage of tumors in Pennsylvania (Witter, 1987). REV was isolated or REV antigen was demonstrated from most affected turkeys or their eggs. An eradication program has had to be considered and has been practically tried in the commercial turkey company for the first time.

In the other parts of the world, serological surveys also indicated the existence of natural infection of REV in commercial poultry industry. A retrospective survey of 586 commercial poultry serums collected during 1973-1975, prior to the use of avian vaccines known to

be contaminated with REV, was made for REV infection by use the FA test in Australia (Bagust and Dennett, 1977). Antibody to REV was detected in two of 14 breeder-layer flocks, one of 30 broiler flocks and a closed flock of turkeys. In Japan, Wakabayashi et al (1976), Wakabayashi and Kawamura (1977) reported that 33 of 480 (6.9%) chicken sera collected from eight prefectures in 1965 were posivtive in antibody against REV by AGP test. Among the samples brought in for diagnosis during 1973-1976, 206 of 1,148 samples (18%) were positive in antibody against REV in AGP test, and REV were isolated from 19 of 322 samples (5.9%) tested. Yamada et al (1977) made another serological surveys and indicated that 5.2% of the 309 chicken sera from 25% of 32 flocks tested in 1964-1965 and 2.6% of 430 chicken sera from 11.9% of 42 flocks tested in 1974-75 were positive in antibody against REV strain T in AGP test and FA.

As the third avian oncogenic virus, REVs had some biological significance as a naturally occuring virus-induced tumor model. The transformation ability of replication defective prototype REV strain T was already observed in different avian species (Sevoian et al, 1964; Olson, 1967; Mussman and Twiehaus, 1971; Taylor and Olson, 1972; Robinson and Twiehaus, 1974) and in cell culture (Hoelzer et al, 1979). A number of transformed cell lines were established from bone marrow cells (BMC) of REV strain T-infected chickens (Franklin et al, 1974; MacCubbin and Schierman, 1986), in vitro REV strain T-infected BMC (Beug et al, 1981; Weinstock and Schat, 1986) , spleen or bursa cells (Lewis et al, 1981) and CEF (Franklin et al, 1977), REV strain T-infected chicken embryo liver or spleen tumors (Koyama et al, 1981), spleen lymphoma of REV-infected chickens (Ratnamohan et al,1982) and

liver lymphoma of CSV-infected chickens (Nazerian et al, 1982). The genome of transforming REV-T was compared to other non-defective and non-transforming members of REV group and the oncogene (rel) was identified and thought to originate from normal avian genome, most probably from turkey (Wong and Lai, 1981; Rice et al, 1982; Wilhelmsen et al, 1984).

Except the above, it was also found that the non-defective REVs could cause chronic neoplasis. Chickens inoculated as embryos or at hatching with CSV developed a high incidence of lymphoid neoplasms between the 17th and 43th weeks of age, involving principally the liver and bursa of Fabricius (Witter and Crittenden, 1979). Witter et al (1981) further indicated that chickens inoculated as embryos with non-defective REV strain T developed lymphomas involving the Bursa and other visceral organs sarcoma, and carcinoma.

Noori-Daloii et al (1981) showed that nondefective REVs were capable of inducing lymphomas in chickens and proviral DNA of the virus was integrated next to C-myc gene in over 90% of the tumors tested. This finding strengthened the hypothesis that the c-myc and its adjacent sequences were important in B-lymphocyte transformation. REV induced tumors were also used as model for serotherapy. Hu and Linna (1976) reported that the passive administration of immunoserum obtained from animals having undergone regression of RE visceral tumors had a significant protective effect on already-detectable tumors caused by REV-T, and could induce regression of established REV-induced tumors and reduction of tumor mortality. It substantiated the host-protective role of the antibody-forming system in the malignancy.

Another interesting aspect in studying REV was its revolutionary

lineage with mammalian retroviruses. In addition to the cross-reactivities of the internal structure protein p30 between REVs and many kinds of mammalian C-type or D-type retroviruses as mentioned before, the RNA-dependent DNA polymerase of REV was also very closely related to that of mammalian retroviruses. Moelling (1977) has reported that the RNA-dependent DNA polymerase from REV had no resemblence in molecular structure to other avian viral reverse transcriptases which all consisted of two polypeptides and prefered Mg^{++}, but was similar to the murine viral reverse transcriptase which consisted of a single polypeptide of 84K and prefers Mn^{++}. DNA polymerase of REV serologically cross-reacted with murine viral polymerase but not other avian viral polymerase. Allen et al (1980) further proved the close relationship serologically of DNA polymerase between REVs and mammalian C-type retrovirus. Antiserum to the DNA polymerase of SNV inhibited the polymerase activity of reverse transcriptase from REV and from mammalian C-type retroviruses of murine, feline, and primate origin, but did not inhibit reverse transcriptases of avian myeloblastosis virus (AMV). Conversely, antiserum to DNA polymerase of a mammalian C-type retrovirus, Rauscher murine leukemia virus, inhibited the polymerases of mammalian C-type viruses and REVs but was ineffective against AMV polymerase.

Nucleic acid homology between REV and mammalian C-type viruses was studied by Rice et al (1981). They have demonstrated the relatedness of REV cDNA to cloned proviral DNA of the colobus monkey endogenous virus (CPC-1) which was highly related to the macaque viruses. Related regions occur within both the pol and the gag genes of the colobus viral genome. Thus they speculated that the REV group appeared to be

descended from an ancestral virus which also gave rise to Macaque viruses (MAC-1 and MMC-1) and CPC-1 group in primates. This might be the first example of interclass transmission among the retroviridae by crossing the interclass barrier between mammals and birds.

The third biological significance of REV was its potential uses in poultry breeding by molecular biology technique. Because of its ability to be inserted into the avian germline, REV probably could be used as vectors capable of introducing foreign genes of intrest into avian germline to improve genetic characteristics of birds (Salter et al, 1986).

Detection of REV infections

Existence of REV infection among birds could be demonstrated by detecting either REV itself or antibodies against REV. Several different assays have been used for detecting antibodies against REV. Witter et al (1970) first conducted an IFA to test sera of experimentally infected chickens for REV antibodies on REV-infected CEF culture coverslips. The assay was successfully used for further epidemiological or serological surveys in commercial chicken and turkey flocks (Bagust and Dennett, 1977; Witter et al, 1982; Witter and Johnson, 1985) and REV transmission studies (Peterson and Levine, 1971; Ianconescu, 1977; Bagust and Grimes, 1979; Bagust et al, 1981; Witter et al, 1981).

Using concentrated REV as an antigen, Ianconescu (1977) developed AGP assay to detect antibodies against REV in chicken and turkey sera. The

test was specific to anti-REV sera; there were no cross reactivities to anti-NDV, IBV, MDV, or HEV sera. The AGP was also used by other workers (McDounagall et al, 1980; Motha, 1984; Motha et al, 1984) for detecting REV antibodies. Its procedure was simpler, but probably less sensitive than IFA. Although Witter and Johnson (1985) successfully used AGP for primary screening of the plasma samples for REV antibody to make epidemiological studiy in broiler-breeder flocks, they also indicated that while the AGP flock status was negative but a confirmatory IFA turned out to be positive.

Neutralizing antibody to REV was also detected by using a plaque reduction test in cell culture. McDougall et al (1980, 1981), Motha and Egerton (1983), and Motha et al (1984) studied the transmission of REV in turkeys and chickens by neutralizing tests.

Smith and Witter (1983) developed an indirect enzyme-linked immunosorbent assay (ELISA) for antibodies against REV and reported that it was consistently more sensitive than IFA tests. The limits of antibody detection by ELISA were comparable to those obtained in virus neutralization but ELISA was simpler than the neutralization test.

No matter what kinds of assays were used, antibody-positive reactions only demonstrate that the individuals or flocks tested had been infected. We could not relate the results to the existing symptoms or lesions in individuals or flocks. The antibody to certain specific agents appear only a period after infection and may last a long period after animals or birds recover from the infection, so it was not enough to diagnose a infectious disease only by detecting specific antibodies. It was especially apparent in the case of REV infection because: (1) the major defects caused by REV infection were nonspecific runting

syndromes, immunodepression and tumors, making it meaningless to detect only REV antibodies for the differential diagnosis until the viremia was proved; (2) REV-infected birds with symptoms and lesions may not show any antibodies against REV due to immunodepression of REV. Bagust and Grimes (1979) studied serological responses in chickens experimentally infected with Australian strain REV/Q/1/73 at 1 day of age and found that some infected birds never developed FA and AGP antibodies against REV during the period of 26-56 weeks even though persistent viremia and some tumors or other lesions were detected in these birds. Witter et al (1981) further indicated that chickens inoculated as embryos with nd-REVs generally developed a "tolerant" infection characterized by lack of FA antibody and by a viremia that was persistent through 93 weeks although chickens developed tumors and other lesions. This may explain partially the fact that although the serological surveys have already shown that REV infections actually were spread widely in the world among different avian species, its economic significance has yet not been recognized so far.

For the eradication program of REV, assays for REV antigen seem also to be much more important than those for REV antibodies . In some infectious diseases, carriers with positive antibody response could still shed infectious agents such as the case of Salmonella sp. infection. The blood-agglutination tests were used as a very powful assay to pick up all antibody positive chickens or hens as Salmonella sp. carriers. But it was quite different in the case of REV infection. All published data showed that REV viremia or antigenemia did not coexist with antibody to REV in the same individuals. In the birds with antibody response, there usually was no viremia or only transient

viremia. REV viremia orantigenemia appeared mainly in "tolerant" birds which were infected early in life and never developed antibody response (Paul et al, 1977; Ianconescu, 1978; Bagust and Grimes, 1979; McDougall et al, 1980; Bagust et al, 1981; Witter et al, 1981). Ianconescu (1978) noticed that immunodepression of REV-infection on NDV antibody only happened in chickens which showed REV-antigenemia but no REV antibody response and the HI titer to NDV was normal in REV-infected chickens which had REV-antibody response but no REV antigenemia.

It was experimentally demonstrated that REV could be transmitted from bird to bird either vertically by shedding virus particles into eggs and semen, or horizontally by close direct contact. Usually only infected birds which had viremia or REV antigenemia and were REV-antibody negative could shed virus. Witter et al (1981) reported that most nd-REV strain T- or CSV-infected chickens which developed "tolerant" infection with persistent viremia but without antibody response could shed infectious viruses into cloaca and eggs, the virus transmitted to their progeny chicks even though at low frequency. But no infected birds which developed antibodies against REV were able to shed infectious virus into cloaca or eggs. The same phenomenon was also reported by Bagust et al (1981). Among five hens infected with REV/Q/1/73 at 2 days of age by inoculation or contact, no REV was detected in vaginal swabs and eggs from four hens which developed antibodies against REV. Only one hen which had persistent viremia but no antibody response could shed virus from vagina, eye, mouth and feather pulp to infect other hens in contact with her in the same case and also shed viruses into eggs from which the REV-infected embryos developed.

McDougall et al (1980) compared the infectious status of different turkey groups infected with REV at different ages and in different ways for their viremia, antibody response and virus shedding into eggs or semen. Within the period of 26-40 weeks, all turkeys inoculated with REV at 1-day-old developed a viremia which persisted through the period of 40 weeks but almost no antibody-reaction. REV was found to be shed into semen from a 35 week old male turkey inoculated with REV at 1-day-old. In turkeys infected with REV by contact at 1-day-old or inoculated with REV at 4 weeks of age, most developed precipitating or neutralizing antibodies but almost no viremia. Also, no virus was detected in eggs from the latter turkey groups but passive antibodies were found in 30-55% of eggs tested. About 27.5% of embryos derived from hens inseminated with the above REV-infected semen were demonstrated to be infected when TEF culture from the embryos were examined by IFA. The mortality and the incidence of viremia and leukosis in progenies of turkey hens inseminated with REV-infected semens were much higher than controls.

The same results were also shown in ducks (Motha, 1984). When ducks were inoculated with REV/Q/1/73 at 1-day-old, both viremia and antibody response were developed in certain percentage within a period of 38 weeks; and 85% of the eggs from these ducks were infected with the virus. When infected by contact at 1-day-old, however, all ducks developed the antibody response but neither antigenemia nor shedding REV into eggs were detected.

Motha et al (1984) reported the possible role of mosquitoes in the mechanical transmission of REV in chickens. REV were isolated from mosquitoes from pens with persistently viremic chickens. The virus was

experimentally transmitted from persistently viremic donor chickens to a recipient chicken by Culex annulirostris.

It becomes apparent on the basis of the above finding that detection of REV itself was much more important than detection of REV-antibody in individuals or flocks for both differential diagnosis and eradication programs.

The most reliable test for detecting REV from materials to be tested was to inoculate the suspected samples into cell cultures and examine the infection of cell cultures with REV antibodies by IFA test several days after virus replication. Bagust and Dennett (1977) developed the procedure for detection of REV antigen in CEF coverslips which had been previously inoculated with different suspected samples. The procedure was routinely used to determine the viremia in chickens and turkeys (Bagust and Grimes, 1979; McDougall et al, 1980; Motha, 1984), and to detect virus shedd into eggs and semen (McDougall et al, 1981; Motha, 1984, 1987), embryos (McDougall et al, 1981), swabs from eye, mouth, cloaca, nostril, rectum and vagina (Bagust et al, 1981), and mosquitoes (Motha, 1984). Smith et al (1977) developed a micro-complement fixation procedure for REV antigens, designated as COFAR, which was possibly more sensitive than IFA for detecting infection in cell cultures inoculated with suspected plasma. COFAR could detect REV in samples only after amplification of REV in cell cultures. It failed to directly detect REV gs-antigens from egg albumen (Witter, 1982).

Based on the virus replication, although the virus assay in cell culture was the most sensitive test for detection of infectious virus particles, it was labor-intensive and costly in terms of materials. In

addition, it would take several days to complete the test. It is not therefore, practical for epidemiological surveys or eradication programs in which a large number of samples should be tested.

Ianconescu and Aharonovici (1978) developed a AGP assay with anti-REV serum to directly detect REV antigen in sera of chickens infected with REV as a embryo or at hatching and some turkeys or chickens of infected field flocks. The assay was proved by Bagust and Grimes (1979) and Motha (1984a), they also successfully detected REV antigenemia in sera of chickens or ducks infected at 1-day-old with REV respectively. The sensitivity of the AGP for REV antigen was not indicated in their works, but it was usually very low. When used as an antigen preparation in AGP, in fact, REV-infected CEF culture fluid had to be 6-10 fold (Ianconescu, 1977) or 20-fold concentrated (Yuasa et al, 1976) to show up the precipitate line for detecting REV antibody. The experiment indicated (Maldonado and Bose, 1976) that 2 ug of purified group-specific antigen p29 in 10 ul per well had to be used for demonstrating a precipitate line in AGP. It suggested that only serum samples with REV titer higher than REV-CEF culture fluid could be found positive for REV antigen in AGP. In most infected birds in the fields, REV viremia or antigenemia is unlikely to be detected at so high level . In addition, the AGP was not reported to detect REV antigens from other kinds of samples. For eradication programs, there is need to identify as many REV-shedding birds with viremia as possible. Apparently, AGP is not sensitive enough for the tasks. Some more sensitive and simpler assays would be required.

For direct detection of ALV, another retrovirus which caused lymphoid leukosis in chickens, Smith et al (1979) established an ELISA procedure

which could detect as little as 2-3 ng of ALV protein. When a biological assay, i.e., phenotypic mixing (PM) was the criterion for the infectious status of specimens, the ELISA consistently identified a greater percentage of virus-positive specimens than direct CF tests. Over 95% concordance was obtained between the ELISA and PM bioassays when meconia and whole blood samples were tested. It could be used to detect the virus in different kinds of samples, such as meconia, egg albumems, cloacal swabs, blood and sera. They assumed that the ELISA would detect about $5x10^3$ infectious units per ml.

In the last decade, more and more experiments have shown that ELISA is a very sensitive, rapid and inexpensive assay for detecting different kinds of antigens. It has been successfully used for detection of hemorrhagic enteritis viruses (HEV) in infected turkey samples (Ianconescu et al, 1984), hepatitis B surface antigen (Deepak et al, 1985; Gadkari et al, 1985), rotaviruses in faecal samples (Kjeldberg and Mortensson-Egnund, 1982; Chernesky et al, 1985) and sewage sludge (Agbalika et al, 1985), respiratory syncytial virus (RSV) in infants and small children (Hornsleth et al, 1986), adenovirus in faeces extracts (Johansson et al, 1985; Mortensson-Egnund and Kieldsberg, 1986), and SV40 virus antigen in contaminated poliovaccine (Edevag et al, 1985). Hornsleth et al (1986) reported that the ELISA would detect 0.5-1.0 ng RSV-protein. Binnema et al (1986) also developed an ELISA for urokinase with a detection limit of 100 pg/ml. By use of MCA to specific antigens, ELISA has been used for detection of feline leukemia virus p27 antigen in cat serum (Lutz et al, 1983), Semlik forest virus in virus-infected cells with threshold between 10^5-10^6 pfu/ml (van Tiel et al, 1984 and 1985), and canine

parvovirus antigen in fecal samples with adetection limit of 1.5 ng of virus (Middbraund, 1984). Because of its high sensitivity and specificity, ELISA with MCAs has been further used to identify or quantify some biochemical molecules such as PiZ alpha 1-antitrypsin (GFAP) in human serum (Wallmark et al, 1984), soluble human glial fibrillary acidic protein with a working range of 1-600 ng GFAP /ml in four layer system or with a working range of 0.5-60 ng GFAP /ml in five layer system (Albrechtsen et al, 1985), human lysosome alpha-glucosidase (Henkel et al, 1985), some hormones (Hanquez et al, 1987) and tissue type plasminogen activator (t-PA) with a working range of 0.4-15.2 ng/ml plasma (Korninger et al, 1986). Thus, when MCAs against REV become available, it is expected that an ELISA procedure for REV antigen could also be developed. Such an assya should be more sensitive than AGP for the direct detection of REV antigen in various samples. In addition, it should be possible to identify positive individuals in a flock by testing the same samples in ELISA for ALV and REV simultaneously and greatly facilitate eradication programs against ALV and REV.

Monoclonal antibodies and their application in biology

Hybridomas growing in cell culture or mouse peritoneal produce homogeneous immunoglobulin species, the antigen-binding variable region of which is reactive with only the same antigenic determinant or epitope. Because of its high specificity and titer, MCAs have been widely used in almost all aspects of biological research since Kohler

and Milstein (1975, 1976) developed the hybridoma technique. The following applications are conducted and included in this dissertation:

MCA as a specific and sensitive reagent for differentiation or definition of antigenical molecules in small percentage of the biological complexes

MCAs have often been used for identifying tumor-specific or tumor-associated antigens, such as lung cancer markers (Hirota et al, 1985), mammary tumor markers (Colcher et al, 1981; Schlom et al, 1985), melanoma tumor-associated antigens (Kan-Mitchell et al, 1986; Yamaguchi, 1987), a human tumor-associated glycoprotein (Johnson et al, 1986), a Burkitt's lymphoma-associated antigen (Lipinski et al, 1982). In animals, MCAs defining chicken Marek's disease tumor-associated surface antigen were developed (Lee et al, 1983; Liu and Lee, 1983; Itkata et al, 1984). Artus et al (1986) developed MCAs recognizing tumor-associated antigens in X-irradiated C57BL/6 mice. Also, MCAs would be used for differentiation or defining other cell components which could not be easily detected by polyclonal antisera otherwise, such as or Con-A rat lymphocyte activation antigen after stimulation by Con-A (Uede et al, 1986).

In veterinary medicine, MCAs were successfully developed for differentiation of virus strains with minimal antigenic differences. Lee et al (1983) established a panel of MCAs which could be used for distinguishing three serotypes of the herpes viruses including pathogenic strains of MDV, nonpathogenic strains, and herpes virus of turkeys. For Newcastle disease viruses (NDV), Srinivasappa et al (1986) generated a MCA which reacted to high titer in

heamagglutination-inhibition tests with only lentogenic vaccine strains commonly used in the United States. It would help differentiate flocks vaccinated or infected by velogenic or mesogenic virus strains. Lee et al (1986) established a hybridoma cell line which reacted with exogenous ALV subgroups A, B, C and D at an antibody titer up to 1,000 fold higher than with endogenous subgroup E RAV-0 strain in indirect ELISA. It offered the potential for developing immunological test to differentiate exogenous and endogenous ALV strains. Lutz et al (1983) screened three MCAs against major core protein p27 of FeLV, capable of distinguishing all FeLV isolates from other retroviruses (MuLV, MpMV, MmTV, SMRV, BHEV). By using MCAs, Stanley et al (1987) showed that the variants of Visna virus, a retrovirus which caused encephalitis, pneumonias and arthritis in sheep and goats, might emerge more frequently during persistent infection than could be detected by polyclonal immune sera.

It is expected appropriate MCAs would be able to distinguish different REV strains, otherwise undistiguishable by use of polyclonal antiserum.

MCA as an immunological reagent in developing more specific and sensitive diagnostic assays for infectious diseases

Taylor (1984) prepared a fluorescencein-conjugated MCA to detect chlamydial eye infection. The assay might be even more sensitive than culture and detect lower levels of infection. It could be a rapid, efficient and inexpensive method of diagnosing ocular chlamidial infection. Morris et al (1985) found that MCAs to the K99 fimbrial adhesin produced by E.coli enteropathogenic for calves, lambs and

piglets could be used as diagnostic reagent. Using the slide agglutination test, the reaction of MCAs was identical to those of a polyclonal antiserum to K99 when both were used in parallel to examine 1,408 $K99^+$ E.coli. When MCA was established, it would be a much cheaper reagent than polyclonal antiserum for diagnostic purpose. MCAs were more widely and successfully used in ELISA to detect small number of antigens as mentioned in detail just above.

MCA as a potentially powerful tool to characterize structural and functional properties of virus protein components.

Although polyclonal antisera had been used for analyzing protein components of many kinds of viruses, their multiclonal nature prevents precise identification of cross-reactive antigenic determinant and determining the relationship between protein structure and their functions. As an example, Ikuta et al (1981) and van Zaane et al (1982) identified 46 MDV viral proteins ranging from 19-350 KD or 35 MDV viral proteins ranging from 20-160 KD in immunoprecipitation with anti-MDV sera, but they could not demonstrate any relationship among these proteins. By using a panel of MCAs against MDV, Silva and Lee (1985) have shown that MDV and HVT glycoprotein gp100, gp60, gp49 might belong to the same protein family antigenically, and three non-glycoproteins p41, p38 and p24 belong to another family antigenically. Similarily, Pereira et al (1984) identified human cytomegalovirus (CMV) glycoproteins by MCAs and divided them into four antigenically distinct groups: gA, gB, gC and gD. Each group except gB formed several bands when immune precipitated from infected cell extracts. They showed that gp160-148K, gp142K, gp138K, gp123-107K, gp95K and gp58.5K belonged to

the same family. The partially glycosylated precursors of these proteins were also identified.

Especially, MCAs could help to relate protein components of virus to their corresponding immunological functions. Roehrig et al (1982) reported by using MCAs against Venezulan equine encephalomyelitis virus (VEEV) that the biological function of heamagglutination and virus neutralization were primarily associated with only one antigenic epitope present on the virus glycoprotein gp56. Collins et al (1984) defined neutralizing determinant of bovine herpes virus I polypeptides and showed that two MCAs which were the most efficient in neutralization recognized a non-glycosylated protein of 115 KD. Neutralizing epitopes were also located on a glycoprotein of 82 KD and a five-glycopolypeptide-group ranging in size from 102 to 55 KD, but neutralizing ability was limited on a non-glycosylated polypeptide of 91 KD. By combined use of MCAs and EM, Taniguchi et al (1985) demonstrated that human rotavirus VP3 protein of 82 KD was located on the outer shell of the virus particles and neutralizing MCAs were found to agglutinate exclusively double-shelled particles and be directed to the outer capsid protein. According to antigenic analysis of equine infectious anemia virus (EIAV), Hussain et al (1987) showed that neutralizing MCAs apparently reacted with strain variable regions of the virus envelope gp90 but the MCAs which reacted with conserved epitopes on gp90 to gp45 failed to neutralize EIAV. They also found that the conformation of different neutralization epitopes appeared to be continuous as they resisted treatment with SDS and reducing reagents. After testing a panel of ecotropic and xenotropic MuLVs by MCAs, Gambke et al (1984) revealed that ecotrop-specificity was

related to p15E/p12E, xenotrop-specificity to p15E, group-specificity to p30 and p15E of virus protein components respectively. The cytotoxic determinants localized on p12.

It was also expected to further analyze molecular structure of REV by MCAs when available. It could help to understand which protein(s) would be responsible for strain-specificity or group-specificity and neutralization ability.

MCA as the exclusive reagent to map epitopes on protein components and relate the epotopes to their biological functions.

All kinds of proteins or antigenil molecules consist of many different antigenic determinants or epitopes on the same molecule. In most cases, only some critical epitopes or domains are mainly responsible for their specific biological activities. It is impossible to recognize or identify different epitopes on specific molecules unless MCAs are to be used. As MCAs to different epitopes on the same molecule become available, it would be possible to topologically map antigenic sites on the same protein molecules and define the relationships between structural domains and biological functions of the molecules.

By using six MCAs specific for the hemagglutinin-neuraminidase (HN) molecule of the parainfluenza type 1 virus and competitive binding assay (CBA) in radioimmunoassay (RIA), Yewdell and Gerhard (1982) detected four distinct antigenic sites on the HN molecule. Although antibodies to each site had similar potencies in hemagglutination inhibition tests, antibodies to sites A and C or D differed approximately 100-fold in their potency to neutralize the virus. Also,

the antibody to site A strongly inhibited viral neuraminidase activity, whereas antibodies to sites C and D enhanced the neuraminidase activity. Only antibodies to sites C and D formed precipitates in Ouchterlony double diffusion against detergent-disrupted virus.

Roehrig et al (1983) identified eight epitopes on the E glycoproteins of Saint Louis encephalitis virus using MCAs on the basis of hemagglutination-inhibition and virus neutralization tests. Analysis of the spatial arrangements of these epitopes using competitive binding assays with representative MCAs indicated that the E glycoprotein of the virus was a continuum of six overlapping domains.

Heinz et al (1983) analyzed topological and functional relationship among epitopes on the structural glycoprotein of tick-borne encephalitis (TBE) virus in haemagglutination inhibition (HI), neutralization and antibody blocking assays with MCAs. Seven out of the eight distinct epitopes were shown to be partially linked and to cluster in two antigenically reactive domains (A,B). Domain A was defined by three HI antibodies, two of which were flavivirus group-specific, whereas the third was TBE virus subgroup specific. Within the domain, only the subgroup-specific antibody was involved in virus neutralization. Domain B was composed of three TBE-complex reactive epitopes, and corresponding antibodies inhibited HA and neutralized the virus. By using corresponding MCAs, Kimura-Kuroda and Yasui (1983) made a topographical analysis of antigenic determinants on envelope glycoprotein V3 (E) of Japanese encephalitis virus in the relationship to their biological functions. Their results suggested that the hemagglutination inhibition (HI) sites on the protein were seperated from the neutralization sites and there were two distinct HI

sites, one of which was flavivirus cross-reactive, the other subgroup specific.

The relationships between antigenic epitopes and their neutralization or heamagglutination abilities have also been analyzed with vesicular stomatitis virus (Bricker et al, 1987), avian infectious bronchitis virus (Niesters et al, 1987), bovine coronavirus (Deregt and Babiuk, 1987) foot-and-mouth disease virus (Pfaff et al, 1988), and Simian rotavirus SA11 (Burns et al, 1988) by using the corresponding MCAs. Competitive inhibition studies demonstrated that 57 MCAs to tetanus toxoid recognized respectively at least 20 different epitopes on the toxoid molecule. All neutralizing antibodies bound to epitopes on the heavy chain of the tetanus toxin. Neutralization of toxicity was affected by nine distince MCAs. Mixtures of two, three, and four different MCAs experted a synergistic effect of 200-fold over that observed with individual MCA, indicating that efficient neutralizing might involve the simultaneous binding of at least two antibody molecules to different specific regions of the toxin molecule.

The topological mapping of antigenic epitopes or antigenic sites with corresponding MCAs were also made on other virus structural proteins, such as yellow fever virus envelope protein (Schlesinger et al ,1984), Dengue-2 virus NS1 protein (Henchal et al, 1987), sheep or goat Visna virus envelope glycoprotein (Stanley et al, 1987), bovine leukemia virus envelope glycoprotein gp51 (Bruck et al, 1982), murine leukemia virus proteins (Stone and Nowinski, 1980), influenza A/PR/8/34 virus hemagglutinin (Lubeck and Gerhard, 1981), feline leukemia virus core protein p27 (Lutz et al, 1983), surface glycoproteins of Venezuelan equine encephalomyelitis virus (Roehrig et al, 1982) and bovine

herpesvirus (Collins et al, 1984). Even on the hapten penicillin, at least three epitopes were recognized by MCAs (de Haan et al, 1985).

Determination of the epitope-specificities of MCAs

As a prerequisite for mapping epitopes on protein molecules, the epitope-specificity of MCAs produced by each individual hybridoma clone needs to be determined or compared to each other. For this purpose, the competitive binding assay (CBA) has been used in either radioimmunoassay (RIA) or ELISA. No matter which assay is used, the mechinism for CBA remains the same. If two MCA samples are against the same epitope or closely related epitopes, they would demonstrate competitive binding properties. On the other hand, if the two epitopes are at sufficiently distant sites on the same protein molecule, their corresponding MCAs would not bind competitively (Stone and Nowinski, 1980). Further, Luberck and Gerhard (1981) interpretated the CBA by a number of different mechanisms: (1) two competing antibodies might bind to structurally overlapping epitopes; (2) two antibodies might recognize structurally nonoverlapping epitopes situated in close proximity (binding of a probe might thus be hindered due to steric constraints resulting from the size of the competing antibody molecules); and (3) binding of an antibody might allosterically alter a second antigenic site.

The basic principle for CBA in either RIA or ELISA is similar. The only difference is that ^{125}I and enzyme-substrate system are used as indicators in RIA and ELISA respectively. Stone and Nowinski (1980) developed a CBA in RIA for identifying the epitope-specificities of

MCAs to MuLV proteins. Briefly, the 96-well micro plates are previously coated with the corresponding virus proteins. The CBA test was carried out as follows: (1) each MCA to be identified in certain dilutions was added into wells of plates as competing antibodies and then washed away after a certain incubation period; (2) ^{125}-radiolabeled antibodies of each sample to be tested were added into the wells and then washed away after a certain incubation period; (3) the immune reactions were detected by autoradiography of the plate on Kodak films. If non-labeled competing antibodies and ^{125}I-labeled antibodies were against the same epitope or spatially-related eitopes, immune reaction would be inhibited according to the darkness on the film. Otherwise, competing antibodies would not give any effects on the immune reaction of radiolabeled MCAs with the antigen coated on the plates. Similar procedures have also been used by other workers for determination of epitope-specificities of MCAs against influenza A/PR/8/34 virus hemagglutinin (Lubeck and Gerhard, 1981), bovine leukemia virus (Bruck et al, 1982), paramyxovirus glycoprotein (Yewdell and Gerhard, 1982), yellow fever virus envelope protein (Schlesinger et al, 1984), Dengue-2 virus NS1 protein (Henchal et al, 1987) or tetanus toxin (Volk et al, 1984), but radioactivity of bound labeled antibodies was detected in gamma counter instead of by exposure to the film. Heinz et al (1983) determined epitope-specificities of MCAs to tick-borne encephalitis virus by CBA in RIA with a modification in which polystyrene beads instead of plates were coated with virus antigens. No matter which kinds of modification were used for CBA in RIA, each MCA had to be purified and radiolabeled with ^{125}iodine. The unlabeled MCAs that reduced binding of the ^{125}I-labeled antibody by a certain extent were

assumed to be recognizing an identical determinant or those in close enough proximity to sterically hinder the binding of the ^{125}I-labeled antibodies.

Similarly to the above competitive RIA, Roehrig et al (1982) developed a competitive ELISA to determine the epitope-specificities of MCAs against Venezuelan equine encephalomyelitis virus. They conjugated alkaline phosphatase to each purified MCAs to be tested and color reaction of enzyme-substrate instead of ^{125}I-labeled MCA was used as indicator of immune reactions. Briefly, starting with a concentration of 1 mg/ml, 50 ul of two-fold dilutions of each competing nonconjugated MCA IgG was mixed with 50 ul of a certain dilution of MCA IgG-enzyme conjugates. The mixture was allowed to equilibrate for two hours at 37^{o}C in 96-well plates precoated with the corresponding virus antigen. The plates were rinsed, 100 ul/well of substrate was added, and immune reaction was recorded by measure of O.D. of solution in each well. The O.D. reading could be ploted in curves. As for competitive RIA, the color reactions of enzyme-conjugated MCAs would be inhibited by the homologous competing MCAs or closely related competing MCAs. In contrast, there would be no inhibition between MCAs recognizing spatially unrelated different epitopes. Kimura-Kuroda and Yasui (1983) also used competitive ELISA for analysis of epitope-specificities of MCAs against Japanese encephalitis virus. In their study, the horseradish peroxidase was used for conjugation of MCAs. A formula, [100(A-n)]/(A-B), was proposed for determining the percentage of competitions, where A was OD in the absence of competing antibody, B was OD in the presence of homologous antibody, and n was OD in the presence of competitor. The competitive ELISA had been used to

determine epitope-specificities of MCAs against other antigen system, such as feline leukemia virus (Lutz et al, 1983), Saint Louis encephalitis virus (Roegrig et al, 1983), and bovine herpesvirus (Collins et al, 1984).

For comparing epitope-specificity of MCAs against sheep and goat Visna virus, Stanley et al (1987) made a minor modification in competitve ELISA. In their study, the purified MCA IgG was biotinylated. The bound biotinylated MCA IgG in the competition binding assay was detected by using streptavidin-horseradish peroxidase and substrate.

No matter what kind of modulations in the assays is used, however, MCA sample to be identified has to be radio-labeled with ^{125}I or conjugated to enzymes. Neither competitive inhibition RIA nor competitive inhibition ELISA could determine the epitope-specificities of hybridomas by using hybridoma culture fluids in the primary screening stages. Each positive hybridoma has to be recloned, kept in liquid nitrogen for several months, injected into mice for ascitic fluid with high antibody titers befor preparing the competition binding assays. They are intensive and require a large amount of reagent for testing a large number of samples. They are thus not well adapted for wide use in biological studies. Friguet et al (1983) discribed a simple ELISA procedure for identification MCA epitope-specificity on the basis of additive effect of different epitopes. Their assay did not require conjuating each MCA sample to enzymes, although still required the asctic fluids. However, the procedure was rarely reportedly repeated by others. Obviousely, it would be helpful if hybridomas could be

identified for their epitope-specificities during the primary screening period by an assay which does not require purified MCA IgG from asitic fluid, labelling or conjugating MCA samples with ^{125}I or enzymes.

MATERIALS AND METHODS

Propagation and purification of viruses. Nondefective REV strain T (Robinson et al, 1974) and strain CS (Cook, 1969) that had been cloned three times (Witter and Crittenden, 1979; Witter et al, 1981) were used. These viruses were propagated in chicken embryo fibroblasts (CEF). Briefly, CEF from 11-day-old line O embryos were cultured in 150 mm Falcon plastic plates containing Leibovitz-McCoy medium supplemented with 4% calf serum. When the culture became confluent, the concentration of calf serum was reduced to 1% for maintenance of CEF growth, and 0.2 ml of supernatant fluids of REV infected CEF culture was inoculated into each plate. Medium was changed every other day. For large-scale production of virus, strain T or CS was cultured in roller bottles as described by Smith et al (1977). Culture fluids were collected every other day and were centrifuged at 21,000 rpm for 45 min by using a Beckman SW 27 rotor in a Model L2-65B ultracentrifuge. The virus pellet was collected, and suspended in 0.01 M Tris buffer (pH7.5), and was stored at -20^{o}C (Smith and Witter, 1983). Virus was purified through a continuous sucrose gradient of 10-52% (W/W) by centrifugation at 45,000x g for 1 hr. as described by Lee et al (1971). The purity of the preparation was verified by electron microscopy in negative staining with 2% PTA, pH 6.8. The protein concentration of the purified virus was measured by the method of Lowry et al (1951).

Immunization, fusion, and selection of hybridomas. Inbred BALB/c mice were immunized i.p. with purified virions of strain T (0.5 mg protein per immunization) or with strain T-infected CEF (2 X 10^{7} cells). The mice were reimmunized i.p. after 28 days, followed by

another i.p. boosting immunization 21 days later. Three days after the final i.p. immunization, spleens were removed, and the splenocytes were fused withNS-1 myeloma cells at a ratio of 5:1. Fusion procedures and cell culture conditions were according to published methods(Lee et al, 1983). The hybrid cells were dispensed into 96-well Costar 3524 tissue culture plates. Beginning between days 8 and 12, the medium from wells showing cell growth was screened for antibody activity against strain T-infected CEF (T-CEF) or purified strain T virus by indirect enzyme-linked immunosorbent assay (ELISA). Hybridomas that produced antibody positive for REV were transferred into 24-well plates for cell expansion and additional testing against strain CS-infected CEF (CS-CEF) or purified CS virus for strain specificity. Hybridomas producing antibodies of interest were cloned by limiting dilution in 96-well plates. Ascitic fluid was produced by the i.p. injection of 3 x 10^6 from each cloned hybridoma into BALB/c mice primed 10 to 14 days previously with 0.3 ml of pristane (2,6,10,14-tetramethyl pentadecane, Aldrich Chemical Co., Milwaukee, WI). Ascitic fluids were harvested, were clarified by centrifugation, and were tested for antibody titers by endpoint dilution in ELISA and fluorescent antibody tests.

ELISA procedure of screening hybridomas. Hybridoma culture supernatant samples were screened by inderect ELISA by using REV-infected CEF (REV-CEF). The procedure for ELISA was as described (Chen et al, 1984). Briefly, 96-well microtiter plates (micro ELISA plates, Dynatech, Alexandria, VA) were coated with 3 to 4 x 10^4 REV-CEF or normal CEF by centrifugation, or were coated with 200 ng of sucrose gradient purified virus in pH 9.6 carbonate buffer in a vol. of 100 ul per well overnight at room temperature. Plates coated with

purified virus were blocked with 3% bovine serum albumin (BSA). Hybridoma culture supernatant (100 ul) or different dilutions of ascitic fluid were added, and were incubated of 1 hr. at 37°C. Unbound antibodies were removed by washing three times with washing buffer (phosphate-buffered saline with 0.1% Tween-80). Anti-moused IgG(H+L)-peroxidase conjugate (100 ul) (Miles Scientific, Naperville, IL) in a dilution of 1:1000 with 3% BSA was added to the wells and was incubated for another 1 hr at 37°C. Wells were washed three times again to remove unbound conjugate. Freshly made substrate (100 ul of 0.08% aminosalicylic acid and 0.005% hydrogen peroxide in 0.02 M phosphate buffer, pH 6.0) was added to each well. Plates were kept at room temperature for 1 hr. Absorbancies were measured in a ELISA minireader (Dynatech, Alexandria,VA). The reading was adjusted to zero absorbancy with a control well containing substrate only. The wells were considered positive if absorbancy obtained with REV-CEF or purified REV was 2.5 times higher than that with normal CEF.

Anti-REV rabbit serum. Approximately 2 mg sucrose-gradient-purified strain T virus protein were emulsified 1:1 (v/v) in Freund's complete adjuvant and were injected s.c. at multiple sites at the back of rabbits. Tweenty-one days later, three more boosters with the same amount of virus protein in Freund's incomplete adjuvant were administered in 2-week-intervals. Two weeks after final immunization, rabbits were bled, and serum was separated. The hyperimmunized anti-REV antiserum was adsorbed with normal CEF cells to remove antibody activity to normal CEF as described by Smith et al (1977). The adsorbed antiserum gave an endpoint titer of 1:4,000 to 6,000 in ELISA against REV-CEF.

Competitive inhibition ELISA. Ascitic fluid was purified by precipitation twice with an equal volume of saturated ammonium sulfate and was dialyzed against PBS overnight at 4°C. Purified IgG thus obtained were used as competing MCA, as well as for conjugating with horseradish peroxidase (Sigma Chemical Company, St. Louis, MO) as described by Nakane and Kawaoi (1974). The concentration of IgG was measured by the method of Lowry (1951). The optimal dilutions of the different MCA conjugates were determined in an indirect ELISA. For the competition experiment, 100 ul of purified MCA in different concentrations on PBS were first added into wells precoated with sucrose gradient-purified REV strain T and were incubated for 1 hr. at room temperature. Plates were washed once with PBS. Different MCA conjugates (100 ul) diluted in 3% BSA were added and were incubated for 1 hr. at room temperature. The remaining procedure followed that ELISA descrbibed above.

FA test. The secondary CEF cells grown on coverslips were infected with strain T or CS. After 5 to 6 days, the coverslips were harvested, and were fixed in cold acetone:alcohol (6:4) for 2 min., and were dried at room temperature. Hybridoma culture fluids or ascitic fluids in different dilutions were tested for REV by using an indirect fluorescent antibody procedure similar to that described by Witter et al (1970).

Labeling of REV-CEF and immunoprecipitation. Labeling of REV-CEF with [^{35}]S-methionine and immunoprecipitation of cell lysate with S.aureus was conducted as described by Silva and Lee (1984). Briefly, Strain T- or CS- infected CEF cultures at 5 to 6 days after infection were labeled with medium containging 50 uCi/ml of [^{35}S]-methionine

(Amersham Corp., Arlington Heights, IL) for 4 to 6 hr. The labeled cells were lysed in lysis buffer containing 150 mM NaCl, 1% sodium dodecyl sulfate (SDS), and 10 mM Tris-HCl, at pH 7.5. The labeled cell lysate was aliquoted and were frozen at -70^{o}C. [^{35}S]-methionine labeled normal CEF lysate was used as a negative control. For experiments for glycoprotein determination, strain T-infected cells were labeled with [^{3}H]-glucosamine (Amersham Corp., Arlington Heights, IL) at 50 uCi/ml for 6 hr. in F10-199 medium containing fructose instead of glucose. For some experiments, T-CEF were incubated in labeling medium with 2 ug/ml of tunicamycin to inhibit glycosylation. The Cowan I strain of S.aureus was used for immunoprecipitation, and a 7.5% to 20% SDS-polyacrylamide linear gradient gel was prepared for electrophoresis as described by Silva and Lee (1984).

Protein A-gold immune labelling REV virions for transmission electron microscopic examination. The immuno-labeling of virus particles was conducted as described by Groscurth et al (1987). Grids were covered with colloidian film and coated with carbon (Klomparens et al, 1986). A drop of purified REV strain T virus suspension (about 0.8 mg protein per ml) was put on several precoated grids, and kept for 10 min at room temperature, and was drained by touching the edge of grids with filter paper, and air-dried for 2 hr. at 37^{o}C. A drop of 3% BSA was put on and kept for 1 hr. at room temperature, and then was drained with filter paper. A drop of ascitic fluid of MCA 11A25 or myeloma NS-1 cell ascitic fluid (as negative control) in dilution of 1:100 in 1% BSA was loaded on the grids respectively, the grids were kept in petri dishs with water-saturated filter paper and incubated with the ascitic

fluids overnight at room temperature. The next day, ascitic fluids were drained, and the grids were placed in PBS with 0.1% BSA, the buffer were changed several times in 1 hr. before loading a drop of 1:160 gold-labeled protein A (with particles of 5 nm in diameter, Sigma Chemical Company) in 0.1% BSA on the grids and incubating for 2 hr. at room temperature. The grids were washed 3 times with distilled water, then stained with 2% uranyl acetate for 1 min. and air dried. Samples were examined with Philips 201 transmission electron microscope.

Neutralization test. Virus-neutralizing ability of MCAs were tested in two ways of FA or ELISA. By using FA test, a series dilution of REV strain T virus stock (REV-CEF supernatant with ELISA titer of 1:128) were mixed with different MCA ascitic fluids in 1:50 dilution with culture medium respectively and incubated for 20 min at room temperature. 35 mm plates with precultured CEF cell monolayers were infected with each mixtures above and incubated at 37^{o}C for 2 hr. The fluids were poured off from each plate and 2 ml of 0.7% agar solution with fresh medium (prewarmed at 50^{o}C and filtered through 0.45u filters) was placed on the plates. The plates were cooled at room temperature for 30 min. and incubated at 37^{o}C. for 5-6 days. The agar-layer was taken off from plates and plates were fixed with cold acetone:alcohol (6:4) for FA staining as above. The virus infectivity of CEF monolayers of plates infected with different dilutions of virus stock were compared for various MCAs to that for NS-1 ascitic fluid to determine the virus-neutralizing ability of each MCAs. By using ELISA, 96-well plates with precultured CEF monolayers were infected well by well with the mixtures of a serious dilutions of REV strain T stock as

above with different MCA ascitic fluid samples or NS-1 ascitic fluid in dilution of 1:50 with culture medium respectively and incubated at 37^{o}C. Two days later, supernatants of each well were changed with fresh media without MCAs for each wells separately every another day. The culture supernatants of each well were also saved four days after infection and tested for REV antigen by ELISA as described in the followings. ELISA titers of wells infected with each mixtures of virus suspension in different dilutions and various MCA samples were compared to determine the virus-neutralizing ability of MCAs.

Synergistic ELISA (sELISA) for epitope-specificity of MCAs.

96-well micro-ELISA plates were precoated with purified REV or REV-CEF. But proper coating concentrations of antigens had to be determined in the preliminary tests to be certain that the amount of virus antigen coated to the plates was just high enough to keep the plateaus of ELISA reading in the range of about 0.8-1.2 when a single individual MCA sample in series of dilutions was tested. Finally, 96-well plates were coated with 3 x 10^4 REV-CEF per well by centrifugation, or were coated with 150-200 ng of purified REV in pH 9.5 carbonate buffer in a vol. of 100 ul per well overnight at room temperature. Plates coated with purified virus were blocked with 3% BSA. Before running sELISA itself, each MCA samples should be titered in precoated plates individually and a proper dilutions of samples need be chosen in the assay to insure that all samples to be tested would give ELISA readings at the similar level. Otherwise, it would give a large variation to bother the statistical analysis or give a false conclusion in the assay. The key point of sELISA is to set up a number of duplicate wells for adding either each single individual sample or mixtures of

different pairs of samples to be compared. If only one sample would be compared to other different samples in an experiment, for example, 50 ul of the sample on the proper dilution was added to each well of a precoated plate followed by adding 50 ul of the same sample and other different samples in the prejusted dilutions to each well of different columns (8 duplicates for each of 12 comparisons) or rows (12 duplicates for each of 8 comparisons) with multiple-channel pipet. If several different samples should be cross-compared in the assay at the same time, for example eight samples, 50 ul of a sample were added to all wells of one column (1 through 8) for each individual sample respectively followed by adding another 50 ul of a sample into all wells of different rows (A through H) with each individual sample again. In this case, 4 plates would be used to get 6 duplicates of each single sample and 12 duplicates of mixed samples for one pair of 8 comparisons. After samples were set up, the further steps were carried out as the ordinary ELISA as the above. But special attention was require so that each plates should be read at the same time after adding substrate to decrease variations between plates. The numbers of replications of each single individual sample in a pair are not restricted seriously, usually 6-12 replicates should be enough to show up the synegistic effect if there is some. Definition of relationships of different MCA samples in their antigenic determinants was dependent on the analysis of ELISA data by statistical method and each pair of samples were analyzed separately. The mean value of ELISA readings in wells added with the mixture of the two samples was compared to the mean value in wells with the single individual samples of the pair by student's t test for two means (Gill, 1978). If the mean values in

ELISA reading of wells with mixed samples are significantly ($p<0.05$) larger than that in wells with single individual samples of a pair, two hybridoma samples in the pair could be judged to have different epitope-specificity provided that dilutions of each samples were chosen properly and the ELISA readings for each single samples were at a close level. The experiments would not be thought valid for the conclusion if the mean value in ELISA readings of wells with mixed samples are smaller than a mean value of wells with any single sample of a pair. Itprobably happens when dilutions of samples were not chosen properly, i.e. one's readings in ELISA is much higher than another's in a pair. In contrast, two hybridoma samples would be thought to be against the similar or close related epitopes if the mean value in wells with mixed samples is not significantly different from that in wells with single individual samples of the pair ($p>0.05$).

ELISA procedure for detecting REV antigens in various samples. Microtiter plates (Dynatech, Alexandria, VA) were coated with mixture of MCAs 11A25 and 11C237 in dilution of 1:1,000 each in 0.5 M carbonate coating buffer, pH 9.5, 100 ul per well, overnight at room temperature. Plates were washed once with PBS, air dried, and kept at 4^{o}C until use. To detect REV antigen in samples to be tested, 100 ul of plasma or infected CEF culture supernatant with or without PBS dilution were added into wells precoated with MCAs 11A25 and 11C237. The mixture was then incubated for 2 hrs at room temperature. Plates were washed three times with washing buffer (pH 7.2 PBS with 0.1% Tween-80). The adsorbed anti-REV rabbit serum at a dilution of 1:600 in PBS in a volume of 100 ul was added to each well. Incubation was for 1.5-2 hrs at room temperature. To remove unbound rabbit serum, plates were washed three

times with washing buffer. 100 ul of anti-rabbit IgG peroxidase conjugate (Miles Scientific, Neperville, IL) at a dilution of 1:800-1000 in 3% bovine serum albumin (BSA) were added into each well and incubated for another 1.5-2 hrs at room temperature. The plates were washed three times again to remove unbound conjugate, 100 ul of freshly-made substrate (as the above) was added to each well, and the plates were kept for 40-60 min at room temperature. Absorbancies were measured in a ELISA minireader (Dynatech, Alexandria, VA). The reading was adjusted to zero absorbancy with a control well containing substrate only. In each experiment, uninfected chick plasma or CEF supernatant were used as a negative control. The wells were considered positive if absorbency obtained from suspected samples was greater than mean values of uninfected controls plus 2.5 times the stsndard deviation.

Determination of fluorescent antibody focus-forming units (FFU) by FA. Indirect fluorescent antibody (IFA) test was conducted as published previously (Witter et. al.,1970). Infectious units of plasma or infected

CEF culture fluids were expressed in fluorescent antibody focus forming unit (FFU). 35-mm plates with growing CEF cells were inoculated with 2 ml of infected CEF-supernatant or chick plasma diluted from 10^{-1} to 10^{-4} in CEF medium and incubated at 37°C for 2 hr. The inocula were then aspirated from the plates and 2 ml of CEF media with 0.6% agar at 50°C was to cover the CEF monolayer. Plates were incubated for 1 week at 37°C. This was followed by removal of agar gel from plates and the addition of 1 ml of cold alcohol-acetone (4:6) mixture to fix CEF monolayer for 2 min. The alcohol-acetone mixture was poured

off. The cell monolayer was allowed to dry naturally. One ml of MCA 11A25 in PBS at a dilution of 1:400 was added and the plates was incubated for 1 hr at 37C. After washing with PBS, one ml of flourorescein isothiocyanate conjugated anti-mouse IgG (Miles-Yeda Ltd. Kiryat Weizmann, Rehovot, Israel) was diluted 20-fold and incubated for 40 min at 37°C. Plates were washed three times with PBS to remove unbound conjugate. Fluorescent foci were seen with an FA microscope and expressed on the basis of FFU/ml (focus forming unit in a plate times the dilution of the inoculum used).

Complement fixation test. It was conducted according to the procedure published by Smith et al (1977). Infected CEF culture supernatant and chick plasma were also tested. Inactivated (30-60 min at 56°C) and non-inactivated chick plasma samples were tested.

REV-infection in chickens. Chickens of line 7_2 from the Regional Poultry Research Laboratory were infected with 1 ml of strain T-CEF culture supernatant (at an ELISA titer of 1:128) at 1 day of age. Uninoculated chickens were kept and raised in separate isolators as negative controls. Two to three chickens from each group were bled at scheduled intervals. Plasma samples were collected by centrifugation of heparinized whole blood and immediately stored at -70°C. At the same time, spleens, the Bursa and thymus were collected and their weights were recorded respectively.

Detection of congenital shedding of gp62 in albumin of eggs. Eggs were obtained from seven RPRL cross $15I_5 \times 7_1$ hens as 1-day-old embryos infected with REV strain CS (provided kindly by Dr.D.W.Salter). No.1-6 were in viremia during egg-laying time, but No.7 showed temperary viremia only before egg-laying. The negative

control eggs were from SPF flocks (Regional poulry research laboratory). Duplicate samples of 100 ul of albumen were taken from each egg for ELISA.

Determination of the time required for detecting REV antigen in CEF culture fluid after infection with one infectious particle. A set of 35 mm plates were precultured with line O CEF. When monolayer of CEF formed, plates were inoculated with 0.2 ml of REV strain T stock fluid (with ELISA titer of 1:128) in a series ofdilutions. Each dilution of virus stock infected four plates. In Exp.I, a pair of CEF plates infected with each dilution of virus stock were used for collection of culture fluids in the first half period and the medium of another pair of plates was changed at day 5 after infection for fluid collection in the second half period. 200 ul of supernatant were collected everyday from each plate and then 200 ul of fresh medium was supplemented from days 1 through 6 after infection. The fluids of another pair of the plates infected with each dilution of virus stock were collected from day 7 through 12. In Exp.II, the same virus stock was used but experienced one more freezing and thawing, fluids were collected everyday at day 6 through 9 and medium was changed at day 9, fluids were collected at day 18 after infection. All fluid samples were kept in freezer until testing. Each fluid sample was tested in duplicates by ELISA.

Statistics. Student's t test was used for the synergistic ELISA. The relationships between REV FFU and ELISA titers in cell culture supernatants, or between antibody titers and antigen titers in chick sera after infection were analysed by estimated correlations. The effects of REV infection on body weight, the Bursa weight and spleen

weight at different ages were analysed by the unbalanced 2-way analysis of variance (Gill, 1978).

RESULTS

Production and characterization of monoclonal antibodies against REV

Hybridomas secreting MCA against REV. From the initial 3600 hybridomas produced in six separate fusions, 232 were found to be positive in ELISA against REV-CEF or cell-free REV, but not against uninfected CEF. Of these, 176 were secreting MCA equally reactive with both strains T and CS, whereas 56 reacted with strain T but not strain CS. Some hybridomas were selected for cloning and additional study.

Specificities of cloned MCA in ELISA and FA. Table 1 summarizes the ELISA and FA reactivity of 11 cloned MCA with REV. Nine MCA reacted with both strains T and CS. The titers of some MCA to homologous strain T were greater than to heterologous strain CS. MCA 11B118 and 11D78 had similar titers to both strains. MCA 11C100 and 11F667 were strain T specific. Their ascitic fluid antibodies had an ELISA titer of 1 to 8 x 10^5 to strain T, but did not react to strain CS even at a 1/10 dilution (table 1 and Fig.1). Reactivity to CS-CEF was similar to an uninfected CEF control at all tested dilutions of ascitic fluid. Figure 2 shows the reactivity of MCA recognizing type-common antigens. MCA 11D175 reacted with significantly lower titers to infected CEF than to purified virus of both strains (Table 1). The lower titered hybridoma culture supernatant reacted with purified virus but not with REV-CEF(data not shown).

MCA that reacted in high titers with strains T and CS in ELISA also cross-reacted with both strains in FA. Generally, the two stain T-specific MCA, 11C100 and 11F667, reacted with T-CEF but not with

CS-CEF (Table 1).

Epitope specificity of MCA by competitive ELISA test. The results of a series of reciprocal competition experiments are shown in Fig. 3, 3a and 4. MCA 11A25 and 11B118 reacted to similar type-common epitopes with a tiny difference, whereas MCA 11C237 recognized a different epitope. The strain T-specific MCA 11C100 and 11F667 were recognizing closely related or identical epitopes. Some cross-inhibition was observed among MCA 11A3, 11A25, 11B118, and 11D78. However, no inhibitory effect was detected among the remaining four MCA (data not shown). The data from the above experiments indicated the presence of at least three REV epitopes, one type-specific and two type-common.

Immunoprecipitation of REV proteins with MCA. All nine type-common MCA immunoprecipitated a virus protein with molecular weight of 62,000 dalton from [^{35}S]methionine-labeled REV-CEF lysates. In addition to the 62,000 dalton polypeptide, four of these MCA immunoprecipitated a 21,000 dalton protein as well. The viral protein immunoprecipitated by the strain-specific MCA 11C100 and 11F667 produced a broad smear upon polyacrylamide gel electrophoresis (54,000 to 72,000 daltons). Fig. 5 is an autoradiogram from a representative gel electrophoresis of immunoprecipitates obtained with three different antibodies (rabbit anti-REV, MCA 11A25, and MCA 11C100). MCA 11A25 immunoprecipitated two viral-specific proteins (62,000 and 21,000) from T-CEF and CS-CEF. In contrast, MCA 11C100 immunoprecipitated protein (54,000 to 72,000) from T-CEF but not CS-CEF. These data confirm that MCA 11C100 recognizes a strain T-specific epitope, whereas MCA 11A25 recognizes a T/CS strain-common epitope. The rabbit anti-REV immunoprecipitated six viral-specific proteins from both strains T and

Table 1. Titers (Log10)[a] of MCAs in ELISA and FA

MCA	ELISA				Fluorescent Antibody	
	T-CEF[b]	CS-CEF[c]	T[d]	CS[e]	T-CEF	CS-CEF
11A3	5.0	4.7	5.3	4.5	3.4	2.7
11A25	4.7	4.7	5.3	4.4	3.4	2.7
11A301	4.1	3.5	3.5	2.6	2.7	2.0
11B118	5.3	5.3	5.6	5.6	3.4	3.4
11B154	4.7	4.1	4.4	3.2	4.0	2.7
11C100	5.6	<1.0	5.9	<1.0	3.4	<1.0
11C237	5.3	4.7	6.0	3.8	4.0	3.4
11D78	5.9	5.8	5.6	5.3	4.0	4.0
11D175	2.6	2.6	4.2	3.0	<1.0	<1.0
11D182	5.6	3.5	4.7	2.3	3.4	2.0
11F667	5.0	<1.0	5.3	<1.0	3.4	<1.0

[a] ELISA and FA titer are expressed as log of reciprocal endpoint dilution. A titer of less than 1 indicates a negative reactivity. All MCA in ascites fluids were diluted from 1/10 to 1/1,000,000. Several replications were carried out with each MCA.

[b] T-CEF cells.

[c] CS-CEF cells.

[d] Sucrose gradient-purified strain T virus.

[e] Sucrose gradient-purified strain CS virus.

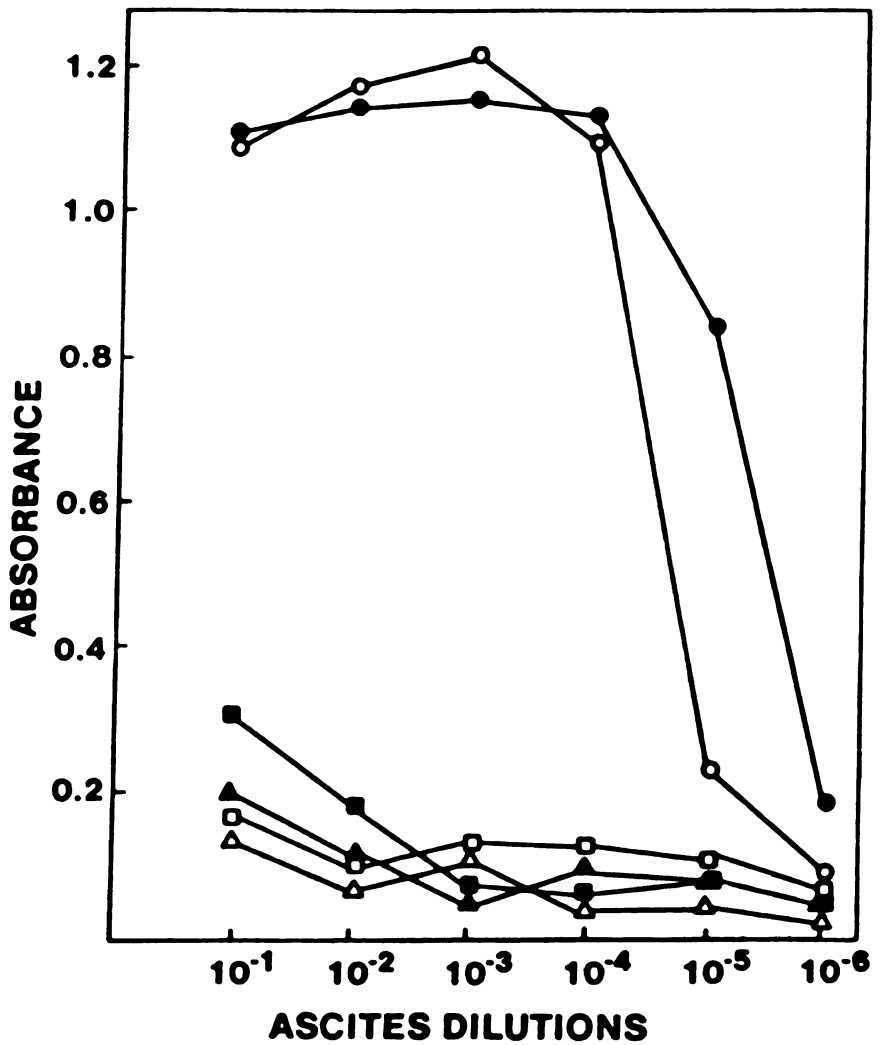


Figure 1. Reactivity of strain-specific MCA 11C100 and 11F667 in ELISA. (○), 11C100 on T-CEF; (△), 11C100 on CS-CEF; (□), 11C100 on uninfected CEF; (●), 11F667 on T-CEF; (▲), 11F667 on CS-CEF; (■), 11F667 on uninfected CEF.

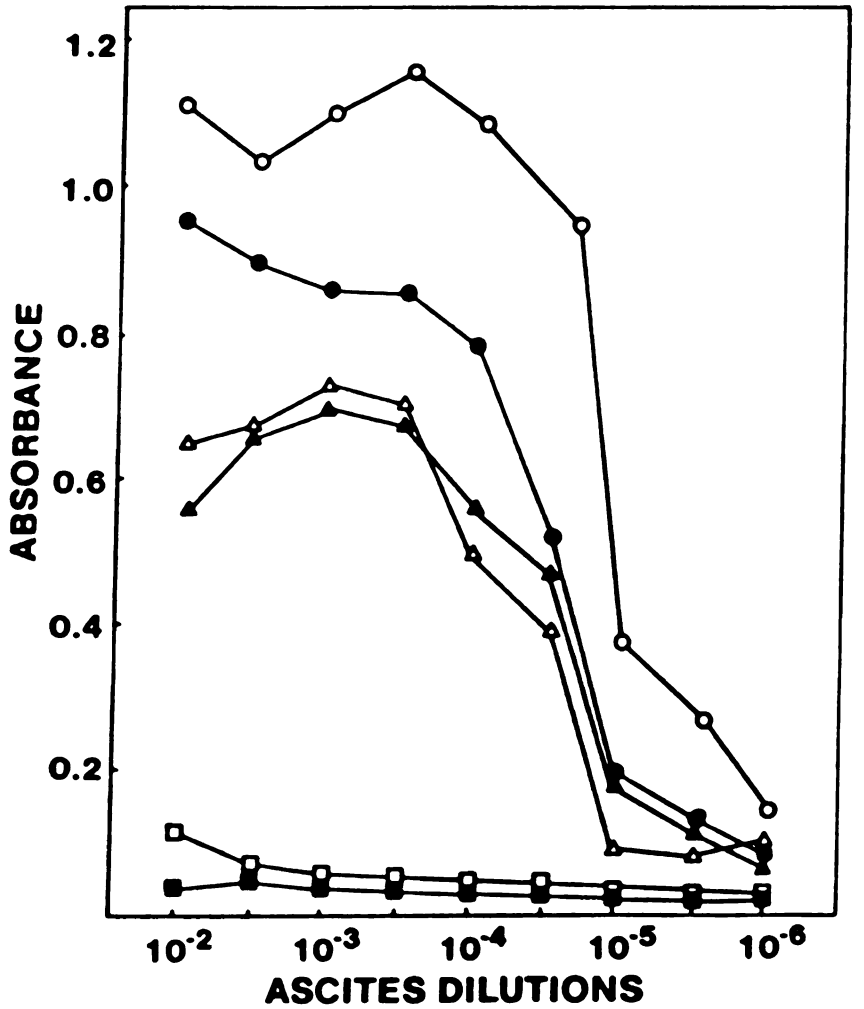


Figure 2. Reactivity of type-common MCA 11C237 and 11A25 in ELISA. (○). 11C237 on T-CEF; (△). 11C237 on CS-CEF; (□). 11C237 on uninfected CEF; (●). 11A25 on T-CEF; (▲) 11A25 on CS-CEF; (■). 11A25 on uninfected CEF.

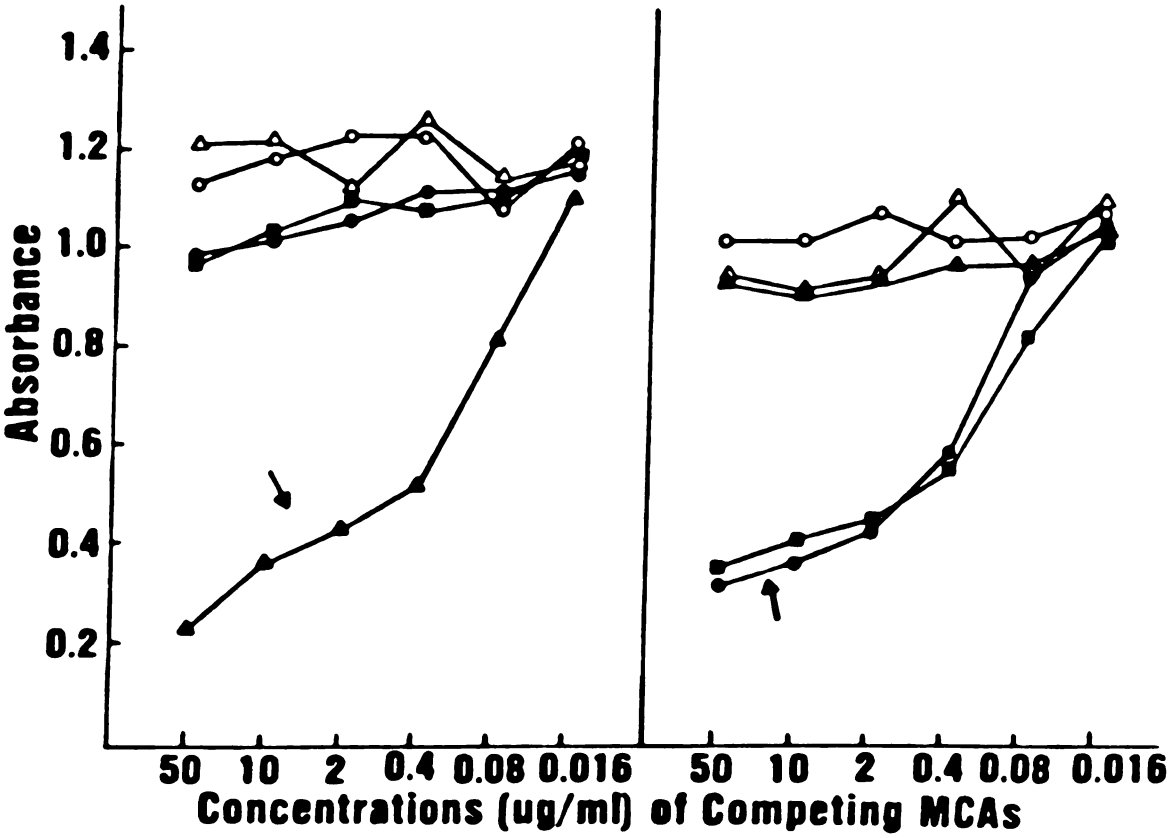


Figure 3. Competitive ELISA immunoassay. Competitive MCA 11A25 (●), 11B118 (■), 11C237 (▲), 11C100 (○), and ascitic fluid from myeloma NS-1 cells (Δ) on type-common MCA 11C237 (*left panel*) or 11A25 (*right panel*) conjugated with horseradish peroxidase. *Arrows* indicate the homologous competing MCA.

Figure 3a. Competitive ELISA immunoassay. Competitive MCA 11A25 (●) and 11B118 (○) on MCA 11A25 (left panel) or MCA 11B118 (right panel) conjugated with horseradish peroxidase. Arrows indicate the homologous competing MCAs.

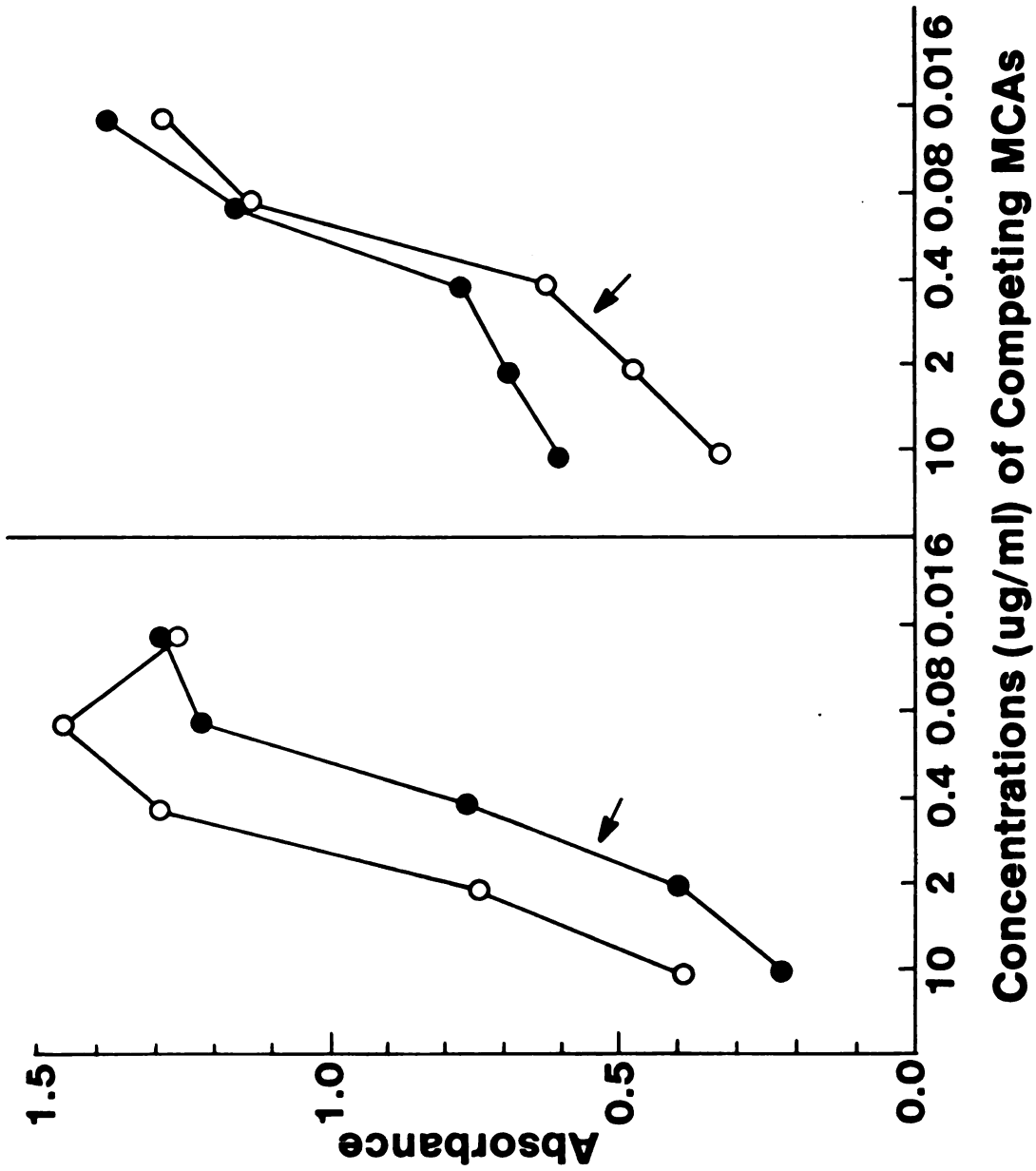

Absorbance
1.5
1.0
0.5
0.0
10 2 0.4 0.08 0.016
Concentrations (ug/ml) of Competing MCAs
10 2 0.4 0.08 0.016

Figure 3a.

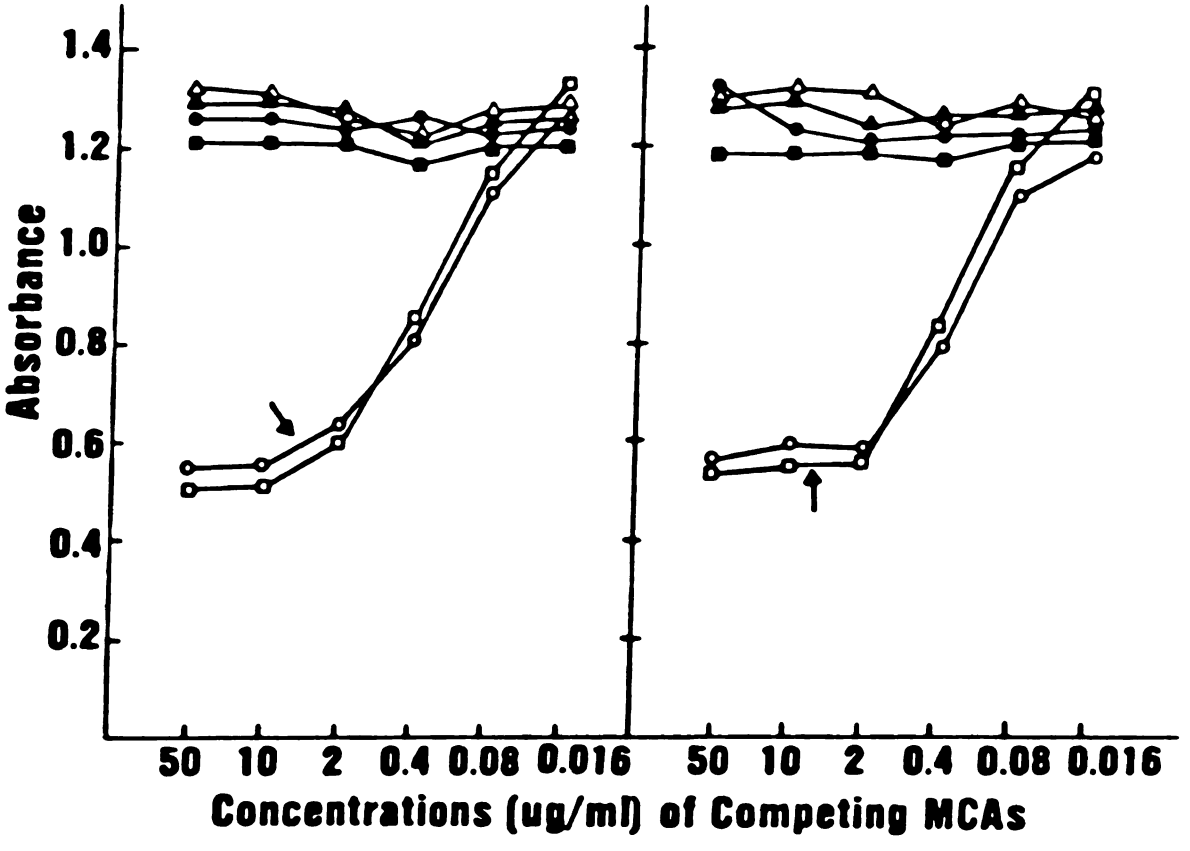


Figure 4. Competitive ELISA immunoassay. Competitive MCA 11A25 (●). 11B118 (■). 11C237 (▲). 11C100 (○). 11F667 (□). and ascitic fluid from myeloma NS-1 cells (Δ) on strain T specific MCA 11C100 (*left panel*) and 11F667 (*right panel*) conjugated with horseradish peroxidase. *Arrows* indicate the homologous competing MCA.

CS, ranging in molecular weight from 21,000 and 62,000. with the polyclonal serum I could not distinguish between the T and CS strains. Table 2 summarizes the MCA reactivity and immunoprecipitation results.

To additionally characterize the three viral polypeptides, REV-CEF was labeled with [^{3}H]glucosamine. As shown in Fig. 6, MCA 11C100 immunoprecipitated a [^{3}H]glucosamine-labeled 54,000 to 72,000 dalton protein. In addition, the 62,000 and 21,000 dalton proteins were also labeled with [^{3}H]glucosamine. Therefore, the three viral polypeptides are glycoproteins. To identify the nonglycosylated precursor polypeptides, T-CEF was labeled when it was being treated with tunicamycin. MCA 11C100 immunoprecipitated a viral polypeptide with a molecular weight of 48,000 from tunicamycin-treated cells, whereas MCA 11A25 immunoprecipitated proteins of 48,000 and 20,000 daltons instead of the 62,000 and 21,000 dalton glycoproteins (Fig.7).

Visualization of the MCA-recognized antigen on the virions using electron microscopy. From Fig.8, numerous gold particles with diameter of about 5 nm were seen surrounding the virus particles in the sample treated with MCA 11A25 against REV, but the same phenomenon was not found for the same virus preparation preincubated with NS-1 cell ascitic fluid as negative contral (Fig.9), indicating that the protein A-gold specifically labeled only MCA-bound virions. The result demostrates that the antigen recognized by MCA 11A25 is located on the surface of virions.

Neutralizing ability of MCA. In preliminary experiment (Table 3, and 4), several MCAs were tested for their in vitro neutralizing ability by ELISA for supernatants and by FA for cell monolayers. Both ELISA and FA gave the same results. Two strain T specific MCAs 11C100

Table 2. Summary in characterization of MCAs

MCA	Source of MCA[a]	ELISA		Immunoprecipitated Polypeptides[b]		Epitope[c]
		T-CEF	CS-CEF	T-CEF	CS-CEF	
11A3	A	+	+	21K, 62K	21K, 62K	ND
11A25	A	+	+	21K, 62K	21K, 62K	B
11A301	T	+	+	62K	62K	ND
11B118	A	+	+	21K, 62K	ND	B
11B154	A	+	+	62K	ND	ND
11C100	A	+	−	54-72K	—	C
11C237	A	+	+	62K	62K	A
11D78	A	+	+	21K, 62K	ND	ND
11D175	T	−[d]	−[d]	62K	ND	ND
11D182	T	+	+	62K	ND	ND
11F667	A	+	−	54-72K	—	C

[a] A = ascites fluid. T = hybridoma tissue culture supernatant.

[b] The size of the immunoprecipitated polypeptides is given in Daltons. ND = not done. − = no viral polypeptides were immunoprecipitated.

[c] The relationships of the different epitopes were determined by competition ELISA assays. Different letters indicate different epitopes. ND = not done.

[d] The hybridoma tissue culture supernatant was negative in an ELISA with T- or CS-infected CEF. However, the supernatant was positive in an ELISA with purified T or CS virus.

Figure 5. Immunoprecipitations of REV-CEF with MCA 11A25, MCA 11C100, and rabbit anti-REV. Immunoprecipitation and electrophoresis were performed as described in Materials and Methods. The arrows indicated virus-specific proteins. Estimation of m.w. was by comparison with ^{14}C-labeled m.w. markers: myosin, 200,000; phosphorylase B, 92,500; bovine serum albumin, 68,000; ovalbumin, 43,000; alpha-chymotrypsinogen, 25,700; beta-lactoglobulin, 18,400. T-CEF (T) and CS-CEF(CS) and uninfected normal CEF (N) were compared.

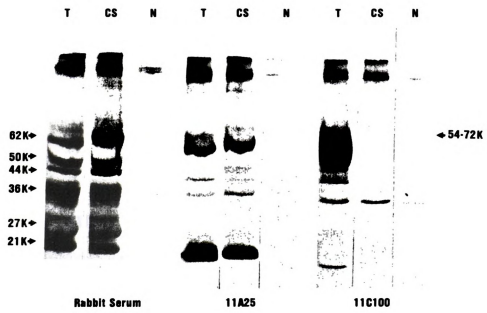

T
CS
N
T
CS
N
T
CS
N
62K
50K
44K
36K
27K
21K
54-72K
Rabbit Serum
11A25
11C100

Figure 5

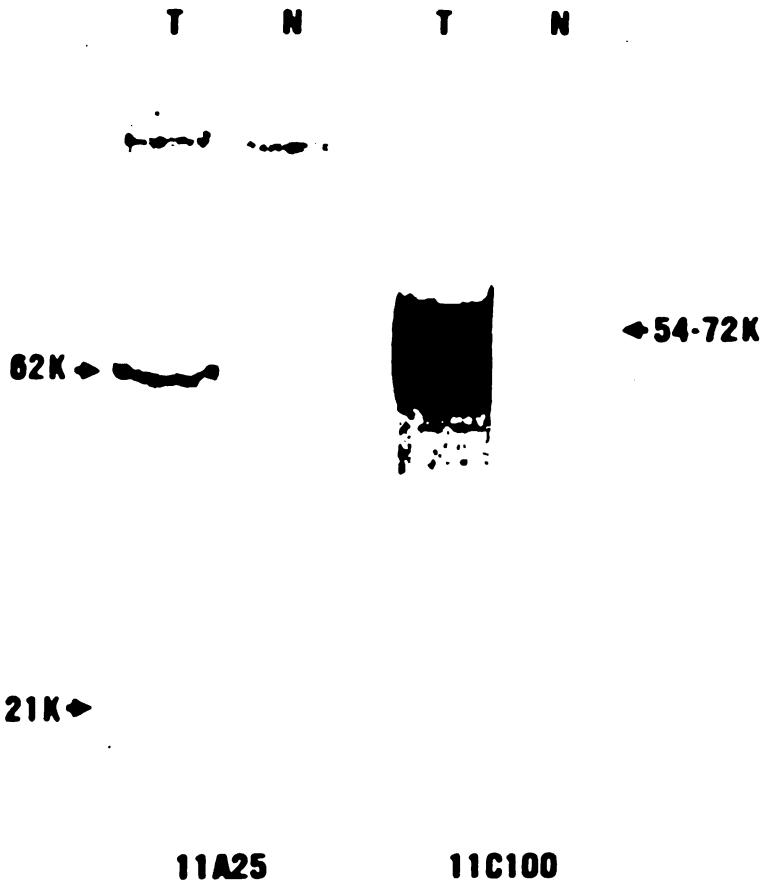


Figure 6. Immunoprecipitation of [^{3}H]glucosamine-labeled polypeptides. T-CEF (*T*) and normal CEF (*N*) were immunoprecipitated with strain T-specific MCA 11C100, and type-common MCA 11A25. The *arrows* indicate virus-specific glycoproteins.

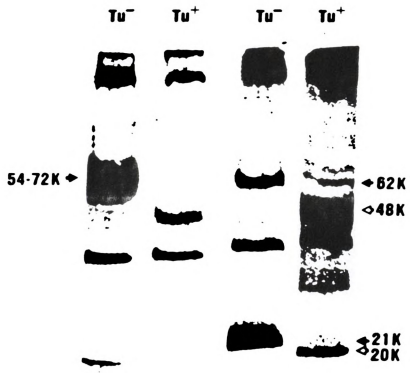


Figure 7. Identification of non-glycosylated precursors. Strain T-infected CEF was pre-incubated for 1 hr in tissue culture medium containing 2 μg/ml of tunicamycin. The medium was replaced with methionine-free medium that also contained 2 μg/ml of tunicamycin and was incubated for another 1 hr. T-CEF was labeled for an additional 6 hr with [^{35}S]methionine in methionine-free medium containing 2 μg/ml of tunicamycin. Both tunicamycin-treated (Tu^+) and non-treated (Tu^-) labeled T-CEF lysates were immunoprecipitated with MCA 11C100 or 11A25 and were electrophoresed in the same gel. Viral glycoproteins are indicated by *solid arrows*, and the precursor proteins of the glycoproteins are indicated by *open arrows*.

Figure 8. Electron microscopic photograph of purified REV strain T virions treated with MCA 11A25 and protein A-gold as described in Materials and Methods. Each virions were surrounded by tiny gold particles in diameter of about 5 nm which are mediated by MCA immunoglobulin and bound to the surface of virion envelopes.

Figure 8.

Figure 9. Electron microscopic photograph of purified REV strain T virions treated with negative control NS-1 cell asitic fluid and protein A-gold. No gold particles were found arround the virions.

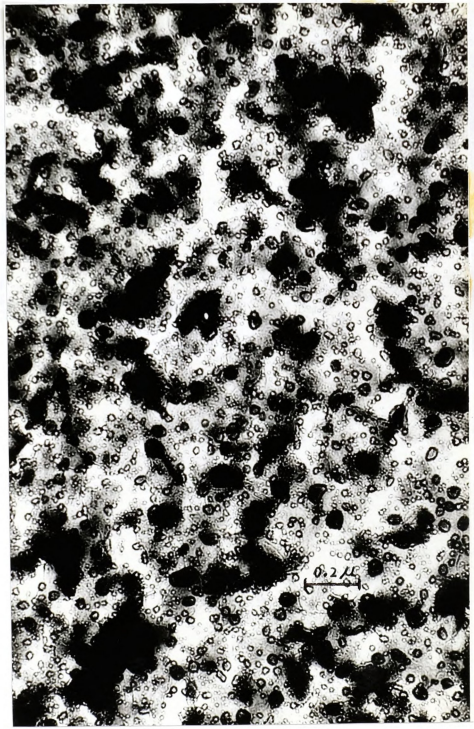


Figure 9.

Table 3. ELISA readings in CEF supernatants 9 days after infection with mixture of diluted virus stock and MCAs for neutralization test

MCA	reciprocals of dilutions of the virus stock (10 x)										
	8	16	32	64	128	256	512	1,024	2,048	4,096	8,192
11C100	.91	.93	.98	.94	.64	.48	.09	.21	.11	.20	-*
11C237	.61	.39	.52	.44	.45	.56	.53	.39	.44	.48	.09
11D78	.86	.83	.82	.84	.85	.90	.84	.96	.80	.99	.80
11F667	.63	.05	.13	.04	.09	.05	.03	.01	.02	.00	.05
11A25	.81	.78	.74	.73	.79	.75	.79	.79	.80	.78	.75
11A3	.75	.76	.78	.77	.85	.83	.90	.86	.82	.83	.68
11B118	.77	.73	.78	.79	.81	.82	.77	.78	.71	.82	.83
NS-1	.77	.78	.79	.84	.74	.80	.80	.74	.74	.66	.66

Readings less than 0.25 were judged as negative for virus antigen.
* the well added with only substrate but no conjugate for blocking the plate.

Table 4. FA results in CEF monolayers 9 days after infection with mixture of diluted virus stock and MCAs in neutralization test

MCA	reciprocal of dilutions of virus stock (10 x)										
	8	16	32	64	128	256	512	1,024	2,048	4,096	8192
11C100	+	+	+	+	+	+	-	-	-	-	-
11C237	+	+	+	+	+	+	+	+	+	+	-
11D78	+	+	+	+	+	+	+	+	+	+	+
11F667	+	-	-	-	-	-	-	-	-	-	-
11A25	+	+	+	+	+	+	+	+	+	+	+
11A3	+	+	+	+	+	+	+	+	+	+	+
11B118	+	+	+	+	+	+	+	+	+	+	+
NS-1	+	+	+	+	+	+	+	+	+	+	+

"+" positive in specific virus plaques
"-" negative in specific virus plaques

Table 5. ELISA readings in CEF supernatants 9 days after infection with mixtures of diluted virus stock and MCAs in neutralization test

MCA	reciprocals of dilutions of virus stock (10 x)										
	2	4	8	16	32	64	128	256	512	1,024	2,048
11C100	1.03	1.08	1.00	1.03	.95	.32	.21	.10	.10	.10	-*
11F667	.74	.12	.10	.10	.10	.11	.10	.11	.10	.10	.10
11E258	1.04	1.08	1.04	1 .01	1.06	1.06	.86	.70	.70	.30	.20
11E197	.98	.92	.80	.58	.28	.16	.16	.13	.10	.10	.10
11F307	1.10	1.07	1.10	1.08	1.09	1.11	1.07	1.03	1.06	.95	1.04
13A208	1.08	1.06	1.10	1.06	1.09	1.06	1.09	1.02	.92	.85	.76
NS-1	1.08	1.09	1.09	1.08	1.10	1.07	1.10	.94	1.03	.85	.98

* the well added with only substrate but no conjugate for blocking the plate.
The readings are averages of two duplicated plates.
The readings less than 0.25 were judged as negative.

Table 6. ELISA readings in CEF supernatants 6 days after infection with mixtures of diluted virus stock and MCAs in neutralizaton test

MCA	reciprocals of dilutions of virus stock (10 x)										
	2	4	8	16	32	64	128	256	512	1,024	2,048
11C100	.49	.42	.23	.21	.19	.13	.12	.12	.11	.11	-*
11F667	.15	.13	.11	.11	.11	.12	.10	.10	.10	.10	.14
11E258	.89	.68	.65	.41	.35	.23	.16	.11	.14	.11	.14
11E197	.28	.21	.16	.14	.13	.12	.10	.11	.11	.11	.11
11F307	1.11	1.12	1.10	1.08	1.00	.92	.88	.56	.41	.34	.25
13A208	1.09	1.12	1.07	.98	.88	.61	.34	.36	.17	.18	.23
NS-1	1.11	1.12	1.10	1.06	.98	.72	.40	.35	.28	.22	.25

* the well added with only substrate but no conjugate for blocking the plate.
The readings are averages of two duplicated plates.
The readings less than o.25 were judged as negative.

Table 7. Relative efficiency of virus-neutralizing ability of MCAs

MCAs	tested 9 days after infection		tested 6 days after infection	
	neutralization effects			
	index[a]	%[b]	index	%
11C100	>32	>96.8	64	98.4
11F667	>1,000	>99.9	>216	>99.5
11E258	>2	>50	8	87.5
11E197	>128	>99.2	216	99.5
11F307	-[c]	0	-	0
13A208	-	0	-	0
11C237	>2	>50	NT[d]	
11D78	-	0	NT	
11A25	-	0	NT	
11A3	-	0	NT	
11B118	-	0	NT	

a: neutralization index was expressed by the ratios of virus dilutions mixted with specific MCA ascitic fluids compared to virus dilutions of mixed with nonspecific NS-1 cell ascitic fluids at the end points detected in ELISA.

b: percentage of virus particles neutralized by MCAs in the mixtures, it equals to (1 - reciprocal of neutralization index) x 100%.

c: negative in neutralization effect.

d: not tested.

and 11F667 showed up a strong neutralizing ability, they could inhibit completely REV-specific plaques in infected cell monolayers or ELISA readings of culture supernatants at virus stock dilutions of 1:5120 (for 11C100) or 1:160 (for 11F667) when negative control was still strongly positive at 1:80,000. Except 11C237, none of group-common MCAs 11A3, 11A25, 11B118, and 11D78 demonstrated any neutralizing ability. MCA 11C237 showed up a very tiny neutralization effect at the virus dilution of 1:80,000. In the further experiment, only ELISA for supernatants was used to test additional MCAs for their neutralizing ability. Two additional group-common MCAs 11E32 and 11E197 appeared to be strongly positive in their neutralizing ability, other two group common MCAs 11F307 and 13A208 showed no neutralizing ability. Two strain T-specific MCAs 11C100 and 11F667 were proved again to be positive, but another strain T-specific MCA 11E258 only had a tiny neutralization effect (Table 4,5,6). The relative extensity of each MCAs in neutralizing ability were summarized in Table 7.

Developing a synergistic ELISA for identification of epitope-specificities of MCA against REV

Grouping of the well identified MCAs against REV by using the synergistic ELISA. Five well identified MCAs against REV in the forms of both culture supernatants or ascitic fluids were tested with different number of duplicates in plates precoated with purified REV or REV-CEF respectively. As a example, Table 8 shows how to organize the experiments and the original mean values with standard errors ($\bar{Y}\pm SE$) of

6-well-duplicates in ELISA readings to each single samples or mixtures of each pair. The reading in the square located in the row and column of 11A25, for example, represents the mean value in ELISA of wells added with only sample 11A25; the reading in the square located in row 11A25 and column 11B118 or in row 11B118 and column 11A25 is the mean value in ELISA of wells with the mixture of samples 11A25 and 11B118, and so on. Table 9 shows how to judge the synergistic effects of mixture of different MCA samples by statistical analysis. The mean values of mixed samples (Y_m) and single samples (Y_s) in each pair were compared, t and p values in student's t test were also listed in the Table 9. ELISA readings of the mixture of MCAs 11C100 and 11F667 was not higher than that of each single sample of the pair indicating there was no synergistic effect between two MCAs and they may have the same or very closedly related epitope-specificity. ELISA readings of all other mixture were significantly higher than that of the single samles of each pair ($p<0.05$), indicating that the two MCAs with synergistic effect in ELISA on each other were against different epitopes on the virus protein. The results proved that MCAs 11C100 and 11F667 belong to the same group and other MCAs to other three different groups. The same five MCA samples were repeatedly tested in the forms of culture fluids or ascitic fluids against REV-CEF or purified REV in different numbers of duplicates respectively. The synergistic ELISA seperately carried out demonstrated the almost same results in grouping their epitope-specificities (Table 10), but a lillte large number of duplicates were needed to show the significant difference between 11A25 and 11B118 when they were tested in the form of ascitic fluids. MCAs 11C100 and 11F667 appear to be against the same antigenic determinant

specific to REV strain T, their mixture did not show any synergistic effect compared to each single sample of them in all seperated experiment (1 through 6) even 96 duplicates were used for single or mixed samples. It seems like that MCAs 11A25 and 11B118 are against different epitopes but there is some relationship between them. MCA 11C237 has its own epitope-specificity.

comparing rusults of synergistic and competitive ELISAs for grouping epitope-specificities of MCAs. The synergistic ELISA was compared to the classical competitive ELISA for identifying epitope-specificities of MCA. The results in epitope-grouping MCA by using synergistic ELISA was quite coincident with that depending on competitive ELISA (Table 11), the 5 MCAs were devided into 4 epitope-groups. As indicated above by synergistic ELISA (Table 10), MCA 11A25 and 11B118 were also against different but related antigenic determinants. The relatioship of them was also proved by the mutual competitive ELISA. Fig.3 and 3a demonstrates that MCA 11A25 and 11B118 inhibited each other's enzyme-conjugate in ELISA, but inhibition was stronger to homologous conjugates than to heterogeneous conjugates.

Grouping some more MCAs against REV by the synergistic ELISA for their epitope-specificity. One more strain T specific MCA 11E258 was compared with other two strain T specific MCAs 11C100 and 11F667 for their epitope-specificity by sELISA. The result showed that MCA 11E258 had its own epitope-specificity different from the other two. In the same assay, MCAs 11C100 and 11F667 still proved to be against a very close related epitope (Table 12). Another set of assay indicated that 4 more REV group cross-reactive MCAs 11E32, 11E197, 11F307 and 13A208 had their own epitope-specificities different from each other and from

Table 8. Mean values ($\bar{Y}\pm$s.e.) of individual MCA samples and their mixures of pairs (absorbancy in ELISA readings)

	11A25	11B118	11C237	11C100	11F667
11A25	.987±.029	1.33±.046	1.20±.021	1.45±.034	1.36±.039
11B118	1.36±.053	1.07±.025	1.46±.023	1.30±.031	1.20±.035
11C237	1.19±.053	1.39±.046	1.14±.063	1.62±.038	1.39±.038
11C100	1.34±.029	1.17±.041	1.31±.060	1.18±.073	1.04±.054
11F667	1.37±.073	1.19±.034	1.36±.029	1.19±.082	1.09±.047

s.e.: standard error of mean values

Hybridoma culture supernatants of each samples in 6 duplicates (in each square) were tested in plates coated with T-CEF.

Table 9. Synergistic effects of mixtures of different individual MCA samples on ELISA

		11B118	11C237	11C100	11F667
	m	1.342	1.193	1.395	1.368
	s	1.037	1.066	1.082	1.038
11A25	m - s	.312	.128	.313	.330
	t	7.68	2.59	5.72	8.42
	p	<0.01	<0.05	<0.01	<0.01
	m		1.427	1.232	1.194
	s		1.109	1.125	1.082
11B118	m - s		.318	.107	.113
	t		7.3	2.08	3.27
	p		<0.01	<0.05	<0.05
	m			1.462	1.376
	s			1.161	1.118
11C237	m - s			.301	.258
	t			4.05	5.74
	p			<0.01	<0.01
	m				1.114
	s				1.133
11C100	m - s				-.019[a]
	t				_[a]
	p				_[a]

a: the mean values of mixed samples was less than that of the single samples, so there was no synergistic effect on ELISA readings between MCAs 11C100 and 11F667 indicating that they have a similar or very closely related epitope-specificity.

m and s : mean values of ELISA readings in wells with mixed samples or single individual samples.

Hybridoma culture supernatants in 6 duplicates were tested in plates coated with T-CEF. The degree of freedom for each comparison is 22.

Table 10. Comparing the results of epitope-grouping of 5 MCA samples in different synergistic experiments

Exp.	Ag-Ab system		N in	epitop-grouping				
	antigen	antibody	Exp.	11A25	11B118	11C237	11C100	11F667
1	T-CEF	super	24	IV	III	II	I	I
2	T-CEF	super	84	IV	III	II	I	I
3	p-REV	super	84	IV	III	II	I	I
4	p-REV	super	192	NT	NT	NT	I	I
5	T-CEF	ascites	84	III	III	II	I	I
6	p-REV	ascites	84	III	III	II	I	I
7	p-REV	ascites	192	IV	III	NT	NT	NT

T-CEF: strain T-infected CEF
p-REV: purified REV

Table 11. Comparisons of grouping epitope-specificities of MCAs with cELISA and sELISA

	sELISA	cELISA
11A25	IV	B2*
11B118	III	B1*
11C237	II	A
11C100	I	C
11F667	I	C

* MCAs 11A and 11B118 were grouped into epitope group B when two MCAs were compared in cELISA with only enzyme-conjugated MCA 11A25. But they showed up differences in their epitope-specificity when two MCAs were mutually compared in the cELISA (Fig.3a).

Table 12. Comparisons of strain T-specific MCAs in their epitope-specificity

		11C100	11F667
	m	0.657	
	s	0.665	
11F667	m-s	-0.008[a]	
	t	_[a]	
	p	_[a]	
	m	0.456	0.628
	s	0.420	0.585
11E258	m-s	0.036	0.043
	t	2.63	3.53
	p	<0.05	<0.01

m: mean values of ELISA readings in wells with mixed samples
s: mean values of ELISA readings in wells with single individual samples
a: m<s indicates no synergistic effects between two samples, random variation could cause tiny negative or positive differences between m and s.
Each MCA sample in the form of ascitic fluids were tested in 21 duplicates in plates coated with T-CEF. The total degree of freedom is 82.

Table 13. Summary of sELISA for comparing some more REV group-common MCAs in their epitope-specificity

	11A25	11C237	11E197	11F307	13A208
11A25	.262±.029*	.271±.074	.354±.042	.325±.049	.256±.037
11C237	.241±.060	.168±.020	.258±.021	.245±.033	.198±.018
11E197	.325±.027	.274±.073	.251±.024	.339±.064	.291±.044
11F307	.291±.031	.245±.069	.349±.093	.210±.038	.271±.050
13A208	.248±.063	.197±.048	.281±.069	.253±.074	.158±.022

* each represents mean values of 16 wells in ELISA readings and their standard errors. MCA samples in the form of ascitic fluids were tested in plates coated with T-CEF.
The differece between two means of wells with single individual MCA samples and mixed samples respectively was ananyzed by student t test for each comparison of a pair separately. The statistical results indicated that all samples were against different independent epitopes on the REV particles.

11A25, 11B118, 11C237 (Table 13).

Developing a MCA-mediated ELISA to directly detect REV antigens in various kinds of samples.

Optimization of antibody concentration. MCAs 11A25 and 11C237, each recognizing a different epitope (Table 2), were used in a combination to coat ELISA plates to enhance the sensitivity of detection. In block titrations, both reacted in an ELISA assay with cell-free virus and REV-infected cells. Titration curves of MCAs 11A25 and 11C237 against strain T-infected CEF and control CEF in ELISA were shown in Fig.10. MCAs 11A25 and 11C237 reacted to a titer of 1.28 x 10^5 against REV-infected cells and did not react nonspecifically against uninfected CEF. These two MCAs were chosen for coating ELISA plates on the basis of their synergestic effect in ELISA. Similarly the specificity and titer of absorbed anti-REV rabbit serum were also determined, the endpoint titer of the adsorbed REV serum was at about 1:5000 in the presence of 0.2 ug of REV protein (Fig. 11).

Specificity of ELISA for detection of REV antigen. The specificity of ELISA for detection of REV antigen is shown in Fig.12. Supernatant from REV-, Marek's disease virus-infected CEF, avian lymphoid leukosis virus -infected CEF and normal CEF cultures were used for testing. All three REV strains T, CS and DIA were highly positive, but supernatant from MDV- and LLV-infected CEF and normal CEF culture were negative.

Other REV strains tested, include SNV (Trager, 1959), MN81 and MN67

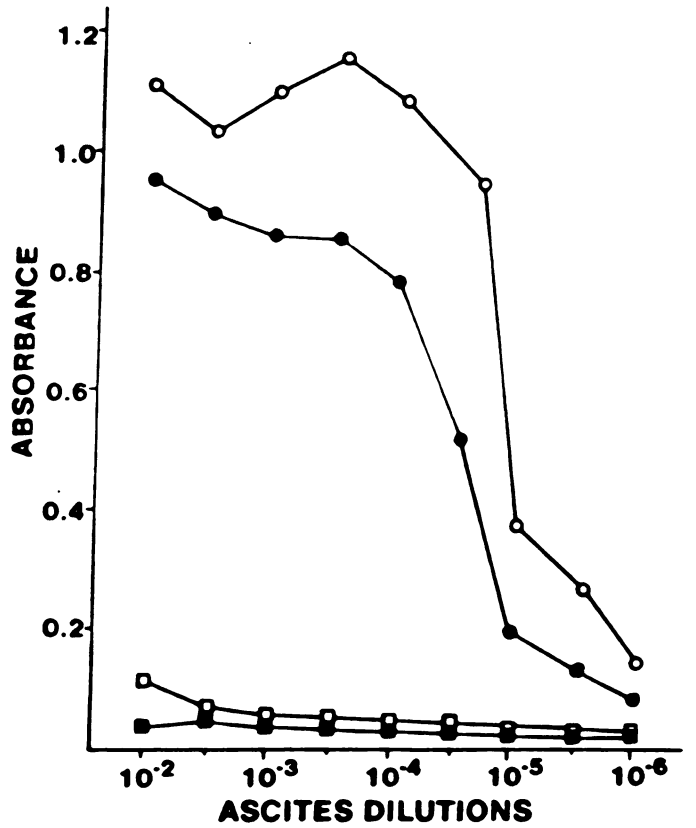


Fig. 10. ELISA titers of MCA 11A25 in infected (O) and control (□) CEFs and of MCA 11C237 in infected (●) and control (■) CEFs.

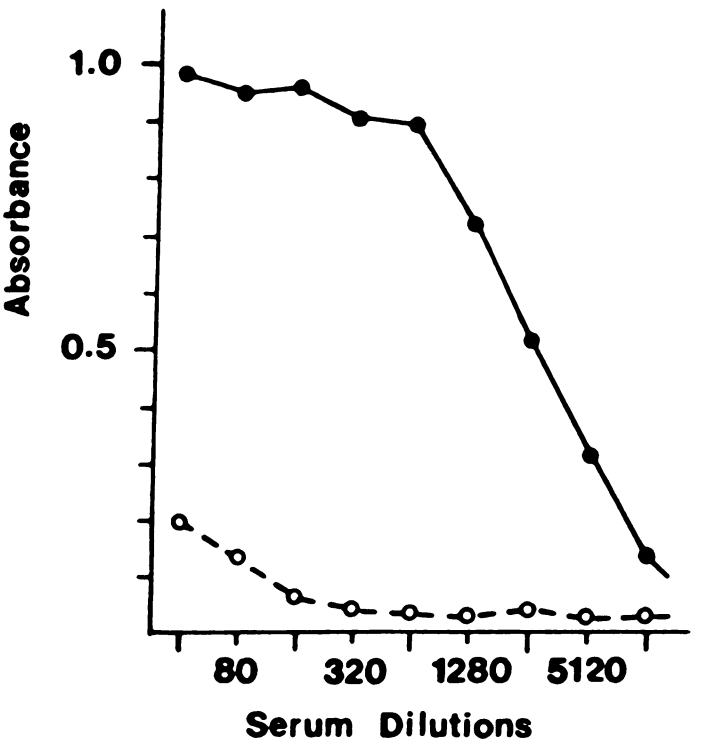
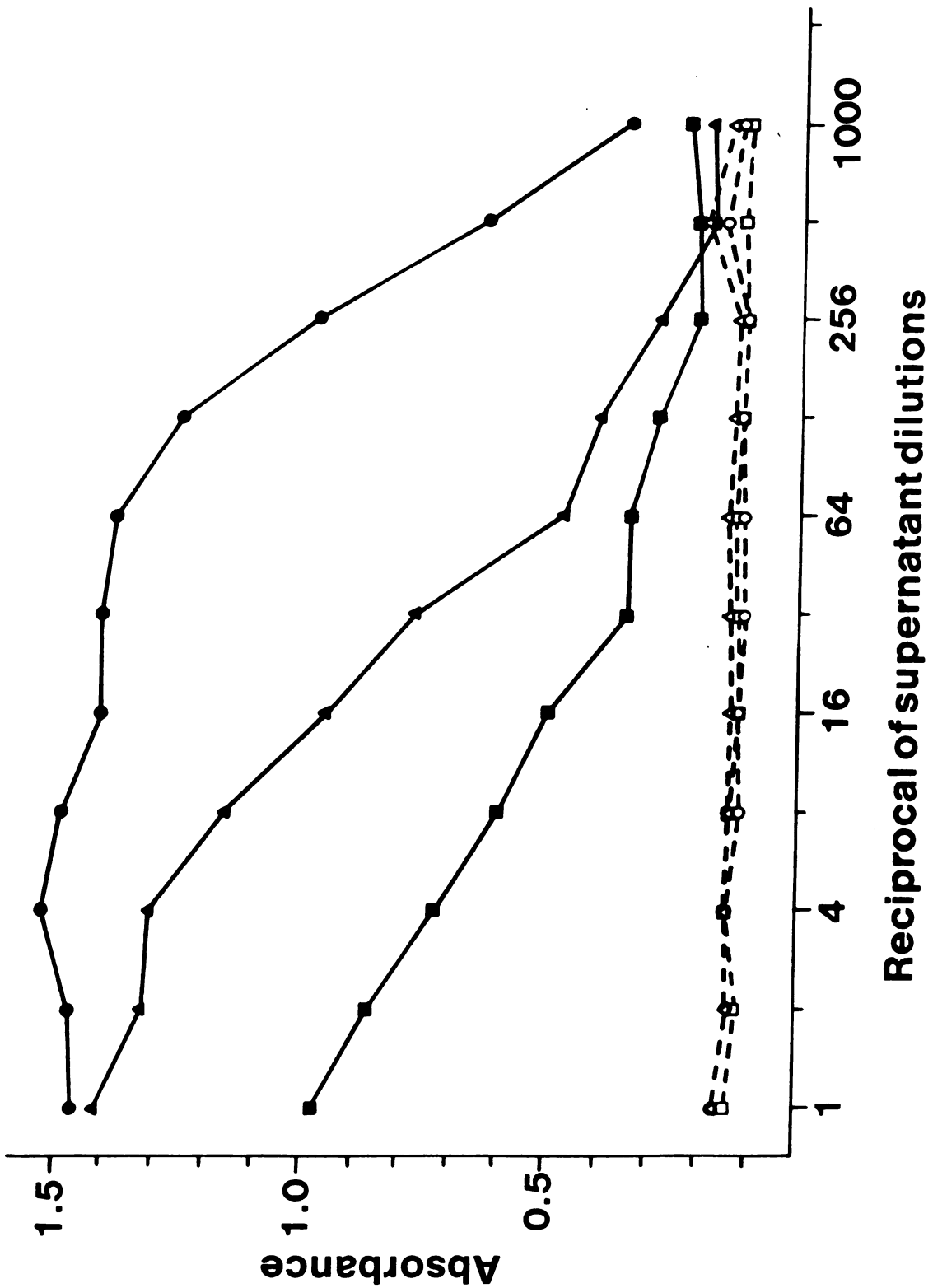


Fig. 11. Adsorbed rabbit anti-REV serum in plates precoated with strain T-infected CEFs (●) or normal CEFs (○). Wells were coated with 5×10^4 cells.

Figure 12. Specificity and sensitivity of ELISA in supernatants of infected CEF cultures with REV strains T (●), CS (▲), and DIA (■). Supernatant samples of ALV- (△) and MDV-infected CEF (□) and normal CEF (○) antigen served as negative controls.


Absorbance
1.5
1.0
0.5
1
4
16
64
256
1000
Reciprocal of supernatant dilutions

(Paul, 1977), were also positively reactive (data not shown).

Comparative sensitivities of ELISA and CF. Sensitivity limits for the ELISA and CF were compared using sucrose- gradient purified virus preparations of three REV strains T, CS and DIA. Table 14 shows that each test gave a very similar sensitivity level to different REV strains. ELISA was about 40-80 times more sensitive than CF.

Sensitivities of ELISA and CF for detection of REV antigen were also compared using REV-infected- culture fluid and chick plasma samples (Table 15). All 21 culture fluid samples were positive in ELISA but only 17 were positive in CF, the ELISA titers were significantly higher than CF titers in the same samples. However, when infected chicken plasma smaples were tested in both assays, all eight infected samples were positive in ELISA with titer from 1:8-1,000. Antigens in sera was not detected by direct CF test. The uninfected samples from either culture fluid or plasma were negative in ELISA and CF as expected. Fig.13 represents the ELISA titration curves of these samples. As shown, plasma from uninfected chicken has no specific reactivity (maximum absorbancy of 0.15), and plasma from REV-infected chickens showed antigen titers as high as 256. Theses results suggest that ELISA has the specificity and sensitivity to detect REV antigen directly in plasma samples. CF results for these same plasma samples were negative.

Comparing ELISA titers to VIF. Table 16 shows ELISA and VIF detection of REV antigen in culture fluid and plasma. For cell-culture antigen, about 50-500 FFUs were required for detection by ELISA. Table 17 demonstrated some more data about correlations of REV titers determined by ELISA and VIF in cell culture fluids. For antigen in plasmas, ELISA endpoint titers ranged from 64 to 1,000 with the

Table 14. Sensitivities of ELISA and CF in detecting purified REVs.[A]

Strain	ELISA	CF	Ratio (CF to ELISA)
T	0.08[B]	3.2	40
CS	0.16	6.4	40
DIA	0.16	12.5	78

[A]Viruses were purified by sucrose gradient, and viral protein concentrations were measured as described in Materials and Methods.

[B]Microgram viral protein per ml.

Table 15. Comparison between ELISA and CF in REV detection.

Virus	Material[A]	ELISA		CF	
		+/total	Range[B]	+/total	Range[B]
REV	Cell culture	21/21	*8–512*	17/21	*2–16*
None	Cell culture	0/21		0/21	
REV	Plasma	8/8	*8–1000*	0/8	
None	Plasma	0/8		0/8	

[A]Samples of cell-culture fluids were collected 5 to 25 days after infection: strain T culture fluid (9 samples), CS (7 samples), and DIA (5 samples). Plasma was obtained from chicks 8–36 days after infection at 1 day with strain T. Samples were tested in both ELISA and CF.

[B]Range titers were expressed as reciprocal of endpoint dilutions.

Figure 13. REV gp62 in chick plasma collected at 7 (●) and 21 (▲) days after infection at 1 day old. Uninfected chick plasma (○) was used as control.

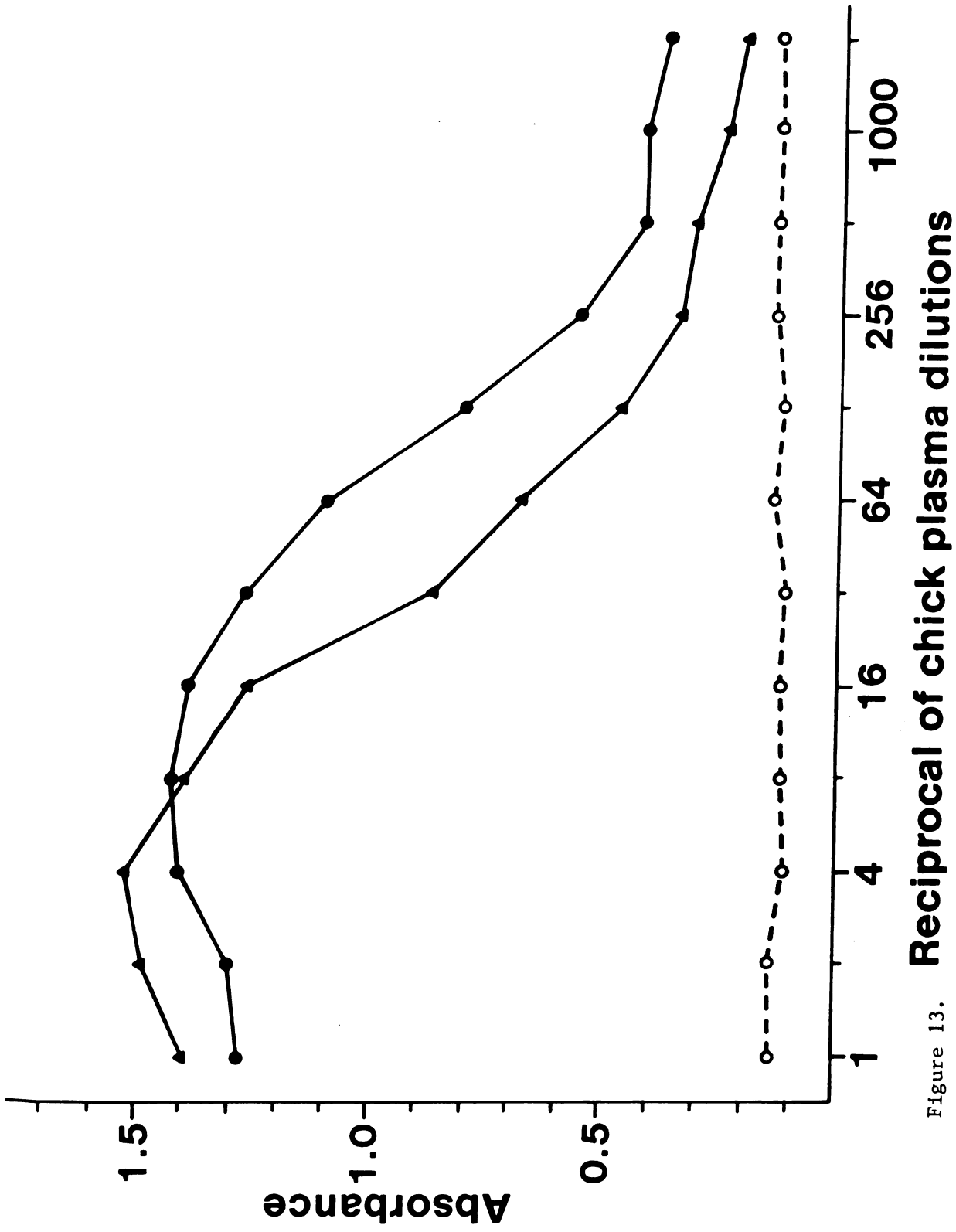

Absorbance
1.5
1.0
0.5
1
4
16
64
256
1000
Reciprocal of chick plasma dilutions

Figure 13.

Table 16. Correlation between VIF and ELISA in REV detection.

Sample no.	Sample type	VIF[A]	ELISA[B]
1	Cell culture[C]	3000	64
2	Cell culture	25,000	49
3	Cell culture	53,000	200
4	Cell culture	70,000	200
5	Cell culture	98,000	490
1	Plasma[D]	230	128
2	Plasma	7.5	64
3	Plasma	0.5	256
4	Plasma	0.5	1000
5	Plasma	0.5	256

[A]VIF values are expressed in immunofluorescent foci, focus-forming units (FFU) per ml.

[B]ELISA titers are expressed as reciprocal of endpoint dilutions.

[C]Culture fluid samples 1 and 2 were collected at days 3 and 17 after infection of CEFs with REV strain T; samples 3, 4, and 5 were collected 6, 7, and 8 days, respectively, after infection with the same virus in tissue-culture plates.

[D]Plasma samples were obtained from chicks at day 7 (Nos. 1 and 2), day 14 (Nos. 3 and 4), and day 21 (No. 5) after infection with REV strain T.

Table 17. Comparisons of ELISA titers and FFU of virus particles in supernatants of CEF culture infected with REV strain T

samples	FFU/100ul	ELISA titer	ratios
1	300	64	4.7
2	2,500	49	51
3	5,300	200	26.5
4	7,000	200	35
5	9,800	490	20
6	1,120	80	14
7	2,720	160	17
8	5,520	160	34.5
9	720	160	4.5
10	5,600	320	17.5
11	480	160	3
12	1,440	160	9
13	40	0	
14	3,750	80	46.9
15	8,000	160	50
16	85	0	
17	2,000	80	25
18	1,250	320	3.9
19	210	0	
20	9,000	160	56.3
21	360	320	1.1

REV strain T infected CEF culture supernatant samples were collected randomly from different batches of cultures and at different days after infection. Samples were frozen as soon as possible after collection and experienced freezing and thawing only once befor testing. There is some correlations (the 95% confidence interval on correlation was about 0.13 to 0.78) between FFU and ELISA titers.

corresponding VIF readings ranged from 0.5 to 230 FFUs. Therefore, ELISA seems to be as sensitive as VIF for detecting REV antigen in plasmas. But ELISA titers of infected chick plasma were not proportional to FFU as that of culture fluids. FFU in plasma was decreased dramatically with age after infection, but ELISA reactivity kept much longer at high titer. (See discussion for explanation).

ELISA titer of REV in culture fluids was more stable than FFU. When a collected fluid sample was kept for 1 and 7 hr at 37°C, ELISA titers were as high as fresh sample (1:128), but FFU decreased from 9.8 X 10^5/ml of fresh sample to 4 X 10^5/ml and 2 X 10^5/ml after being kept for 1 and 7 hr respectively.

Detection of REV antigen in egg albumen from infected hens. Fig.14 indicates that ELISA could effectively detect REV gp62 antigen in egg albumen. All 24 eggs from 6 infected-hens with viremia gave a very strong positive reaction. In contrast, all 17 eggs from 7 SPF hens gave very low ELISA readings. 11 of 12 eggs from infected hen No.7 without viremia were also negative like that from SPF hens except 1 with very weak reaction.

The duration needed for detection of REV antigen in culture fluid after infection with one infectious REV particle. The experiments were conducted to determine how long it will take to detect REV antigen in cell culture after infection with only one infectious unit (Table 18). In Trial I, REV antigen could be detected in fluid by ELISA from day 7 after infection in one of two duplicate culture plates infected with 0.2 ml of virus stock in dilution of 1:2.56 X 10^6 but could not be detected in another duplicate plate in the same pair even at day 12 after infection. In Trial II, REV antigen was detected in one plate at

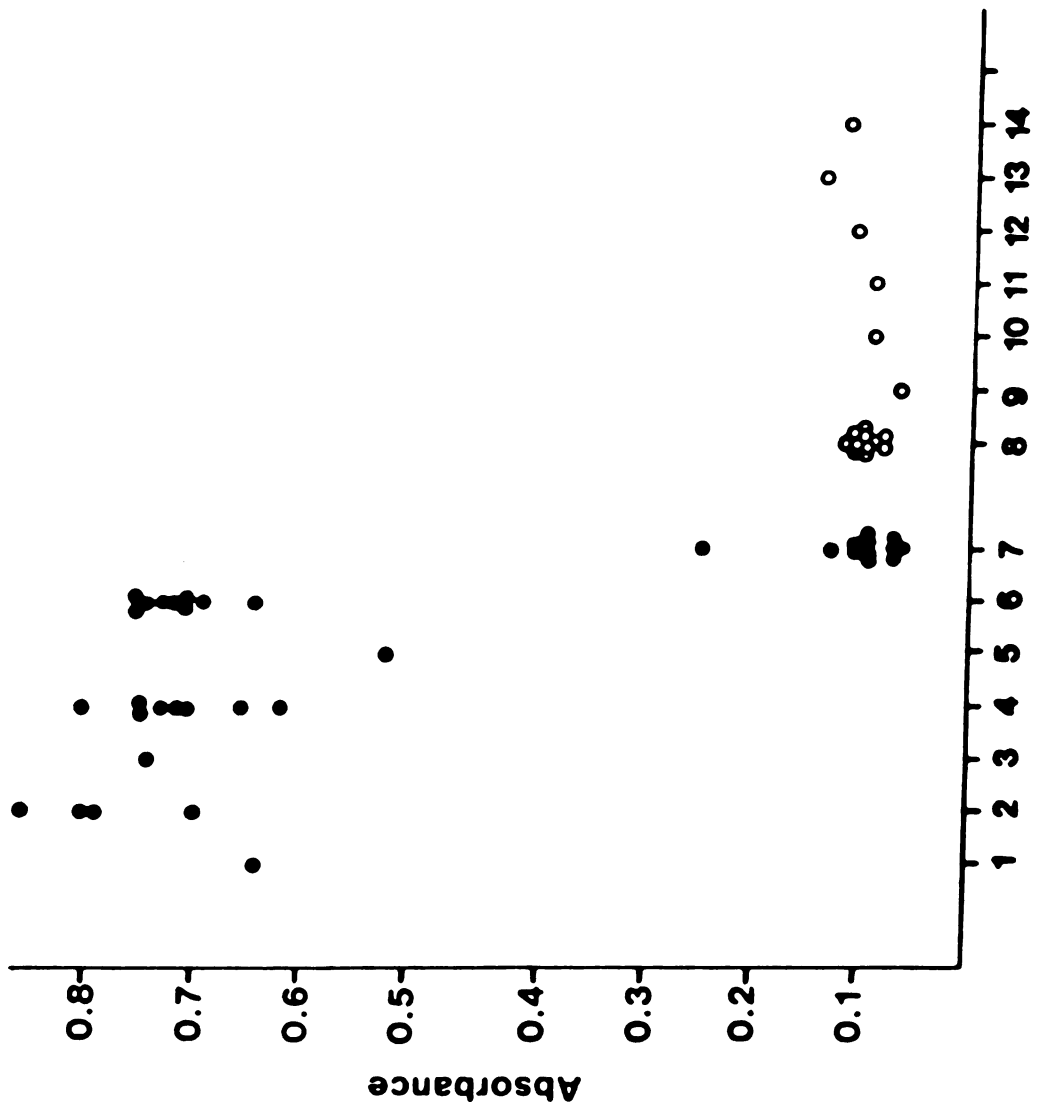


Fig. 14. Detection of REV gp62 in egg albumen from hens infected with strain CS (●) and from uninfected hens (O).

Table 18. ELISA for detection of one infectious REV particle.[A]

Trial	Virus dilution	Days after infection								
		5	6	7	8	9	10	11	12	18
1	8.0×10^{-4}	–	+	+	+	+	+	+	+	+
	1.6×10^{-5}	–	+	+	+	+	+	+	+	+
	3.2×10^{-5}	–	+	+	+	+	+	+	+	+
	6.4×10^{-5}	–	–	+	+	+	+	+	+	+
	1.3×10^{-6}	–	–	+	+	+	+	+	+	+
	2.6×10^{-6}	–	–	+/–[B]	+/–	+/–	+/–	+/–	+/–	+/–
2	10^{-4}	+	+	+	+	+	+	+	+	+
	10^{-5}	–	–	–	+/–	+/–	+/–	+/–	+/–	+/–
	10^{-6}	–	–	–	–	–	–	–	–	–
	10^{-7}	–	–	–	–	–	–	–	–	–

[A]Duplicate CEF cultures in 35-mm plates were infected with 0.2 ml of REV strain T (ELISA titer, 1:128) in serial dilutions. Culture fluids were collected daily and kept frozen until use. Each sample was tested in duplicate.

[B]+/– indicates that one of two duplicate plates was positive and the other was negative at the same dilution, but both duplicate plates were positive in lower-dilution plates.

Figure 15. Relative titers of REV antigen in supernatants of CEF cultures infected with strain T, CS and DIA. T-CEF supernatant samples (as arrows indicate) collected at days 3 and 17 after infection contained 3 x 10^4 and 2.7 x 10^5 FFU/ml respectively. ELISA titers with strain T (●), CS (■) and DIA (▲). CF titers with strain T (○), CS (□) and DIA (△).

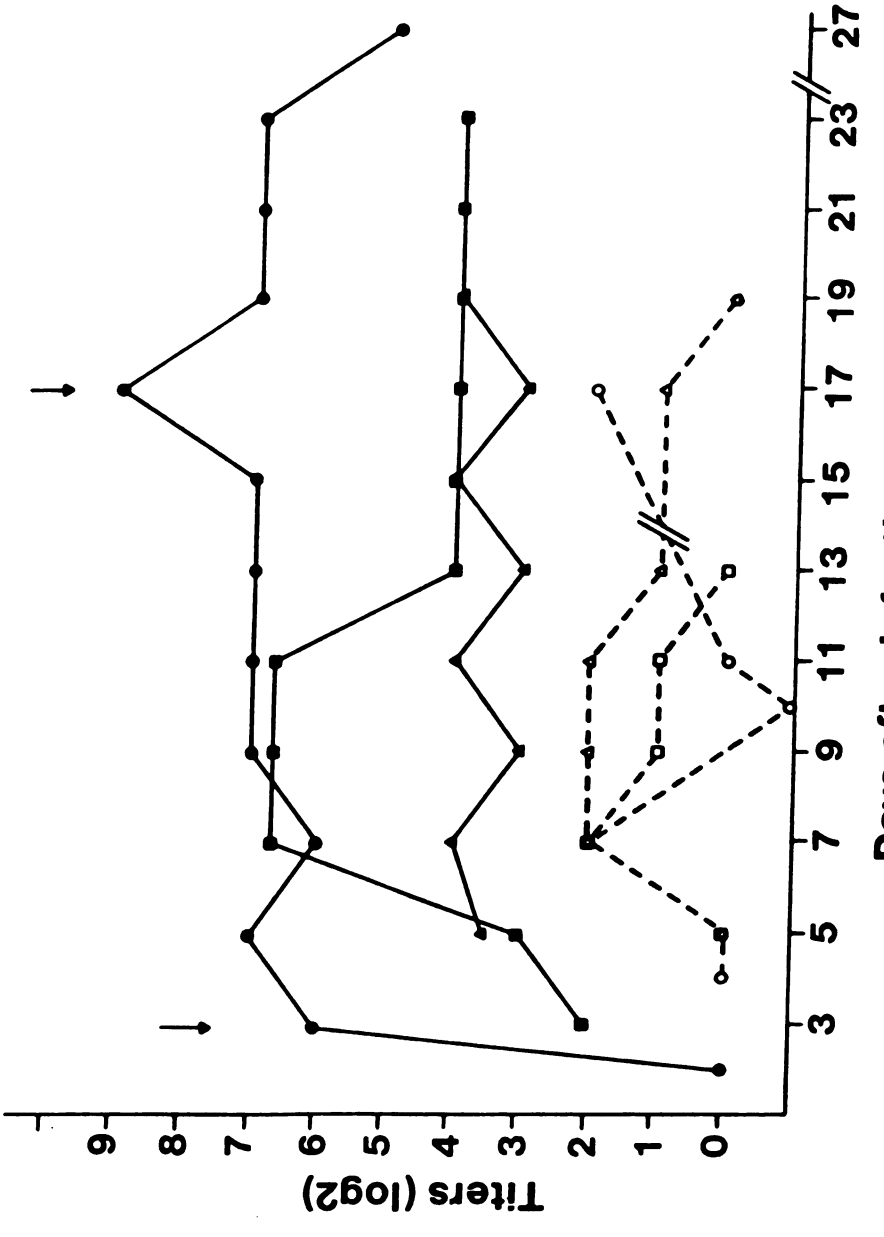

Titers (log2)
0
1
2
3
4
5
6
7
8
9
3
5
7
9
11
13
15
17
19
21
23
27
Days after Infection of CEF

Figure 15

day 8 but not found even at day 18 in another duplicate plate in the pair of plates after infection with 0.2 ml of virus stock in dilution of $1:10^5$. It indicates that it will take 7-8 days for REV antigen to be detected in cell culture fluids after infection with one infectious unit.

Dynamics of REV infection in vitro and vivo

Prolonged infection of CEF with REV and constantly releasing virus from culture. CEF culture could stand REV-infection for a very long period and continuously release the virus into supernatant. As Fig. 15 indecates, CEF infected with 3 different REV strains could constantly release virus for at least 3 weeks, the infection with the virus seems not kill CEF cells and even not affect division of CEF cells. In another experiment, CEF monolayers had grown in roller bottles and constantly released the virus particles into culture supernatants for 126 days, and the infected CEF cells then experienced further 24 passage of trypsinization and reculture in plates in another 74-day period, still released virus particles (Table 19).

Viremia, viral antigenemia, and antibody respones of chicks infected with REV. Birds infected with REV at different ages demonstrated the similar anti-REV antibody responses, but different dynamics of viremia and viral antigenemia. As Table 20 indicates, antibody responses of both groups to REV were detected 3 weeks after infection and lasted for at least 9-11 weeks at high titers. In birds infected at 1-day-old of age, strong viremia appeared at early stage of

Talbe 19. Long-period constantly virus-releasing in CEF culture infected with REV strain T

days after infection	ELISA titer	days after infection	ELISA titer	days after infection	passage	ELISA titer
2	4	53	64	132	1	8
5	64	56	64	141	6	8
7	128	58	64	187	21	8
12	128	78	128	192	23	8
22	>256	85	128	200	24	8
29	256	92	128			
45	256	100	128			
47	64	113	64			
49	64	120	64			
51	64	126	64			

CEF of line O grown in roller bottles were infected with REV strain T when CEF monolayer formed on the wall of roller bottles. Culture supernatants were harvested and supplemented with fresh media every 2-3 days as in Materials and Methods.

ELISA titers: supernatants harvested at certain days after infection were tested for their virus antigen titers by ELISA developed in this dissertation experiment.

Passage: At 126th day after infection and growth in roller blttles, the infected CEF monolayers were trypsinized, and transferred to 150 mm plates for continuous culture. The cultures were trypsinized and transferred to new plates every 3 days. The culture supernatants were saved for ELISA test at certain passages.

Table 20. Comparison of viremia levels and antibody responses of chicks with REV strain T infected at different ages

weeks after infection	bird No	infected at 1 day of age			infected at 2 weeks of age		
		FFU	Ag titer	Ab titer	FFU	Ag titer	Ab titer
1	1	100	64	NT	NT	NT	NT
	2	2,300	128	NT	NT	NT	NT
2	1	<5	128	NT	<5	128	NT
	2	5	64	NT	<5	64	NT
3	1	<5	256	200	<5	-	-
	2	45	2,048	200	<5	1	3,000
5	1	<5	8	3,000	<5	1	3,000
	2	<5	16	200	<5	1	3,000
7	1	NT	4	3,000	NT	-	3,000
	2	NT	-	3,000	NT	NT	NT
9	1	NT	4	3,000	NT	-	3,000
	2	NT	-	6,000	NT	-	400
	3	NT	8	400	NT	NT	NT
	4	NT	4	3,000	NT	NT	NT
11	1	NT	NT	NT	NT	-	1,000
	2	NT	NT	NT	NT	-	1,000
	3	NT	NT	NT	NT	NT	400

FFU: fluorescence plaque forming unit was measured as described in Materials and Methods.
Ag titer: virus antigen titers in serum was measured by ELISA developed in this dissertation.
Ab titer: anti-REV antibody titers in serum was measured by ELISA as described in Materials and Methods.
<5: no plaque was found when serum in dilution of 1:5 was used for infection of CEF.
NT: not tested.
"-": negative.

Antibody titers trend to corelate negatively to antigen titers in sera of chicks infected at 1-day-old, but not significatly ($r = -0.249$, $p = 0.25$).

infection, then decreased dramatically and lasted only for 3 weeks. Viral antigenemia appeared at the same time, but highest titer of viral antigenemia came a little later followed by a low titer period of at least 9 weeks. It seems like that both viral antigenemia and antibody response could coexist for a long period in birds infected at age of 1-day. However, no viremia was detected, and viral antigenemia existed only for a short period after infection in birds infected at age of 2-weeks. Age gave a tremendous influence on the suseptibility of birds to REV infection.

Pathogenic effects of REV infection in chicks. As chicks were raised in SPF conditions, the nd-REV strain T did not cause death and specific lesions, but it did induce some pathogenic effects. Table 21 indicates that nd-REV strain T infection at 1-day-old age significantly decreased whole body weight and the Bursa weight as compared to the controls ($p<0.05$), i.e. caused growth retardedness and the Bursa atrophy, and also caused spleen enlargement ($p<0.05$) indicating some inflamatory or proliferative responses.

Distribution of REV in other tissues of infected birds. In birds infected at 1-day-old with nd-REV strain T, REV antigen could easily be found in all tested tissues, such as the Bursa, kidneys, livers and spleens, by ELISA (Table 22). However, no REV antigen could be detected in the same kinds of tissues from birds infected at age of 2 weeks with the same virus. Virus antigen could be released into cloaca of chicks infected at 1-day-old with nd-REV strain T. Table 23 shows ELISA readings of cloaca swabs. Among swab samles from infected birds, at least 3 would definately be judged as positive, and some more were weakly positive when compared to controls. REV also could be released

Table 21. Effects of infection of chicks at the age of 1 day with REV stran T on the weights (grams) of the Bursa, spleens and the whole body

Age	chick	body		the Bursa		spleen	
weeks		infected	uninfected	infected	uninfected	infected	uninfected
	B1	58		0.11		0.1	
1	B2	48		0.08		0.09	
	B3		55		0.11		0.03
	B4	80		0.25		0.2	
2	B5	95		0.25		0.25	
	B6		105		0.47		0.11
	B7	110		0.38		0.36	
3	B8	145		0.52		0.4	
	B9		135		0.62		0.18
	B10	280		1.1		1.0	
5	B11	240		1.3		1.0	
	B12		340		2.25		0.57
	B13	460		0.4		1.1	
7	B14	630		3.44		1.38	
	B15		580		4.55		1.27
	B16	580		0.78		2.03	
	B17	500		0.70		1.28	
9	B18	470		1.15		2.0	
	B19	670		0.88		1.85	
	B20		950		4.46		1.5

Both body and the Bursa weights in infected chicks were significantly ($p<0.05$) smaller than that of uninfected chicks, and the spleens of infected chicks were significantly ($p<0.05$) larger than uninfected chicks. The data were analysed by the unbalanced 2-way analysis of varieace according to Federer-Zelen method (Federer and Zelen, 1966).

Table 22. ELISA readings for detection of REV antigen in tissue suspention

tissues	dilution	infected birds					uninfected birds		
		B1	B2	B4	B5	B7	B3	B6	B9
Bursa	1:4	.67	.65	.83	.86	.77	.01	.00	.04
	1:8	.62	.56	.65	.75	.86	.00	.00	.01
	1:16	.42	.37	.50	.58	.46	.00	.00	.00
Kidney	1:4	.94	.77	.71	.72	.67	.08	.04	.01
	1:8	.91	.75	.67	.74	.74	.09	.02	.03
	1:16	.93	.62	.72	.74	.59	.08	.04	.04
Liver	1:4	.74	.89	.56	.55	1.03	.04	.03	.06
	1:8	.75	.79	.77	.80	1.02	.05	.03	.04
	1:16	.54	.68	.57	.68	.94	.05	.04	.06
Spleen	1:4	.98	1.02	.87	.95			.03	.02
	1:8	.95	.98	.99	1.01			.00	.02
	1:16	.79	.99	.88	1.02			.01	.02

REV antigens were detected by the ELISA developed in this dissertation experiment. 100 ul of tissue suspension in PBS in dilutions of 1 to 4-16 was added to the wells. The status of each chicks are described in Table 21.

Table 23. ELISA readings for detection of REV antigen in cloaca swabs

bird #	infected										uninfected	
	19	22	25	26	28	29	30	89	92	96	77	78
duplicate	.21	.15	.91	.13	.12	.10	.24	.07	.03	.01	.00	.01
	.20	.14	.97	.09	.10	.09	.29	.05	.01	.00	.00	.00

Each swab was soaked in 0.5 ml of PBS to get sample suspension. 100 ul of suspension of each swab sample was added to well of ELISA plates in duplicates. The ELISA readings seemed to be very constant for each individual samples. Samples from birds #19, 25, and 30 were definitely judged as positive. Some more samples probably were positive but not strong enough.

Table 24. ELISA readings for detection of REV antigens in semen

birds #		dilutions of samples					
		1:1	1:4	1:16	1:64	1:256	1:1024
infected	#1	.40	.26	.16	.11	.12	.13
	#2	NT	.29	.18	.13	.12	.13
	#3	.43	.28	.16	.13	.12	.13
	#4	.33	.26	.17	.14	.12	.13
	#5	.74	.70	.51	.35	.21	.14
	#6	.47	.29	.17	.13	.14	.14
	#7	.09	.10	.10	.10	.12	.13
	#8	NT	.52	.39	.28	.17	.15
uninfected control	#9	.13	.10	.11	.11	.13	NT

The infected birds were "tolerant" male breeders with viremia, they were inoculated as embryos with REV strain CSV. The uninfected control semen was from SPF flocks.
NT: not tested.
Semen samples from all except #7 infected birds were positive for REV antigen. Some sample such as #5 gave a titer as high as 1:256.

into semen of "tolerant" male breeders with viremia. Table 24 shows ELISA readings to detect REV antigen in semen. All 8 except 1 samples appeared positive.

DISCUSSION

Although REV infection has been found in parts of the world among various avian species (Cook, 1969; Dren et al, 1983; Grimes and Purchase, 1973; Li et al, 1983; Ludford et al, 1972; McDougall et al, 1978; Paul et al, 1976; Robinson et al, 1974; Sarma et al, 1975; Solomon et al, 1976; Trager, 1959; Witter and Glass, 1984; Yuasa et al, 1976), its economic role in poultry industry is still not clear as indicated in the literature review. More than 30 isolates of REV were obtained in the world, all of them were antigenically closely related and could not be differentiated from each other by polyclonal antisera (Witter, 1970; Purchase et al, 1973; Maldonado and Bose, 1976; Bulow, 1977; Chen et al, 1987), even though these isolates or strains came from originally different avian species with quite different syndromes and pathogenic lesions. REV structural proteins or polypeptides were recognized by polyclonal anti-REV sera for some strains (Halpern et al, 1973; Maldonado and Bose, 1973, 1975, 1976; Mosser et al, 1975; Tsai et al, 1985), but the relationship among these polypeptides and the relationship of the polypeptides with their biological functions are unknown. By its advantage of high specificity and high titer to be reached, monoclonal hybridoma technique hopefully could help us to further understand these unresolved problems. This dissertation focused on the generation, characterization, and applications of monoclonal antibodies against REV. It was expected that MCAs would be useful in both poultry industry and molecular virology. For example, I attempted to use MCAs for differentiation of various strains of REV group, to develop a MCA-mediated-ELISA for detection of REV antigen for field

surveys or eradication programs of REV infection, to relate some antigenic epitopes to their biological functions.

This dissertation represented the first report on the development and characterization of a panel of MCAs to REV. These MCAs reacted specifically with REV-infected cells and purified REV but not with uninfected CEF or other avian lymphoma-inducing viruses such as Marek's disease virus or avian leukosis virus. Nine of the 11 well identified MCAs were directed against strain-crossreactive epitopes. Four MCAs immunoprecipitated both 62,000 and 21,000 dalton glycoproteins, whereas the remaining five MCAs immunoprecipitated what appears to be the same 62,000 dalton glycoprotein, but not the 21,000 dalton glycoprotein. The tunicamycin findings suggest that the 48,000 and 20,000 dalton polypeptides are the precursors of the 62,000 and 21,000 dalton glycoproteins. Similarly, another 48,000 dalton polypeptide appears to be the precursor of the 54,000 to 72,000 dalton strain T-specific glycoprotein. These results also indicate that the MCAs are directed against epitopes in the peptide chains but not the glycosyl- moiety of the glycoproteins.

In contrast with the results with the MCAs, serum obtained from strain T-hyperimmunized rabbits immunoprecipitated several viral proteins. On the basis of published data, the 29,000 dalton protein is probably the major virus structural core protein responsible for the REV group-specific antigenicity (Maldonado and Bose, 1975, 1976; Mosser et al, 1975; Tsai et al, 1985; Wong et al, 1980). The fact that none of the MCAs in the study recognized this major immunogenic protein was unexpected. However, it is speculated that this may be due in part to the hybridoma screening procedure. The hybridomas in this study were

screened by an indirect ELISA using plates coated with REV-infected cells or purified REV. With this procedure, the positive clones would be the ones that reacted with the viral envelope or with viral glycoproteins on the surface of infected cells. Maldonado and co-workers (1975, 1976) reported finding similar glycoproteins of 71,000 and 22,000 daltons exposed on the external envelope of REV strain SNV. Considering the error inherent in determinging glycoprotein sizes from polyacrylamide gel electrophoresis, there is enough similarity in sizes between their glycoproteins and the 62,000 and 21,000 dalton glycoproteins found in the study to suggest that they may be identical.

A comparison of epitope-specificities of five MCAs in competitive ELISA inhibition experiments revealed the presence of at least three distinct epitopes. The two strain T-specific MCAs, 11C100 and 11F667, were directed against an epitope located on the 54,000 to 72,000 dalton glycoprotein, whereas the type-common MCAs 11C237 recognized an epitope on the 62,000 glycoprotein. The competition ELISA experiments demonstrated that the two type-common MCAs 11A25 and 11B118, reacted with yet another epitope. However, both MCAs immunoprecipitated two glycoproteins (62,000 and 21,000 daltons). Theses results together with the finding that five of MCAs only immunoprecipitated the 62,000 dalton glycoprotein suggest three possible explanations. First, the 62,000 and 21,000 dalton glycoproteins both contain an epitope recognized by four of MCAs (11A3, 11A25, 11B118, and 11D78), whereas five of MCAs (11A301, 11B154, 11C237, 11D175, and 11D182) recognize a different epitope present only on the 62,000 dalton glycoprotein. This possibility could occur if the 21,000 dalton glycoprotein is a cleavage product from the

62,000 dalton glycoprotein. Second, the 62,000 dalton glycoprotein immunoprecipitated by the five MCAs (11A301, 11B154, 11C237, 11D175, and 11D182) is not the same 62,000 dalton glycoprotein immunoprecipitated by the other MCA (11A3, 11A25, 11B118, and 11D78). In this case, the two 62,000 dalton glycoproteins would be unrelated except that they both migrate at similar rates in the denaturing polyacrylamide gels. Third, if the latter case is true, then a third possibility exists. The 62,000 and 21,000 dalton glycoproteins do not share common epitopes but do exist as a complex that only become dissociated in the SDS denaturing gel conditions. In this case, the four MCAs (11A3, 11A25, 11B118, and 11D78) would be recognizing an epitope on either the 21,000 or 62,000 dalton glycoprotein. Answers to theses questions must await additional experiments.

The MCA reported in this dissertation should be useful for diagnostic purposes in the field. REV tumors are not easily differentiated from lymphoid leukosis virus-induced tumors by conventional methods. An additional problem is that conventional polyvalent serum is often not able to differentiate between related REV strains as mentioned above. By using both type-specific and type-common MCAs, it is easy to diagnose REV infections and differentiate between the strain T and CSV. In fact, a combination of MCAs developed in this study has been used for subtyping REV group. Chen et al (1987) tested all 26 nd-REV isolates obtained in U.S.A. with MCAs 11A25, 11B118, 11C100, 11C237, and 11D182 in IFA and found that the panel of MCAs could be easily used to divide all 26 isolates into 3 subtypes. MCAs 11A25 and 11B118 reacted with all 26 isolates, and MCA 11C237 reacted with subtypes 1 and 3, MCAs 11C100 and 11D182 reacted with only subtype 1.

Aside from their practical applications, these MCAs can identify epitopes on viral envelope proteins for studies on the mechanism of viral neutralization. Results demonstrated that virus neutralization activity was related only to certain antigenic epitopes not all epitopes no matter whetger they were strain-specific or strain-crossreactive. Two strain T-specific MCAs 11C100 and 11F667, which recognized an identical or very closely related epitope, showed a very strong neutralizing activity but another strain T-specific MCA 11E258, which reacted to a different epitope from 11C100 and 11F667, had a weak neutralization effect. Among strain-crossreactive MCAs, MCA 11E197, which reacted with two independent epitopes also appeared to be very strong in neutralization test. Whereas, MCA 11C237 which reacted with another independent epitope was very weak in neutralization test. All the remaining MCAs did not show any neutraliaztion activity al (Table 3 to 7). The fact of dependence of neutralization on specific epitopes was similar to what has been reported for other viruses such as Saint Louis encephalitis virus (Roehrig et al, 1983), tick-borne encephalitis virus (Heinz et al, 1983), Japanese encephalitis (Kimur-Kuroda and Yasui, 1983), vesicular stomotitis virus (Bricker et al, 1987), avian infectious bronchitis virus (Niesters et al, 1987), bovine coronavirus (Deregt and Babiuk, 1987), foot-and -mouth disease virus (Pfaff et al, 1988), and Simian rotavirus SA11 (Burns et al, 1988).

Because it was reported that two glycoproteins gp 71 and gp 22 were located on the outer surface of the lipid envelope of the virions, as demonstrated by lactoperoxidase-catalyzed iodination and by bromelain digestion (Mosser et al, 1975). The MCA-recognized glycoproteins may

also be reacted with virus surface antigen. Using protein A-gold, the immuno-labeling technique made it possible to directly observe the location of antigens recognized by MCAs on the virion surface using TEM. MCA 11A25 recognized an antigen surrounding the whole surface of the varions. Unforturenately, I could not indicate whether the antigen would also locate inside of the virions from the results until ultrathinsections of virions were examined in the same way. By using uranyl acetate negative staining, all the four representatives of REV group, strains T, CSV, SNV, and DIAV, showed viral envelopes, which were covered with apparently hollow peplomers approximately 10 $\pm$ 1.5 nm in diameter at the tip by 6 $\pm$ 1 nm long under EM (Kang et al, 1975). There were about 100 of those peplomers per virion. The appearnace of protein A-gold immuno-labeled virions implies that the glycoproteins recognized by MCA 11A25 probably were a part of the peplomers of the virus envelopes.

Development of a MCA-mediated ELISA for detection of REV antigen was one of the major objectives of this dissertation. The optimal MCA combination was chosen on the basis of several criteria: They should be reactive with all or at least most members of REV group; they should recognize epitopes located on the surface of virions and easily to be reached; they should recognize different epitopes from each other and could be used in combination to enhance the antigen-catching ability in the assay; they should be fixed on the plates without losing antigen-catching ability. It seemed to be very difficult to fit all the criteria. Fortunately, the reality of the combination of MCAs used in developing the ELISA was very close to the ideal criterion if not perfect. MCAs 11A25 and 11C237 recognize two quite different epitopes

(Fig.3). They were reactive with glycoproteins in immunoprecipitation (Fig.5 and 6) indicating that the epitopes they recognized were most likely located on the surface of the virions. The EM observation further proved that MCA 11A25 did recognize the epitope on the surface of virus envelope (Fig. 8). Chen et al (1987) also showed that MCA 11A25 reacted with all the 26 isolates of REV collected in U.S.A. and MCA 11C237 was positive with more than two thirds of them. The use of acombination is not only for enhancing the assay sensitivity but also for safely detecting a variety of REV isolates which will appear in the fields and probably have some antigenic mutation. A new MCA candidate reactive with all three subtypes of REV is being tested and may replace MCA 11C237 for use in the combination.

The ELISA developed in this dissertation appeared to be much more sensitive for detection of REV antigenthan AGP and CF. Neither AGP nor CF is sensitive for detecting REV antigen directly. Procedures commonly employed involve immunofluorescent tests with antibodies. The ELISA was 40-80 times more sensitive than CF. Moreover, ELISA detected 100% of REV-infected CEF cultures whereas CF detected only 81% (Table 14 and 15). The ELISA detected antigen in all viremic chickens, but not CF. ELISA can be used instead of immunofluorescence tests, which are time-consuming and the results of whic are subbjective. Although AGP could directly detect REV antigens in sera of some experimently infected birds (Ianconescu and Aharonovici, 1978; Bagust and Grimes, 1979; Motha, 1984), its sensitivity obviously was very poor. As mentioned in literature review, REV-infected CEF culture fluid had to be 6-10 fold (Ianconescu, 1977) or 20-fold (Yuasa et al, 1976) concentrated when used as the antigen preparations to give a

precipitate line in AGP, but the ELISA developed in the study could easily detect antigens when REV-infected culture fluid was at dilution of 1:64-256. Maldonado and Bose (1976) indicated that 2 ug of purified REV group-specific antgen p29 in 10 ul per well had to be used for showing the precipitate line in AGP, but sensitive limits of the ELISA were about 0.008-0.016 ug in 100ul per well (Table 14). Therefore the ELISA is about 100-1,000 times more sensitive than AGP.

ELISAs performed directly on tissue are simpler and faster than biological assays for detecting cell-culture antigens, but it is not as sensitive. The results indicated that at least 50-500 infectious units (Table 16) were required for a positive response in ELISA. For mass-screening of plasmas, the ELISA is the method of choice in terms of both simplicity and sensitivity. The discrepancy between the two methods in sensitivity may be attributed to the fact that, whereas viral assays reflect the presence of infectious REV, ELISA detects gp62 from both infectious and non-infectious REV particles. In REV-inoculated chickens, Bagust et al (1981) observed non-infectious REV antigenemia up to 7 weeks after infection, and Moelling and Gelderblom (1975) showed that at least 20% of REV particles are structurally immature. In addition, REV was heat-labile and infectivity was completely destroyed in 4 min at $56^{o}C$ or in 2 hr at $37^{o}C$ (Campbell et al, 1971). Finally, REV antibodies in plasmas may also interfere in assays that require viral multiplication.

An ELISA for detecting antibodies against REV has been in use for serological surveys of REV infection in commercial chicken and turkey flocks (Smith and Witter, 1983). However, persistent viremias exist in some REV-tolerant chickens (Bagust and Grimes, 1979,1981; Ianconescu

and Aharonovici, 1978). These chickens may transmit REV infection horizontally and congenitally. In the present study, we have detected gp62 directly in albumen samples, suggesting that REV may be congenitally transmitted. In view of the evidence for vertical transmission of REV in chickens (Bagust and Grimes, 1981;Motha and Egerton, 1987; Witter et al, 1970) and turkeys (McDougall et al, 1981) and the occurrence of REV envelope sybtypes (Table 2), the MCA-mediated ELISA may be useful in comparative studies on congenital transmission of REVs.

The ELISA developed in the study has recently been applied for an eradication program practically in some commercial turkey breeder farms with high incidency of REV infection resulted in high rates of tumor. Witter and Salter (1987) directly tested blood, cloacal swabs, and egg albumen samples of 59 hens from the infected farms by the ELISA for REV antigen, and compared the ELISA results with biological test (i.e. inoculation of cell culture with samples). Of the 59 hens, 11 were possitive for REV antigen by the ELISA. All these 11 hens were consistently viremic and positive in dot blot (for hybridization of REV RNA or provirus DNA with probes) , but none of the 11 hens had antibody suggesting that they were viremic-tolerant dams. All the 81 egg albumen samples from the 11 hens were possitive in the ELISA, when all negative control albumen samples were negative in the same assay. Of 4 transmitting hens which gave infected progenies, 3 were positive in the ELISA. Thus they concluded that the direct ELISA test should detect most shedder hens and is of value in an eradication program to remove transmitting hens should according to the direct ELISA testing of albumen samples (or possibly cloacal swabs). As the first time, the

practical eradication program for REV infection is being tried by using the ELISA in these commercial turkey breeder farms in Pennsylvania.

An ALV ELISA based on the group-specific (gs) antigen p27 has been used for detecting antigen in cloacal swabs, meconia, albumen, embryo extracts, and blood (Crittenden et al, 1984). In ALV eradication programs, it has partly replaced CF for identifying dams that congenitally transmit ALV. The REV ELISA described here differs from that for ALV in that MCAs against common glycoprotein gp62 were used instead of a common group-specific antigen, p27. The data also indicated that ELISA for gp62 was suitable for REV detection in shedder hens. It may be feasible to use the same albumen samples for a simultaneous ALV- and REV-ELISA screening for both avian retroviruses. Thus, a program designed for ALV eradication could simultaneously help in REV eradication.

The incidence of REV infection in commercial chicken and turkey flocks has been reported (Witter and Crittenden, 1979). Witter (1984) described that infected chickens may develop proventriculitis, runting syndrome, feathering abnormalities, immunodepression, and lymphomas. Witter and Crittenden (1979) found that chickens infected as embryos or at hatching with REV strain CS developed bursal tumors. These tumors are morphologically and antigenically similar to those induced by ALV, and no simple methods for their differential diagnosis are available. Some field problems thought to have been induced by ALV may be REV-induced. REV ELISA can be of use for testing tumors for REV antigen.

REV and ALV are endemic in some chicken flocks and are potential contaminants of biologics of chicken origin. We have found in this

study that REV-ELISA may detect one infectious virus particle in cultures of infected fibroblasts after 7-8 days of cultivation. This time period corresponds with the data discribed by Crittenden (1987, personal communicaton), who found that with ALVs an endpoint titration was reached after 9 days of continuous cell culture. Thus, REV-ELISA could be used as an adjunct to ELISA for ALV. It could be combined, for example, with ALV-ELISA to assay the same test samples to detect retroviral contamination of poultry-based biologics.

Surprisingly, the MCAs and the ELISA was also successfully used for some research project in molecular biology soon after they had been developed. By using the ELISA and corresponding MCAs, Federspiel et al (1988) detected glycoprotein expressed by REV envelope gene inserted into D17 cells, a canine cell line from an osteosarcoma. It would not be possible otherwise by other available reagents and assays.

The pathogenic effects of REV infection were studied. Infection of chicks at 1-day-old of age did cause growth retardedness and atrophy of bursa of Fabricius, which was in agreement with the results reported before (Mussman and Twiehaus, 1970; Taylor and Olson, 1971). Since Chang et al (1955, 1957, and 1958) reported that bursectomized chicken failed to produce antibody after immunization with Salmonella spp., it has been proven that the Bursa in the early stage of the life takes a critical role in inducing antibody responses to antigens. In the case of REV infection, it was noticed that chicks inoculated as embryo with nd-REV strains did not give antibody response to REV (Witter et al, 1981). But in this study, birds inoculated at 1-day-old with nd-REV strain T still had a high titer antibody to REV, even though the Bursa atrophy happened later on. It was probably because the

infection was given too late to completely inhibit the antibody-inducing ability of the Bursa.

In this study, a prolonged infection of CEF cells with REV was also reported. During a 200-day-period of culture with infection, CEF cells continueously grew, divided and kept release virus particles. Although there was no transformation proved, it could partially explain why nontransforming REV could exist in infected birds for a long period and sometimes cause tumors in birds.

The synergistic ELISA was developed in the study for identifying antigenic epitope-specificities of MCAs, it is much simpler and less labor- or reagent-expensive than the competitive binding assay (CBA) in RIA and ELISA. As a classical method, CBA has been used for analysis of antigenic epitope specificity of MCAs to map topologically antigenic determinants of structural proteins of viruses and compare the relationships between antigenic structure and functions such as heamagglutination and neutralization (Yewdell and Gerhard, 1982; Roehrig et al, 1983; Heinz et al, 1983; Kimura-Kuroda and Yasui, 1983). In CBA, competing antibodies could competitively inhibit reactions of radioreactive isotope- or enzyme-labeled antibodies (congugates) if antigenic determinants, which the competing antibody and labeled antibody react with, are identical (or similar), overlapped or interracted (Stone and Nowinski, 1980; Luberck and Gerhard, 1981). For the purpose, it is necessary to use MCA samples with high titer for preparing competiting antibody and conjugates with certain enzyme. In contrast, the synergistic ELISA depends on a different principle. When a certain epitope on some antigenic material (such as virus proteins, cell surface antigen, etc.) is saturated by a specific MCA, there are

still other unrelated epitopes available for other MCAs with different epitope-specificities. If two MCA samples are reactive with different and unrelated epitopes, the mixture of two samples will give a higher ELISA readings than that of each single sample, due to more antigenic determinants available for binding antibodies. This is called as a synergistic or cumulative effects in ELISA. However, if two seperated samples are reactive with the same epitope or closely related epitope(s), their mixture would give the same level of reaction as each single sample alone, i.e., only one epitope could bind antibody and there is no synergistic effect on ELISA between two seperated samples with the same epitope-specificity. According to whether ELISA reading of mixed samples are increased significantly or not, we can estimate the relationships in epitope-specificities between two MCA samples. However, the relationship between the ELISA readings and the amount of antibodies bound to antigens is not a linear line but a hyperbolic curve. It means that the ELISA readings do not increase linearly with doubling the amount of bound antibodies when they reach a certain level, but do increase reasonablly by synergistic effect. Thus, the ELISA data should be analyzed statistically to determine whether the increased ELISA readings of mixtures are due to synergistic effect of two MCA samples reactive with different epitopes or to variation in ELISA testing.

The study indicated that sELISA gave the same results in grouping epitope-specificity of MCA as cELISA, i.e., 5 MCAs were divided into 4 seperate groups, although the relatedness between 11A25 and 11B118 was demonstrated at some level in both sELISA and cELISA. MCAs 11A25 and 11B118 appeared to have different epitope-specificities when two

samples were compared in the form of hybridoma culture fluids in sELISA. As ascitic fluids were tested (Table 8 and 9), the difference between 11A25 and 11B118 was observed only when a large number of duplicates were used. The same results were also found in cELISA. When mutual cELISA was carred out, competing MCAs 11A25 and 11B118 inhibited each other's labeled antibodies but inhibition to the heterologous antibodies was slightly weaker than that to the homologous (Fig.3a), indicating both relatedness and difference between 11A25 and 11B118. Compared to cELISA, sELISA shows some obvious advantages. In sELISA, only commercially available anti-mouse IgG or IgM antibody-enzyme conjugates are used, and it is not necessary to purify immunoglobulins from ascitic fluids of each MCA samples to be tested and conjugate them with enzyme for sELISA. It is, thus, much simpler and less labor- and reagent-cost than cELISA. Especially, different hybridomas could be analysed for their epitope-specificities with the culture fluids during the early stage of screening specific hybridomas. In addtion, the data in sELISA could be inputted into and analysed by computors. All these advantages of sELISA would make it accepted as a very helpful and convenient assay to analyze or identify a large number of MCA samples in terms of their epitope-specificities.

Although the principle of the sELISA was described by Friguet et al (1983), the assay they conducted has rarely been metioned and not been repeated, even though there are so many papers published about identification of epitope-specificities of MCAs by using competitive ELISA or RIA since then. It could probably be explained as following: a) In the assay described by Friguet et al (1983), they did not metion if they had used duplicates in ELISA, did not consider the variations

from well to well in ELISA itself, ofcause, they did not analyze their data by statistics. It was not reasonable and not easy to be accepted by others. They set up a index to judge if two samples were reactive with the same epitope or not, but there was no acceptable standard to make an objective judgement. So it was difficult to be repeated by others or in other antigen systems. b) They still had to use ascitic fluid samples in their test procedure but not hybridoma supernatants. Both these two problems are resolved in this dissertation by using different assay procedure. And the results of sELISA are also compared to that of the classical cELISA in this study, it makes conclusions more convincing.

SUMMARY AND CONCLUSION

A panel of monoclonal antibodies were developed against REV. The three MCAs 11C100, 11E258, and 11F667 were strain T-specific, and the left were crossreactive with both strain T and CS or group-common. It was the first reagent which were able to differentiate different strains of REVs. The results in immunoprecipitation tests indicated that strain T-specific MCAs 11C100 and 11F667 recognized REV glycoprotein of about 54,000-72,000 dalton, whereas the group-common MCAs tested recognized REV glycoprotein bands of 62,000 or both 62,000 and 21,000 dalton. Tunicamycin treatment demonstrated that the precursors of 62,000 and 54,000-72,000 dalton proteins were polypeptide of 48,000 dalton, the precursor of 21,000 dalton protein was polypeptide of 20,000 dalton. The Protein A-gold immunolabeling technique and TEM observation showed that the proteins recognized by MCA 11A25 were located on the surface of virion envelope.

The synergistic ELISA were developed in this dissertation and it gave the quite same results as the classical competitive ELISA in identification of MCAs for their epitope-specificity. However it showed some advantages over the classical competitive ELISA for determining epitope-specificities of MCAs, sach as its simplicity and being able to test culture fluids in the early stage of hybridoma screening process instead of ascitic fluids. It would greatly stimulate the further wide useness of MCAs in topological analysis of different antigen molecules and establishment of relationships between antigenic epitopes and biological functions.

By using both sELISA and cELISA, MCAs were analyzed for their

epitope-specificities. Among three strain T-specific MCAs, 11C100 and 11F667 reacted with the same or very closely related epitope, but 11E258 was reactive with an independent epitope different from 11C100 and 11F667. The all group-common MCAs tested were reactive with their own epitopes different from each other, althouth there was some relationship between MCAs 11A25 and 11B118. MCAs were also tested for their virus-neutralizing ability in cell cultures. Two strain T-specific MCAs 11C100 and 11F667 which were reactive with the same epitope showed a very strong in vitro neutralizing ability, but another strain T-specific MCA 11E258 reactive with a different epitope just gave a very weak neutralization activity. A group-common MCA 11E197 demonstrated a very high titer in neutralization test, MCA 11C237 was barely positive in the test, and the other group-common MCAs tested did not show any neutralizing ability. So the neutralizing activity of MCAs were depended on their epitope-specificities.

A MCA-mediated ELISA was developed for directly detection of REV antigens by using combination of MCAs 11A25 and 11C237 which were reactive with different group-commom epitopes on the surface of virions. The sensitive limit of the ELISA was about 0.008-0.016 ug of purified REV protein in 100 ul per well. It was about 40-80 times more sensitive than complement fixation test, the standard procedure used currently. More importantly, CF could detect REV antigens from only cell culture supernatants but not any kinds of avian samples. However the MCA-mediated ELISA could directly detect REV antigens from all kinds of avian samples, such as blood, sera, tissue suspensions, cloacal swabs, egg albumin, and semen. Testing the egg albumen for REV antigen by the ELISA would be recomended to be used for epidemic

surveys and eradication programs of REV infection.

The MCAs and the ELISA developed in this study has been successfully used for different purposes by others: to subtype all REV isolates; to differentiate tumors or cell lines transformed by different strains of REV; to study the transmission of REV and make epidemical surveys; to pick up the transmiter hens from REV infected turkey flocks for REV eradication programs in some turkey farms in Pennsylvania; to detect the antigen expressed by REV genes inserted into cell germlines for transgenic animal studies.

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ABSTRACT

PRODUCTION, CHARACTERIZATION AND APPLICATION OF MONOCLONAL ANTIBODIES AGAINST RETICULOENDOTHELIOSIS VIRUS

By

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Reticuloendotheliosis virus (REV) infections have been reported throughout the world, but its economic role is still not clear. More than 30 isolates were obtained from different avian species with various symptoms. They can not be differentiated antigenically. Some molecular studing is done for REV viral proteins recognized by polyclonal sera, but nothing is known about the relationship between antigenic components and biological functions. The monoclonal antibodies are expected to help us to further understand the problems.

In this study, a panel of MCAs against REV strain T are generated and characterized. MCAs 11C100 and 11F667 are strain T specific and recognized a 54-72K dalton glycoprotein. They are useful for subtyping REV isolates. Others are crossreactive with other members of REV and recognized 64K or both 64K and 21K dalton glycoproteins. MCAs 11A25 and 11B118 are strongly reactive with all the REV isolates tested.

To determine epitope-specificities of MCAs, a synergistic ELISA (sELISA) is developed. It is quite coincident with the classical competitive ELISA (cELISA) in the results of identification of epitope-specificities of MCAs. However, it is much simpler than the cELISA and could use culture supernatants instead of ascitic fluid for testing. These two advantages over cELISA should make sELISA very helpful in testing a large number of hybridoma samples and thus stimulate more interests in topological analysis of various antigen molecules. Several independent epitopes on REV glycoproteins are differentiated with both cELISA and sELISA. The neutralizing activity of some MCAs is also tested showing that the neutralization activity is related to only some epitopes on virions.

By using the combination of MCAs, which are REV group-common and recognizing glycoproteins on the surface of virions but different epitopes, a MCA-mediated ELISA is developed for direct detection of REV antigens from plasma, tissue suspensions, egg albumen, cloacal swabs, and semen. The sensitivity limit of the ELISA is 8-16 ng purified REV protein in 100 ul. It is 40-80 times more sensitive than complement fixation test (CF) the standard assay currently used. More importantly, The ELISA could directly detect REV antigens from bird samples, but CF could not do so without amplification of viruses in cultures.