PERSISTENT IN VITRO INFECTION WITH MOUSE HEPATITIS VIRUS TYPE 3

by

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SHORT TITLE

PERSISTENT MHV_3 INFECTION

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SHORT TITLE

PERSISTENT MHV_3 INFECTION

To my husband, JEAN-PAUL whose support and encouragement throughout my studies were essential and deeply appreciated,

and to my son FREDERICK born at the beginning of this work.

ABSTRACT

Various mouse strains display different types of sensitivity to mouse hepatitis virus type 3 (MHV_3) infection: resistance, full susceptibility (acute hepatitis leading to death), and semi-susceptibility characterized by occurrence of paralysis and viral persistence. In order to study the mechanisms of viral persistency, persistent infections were established in vitro in mouse lymphoid cell line cultures and were characterized in terms of virus production, occurrence of cytoplasmic viral antigens and cell lysis. Cell cloning and antibody treatment experiments showed that viral transmission was horizontal and not vertical. These data indicated that persistent infections induced by MHV_3 in lymphoid cell lines were characterized by a viral "carrier state" where production of infectious viral particles remained in equilibrium with cell permissivity. Biological and biochemical properties of MHV_3 variants derived from persistently infected YAC lymphoid cells were characterized. Similar heterogeneous thermosensitive properties were observed when YAC-derived cloned substrains (YAC-MHV_3) were compared to parental-derived cloned viruses, indicating that no selection of temperature-sensitive mutants was induced in persistently infected YAC cells. The capacity, however, of YAC-MHV_3 to induce a lethal acute disease when injected into susceptible mice was lost very rapidly and seemed to be regulated by host factors. The in vivo significance of such results has been studied by MHV_3 infection of normal lymphocytes and macrophages originating from mouse strains exhibiting different MHV_3 sensitivities. A correlation was observed between in vivo susceptibility of mouse strains to MHV_3 infection and the capacity of such cells to restrict virus replication in vitro. In order to study genetically controlled natural resistance mechanisms, MHV_3 infections were induced in primary and

secondary embryonic fibroblast cell cultures. Persistent infection was induced in 4 out of 6 primary and all (6/6) secondary embryonic fibroblast cultures. A high yield of virus was obtained, as determined by viral titers and cell membrane antigen detection. Similar virus titers were observed in embryonic fibroblast cultures originating from various mouse strains. However cytopathic effects , characterized by cell lysis were related to <u>in vivo</u> phenotypes of mouse strains for MHV_3 infection. .

RÉSUMÉ

Le virus de l'hépatite murine de type 3 (MHV_3) se comporte selon différents modes évolutifs en fonction de la lignée des souris: soit une résistance à la maladie, soit une susceptibilité (hépatite aigüe mortelle) soit une semi-susceptibilité caractérisée par le développement de paralysie et une persistance du virus dans l'organisme. Afin d'étudier les mécanismes impliqués dans la virose persistante, des infections persistantes, ont été induites dans des cultures de cellules lymphoïdes murines en lignée continue. L'infection persistante a été caractérisée par la production de virus, la présence d'antigènes viraux cellulaires et la lyse cellulaire. Le clonage cellulaire et le traitement des infections persistantes à l'aide d'anticorps spécifiques ont démontré que la transmission de l'infection se faisait par voie horizontale et non verticale au sein de la population cellulaire. Ces résultats indiquent que la virose persistante induite par le MHV_3 dans des cellules lymphoïdes en lignée continue est maintenue par une infection de type "porteur" où la production de particules virales infectieuses se maintient en équilibre avec la permissivité des cellules. Les propriétés biologiques et biochimiques des virus variants provenant d'une infection persistante dans des cellules lymphoïdes YAC ont été analysées. Une hétérogénéité dans la propriété de thermosensibilité a été observée autant chez les virus clonés provenant de cellules YAC que ceux de la culture parentale. Ce résultat indique qu'aucune sélection de virus mutants thermosensibles n'est induite lors d'une virose persistante sur cellules lymphoïdes YAC. Par contre, la capacité du YAC-MHV_3 à induire une maladie létale après injection à des souris de lignée susceptible est rapidement perdue et semble dépendre de facteurs associés à l'hôte.

L'importance de tels résultats, les mécanismes impliqués dans l'infection <u>in vivo</u>, a été évaluée par l'étude de lymphocytes et de macrophages infectés

provenant de différentes souches murines présentant des sensibilités variables à l'infection causée par le MHV_3. Un phénomène de restriction de l'infectivité virale a été mis en évidence dans ces cellules et est associé au statut génétique de la souche de souris.

Afin d'étudier les mécanismes de résistance naturelle sous contrôle génétique, des infections ont été induites par le MHV_3 sur des fibroblastes embryonnaires en culture primaire et secondaire obtenues à partir de différentes lignées de souris. Une production virale élevée, déterminée par les titres de virus et par le pourcentage de cellules exprimant des antigènes viraux en immunofluorescence, a été remarquée dans ces cultures. Aucune différence dans la production virale n'a été observée entre les différentes cultures de fibroblastes embryonnaires. Par contre, l'effet cytopathique induit par le MHV_3 et caractérisée par une lyse cellulaire, varie selon le phénotype de l'infection virale <u>in vivo</u> de la lignée murine.

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CLAIM OF CONTRIBUTION TO KNOWLEDGE

1. This work is the first demonstration of persistent infection induced by a coronavirus in lymphoid cell lines.

2. The mechanism involved in these persistent infections is related to a "carrier state" type, i.e., infectious virus production in equilibrium with cell permissivity.

3. In contrast to other persistent viral infections, MHV_3 persistency in lymphoid cell lines is not related to factors such as defective-interfering viral particles, virus mutants, antigenic drift or the interferon system.

4. It is the first report of the heterogeneous thermosensitivity properties of coronaviruses produced by various cell lines.

5. The capacity of virus progeny from persistently infected lymphoid cell, to induce a lethal acute disease when injected into susceptible mice is rapidly abrogated.

6. It is the first demonstration of the ability of lymphoid cells and macrophages to modify in vitro virus infectivity according to the in vivo sensitivity of the mouse strain.

7. It is the first report of the establishment of persistent infectins in embryonic fibroblast cell cultures.

8. It is the first demonstration that a persistent infection in embryonic fibroblast cells is related to the in vivo phenotype of mouse strains and is expressed by resistance to virus-induced cell lysis.

9. These studies present different virus-host cell systems under genetic control in which neither defective-interfering viral particles nor interferon seem to be involved.

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INTRODUCTION

The pathogenesis of virus persistency is poorly understood and difficult to study, and such infections are therefore difficult to treat. The use of an animal model should enable us to understand the interaction between viral infection and immune or non-immune mechanisms which lead to susceptibility and virus persistency. Various virus-cell interactions are involved in the production of slow or persistent viral infections. Studies of these relationships in the host reach a high level of complexity because of the mosaic of different cells and the diversity of host responses.

Mouse hepatitis virus 3 infection provides an interesting experimental model since infected mice display various types of sensitivity depending on the inbred strains tested: resistance, full susceptibility leading to acute hepatitis and death in 5-7 days and semi-susceptibility characterized by a chronic disease with progressive neurological involvement, paralysis and subsequent death. In this persistent virus infection, as well as in those induced by other viruses, there is little or no experimental evidence of the mechanism involved. It is important, therefore, to use an experimental model to study the host determinants which may contribute to the persistence of viral infection, e.g., age, degree of maturity and competence of immunological factors, virus-permissive cell interactions and their relation to genetic factors.

Pathogenic, viral and immunological mechanisms involved in the establishment and maintenance of in vivo and in vitro viral persistency were reviewed herein with respect to human and animal diseases. Recently acquired knowledge concerning the coronavirus group, particularly coronavirus-induced murine diseases, were described in relation to viral structure, replication and persistence. The pathological, immunological, virological and genetic aspects of MHV_3 infection in vivo and in vitro were extensively reviewed.

In order to elucidate the mechanisms of MHV_3 persistency and inborn

resistance, persistent in vitro infections were induced in mouse lymphoid cell lines, embryonic fibroblast cells, lymphocytes and macrophages from mouse strains exhibiting various sensitivities. The mechanisms involved in the establishment and maintenance of these infections as well as in the virus-host cell interactions were tentatively identified.

CHAPTER 1 : BIBLIOGRAPHICAL REVIEW

1.1 VIRUS PERSISTENCE

1.1.1 GENERAL MECHANISMS OF VIRAL PERSISTENCY

The persistently infected animal may serve as a reservoir of viral genetic information, but the virus must be intermittently expressed in order to spread the viral infection to other hosts. Persistent states of viral infection are determined by host-virus relationships. For virus to persist following an acute phase of disease in an immunocompetent host requires that the virus be able to evade normal immune surveillance and clearance mechanisms. The following general circumstances can permit the establishment and maintenance of viral persistency:

1. Virus must be sequestered in a "protected site" (e.g., the brain or cellular elements of the immune system) which prevents viral antigen detection by the humoral or cellular immune system.
2. Persistent virus could be less immunogenic or nonimmunogenic.
3. Viruses could elicit large quantities of non-neutralizing antibodies which interfere with neutralizing antibody action. Non-neutralized virus-antibody complexes could be ingested by the reticuloendothelial system, permitting viral replication in these cells.
4. Less cytocidal or noncytocidal viruses, which are able to replicate freely without causing the death of the host, could induce tolerance.
5. Viral persistency may result from viral growth in a less permissive cell type (Stroop and Baringer, 1982).

1.1.2 PATHOGENESIS OF VIRAL PERSISTENCY

The pathological effects of viral persistency are related to the various types of persistency. Human or animal hosts can support persistent viral infections in which viral antigens or virions are produced without being shed externally. These phenomena are seen in the brain of patients affected by subacute sclerosing

panencephalitis (SSPE) or by progressive multi-focal leukoencephalopathy (PML), and in sheep afflicted with maedi-visna (MV). The shedding of virus to the outside world can sporadically occur as the result of reactivation from a latent state such as is the case with herpesviruses. Finally, some viruses can replicate in cells involved in the immune system and are continuously being shed to the exterior, e.g., lymphocytic choriomeningitis (LCM) virus in mice (Mims, 1982).

Persistent virus infection can induce glomerulonephritis, vasculitis and other pathological changes by providing a continuous supply of antigens for the continued generation of circulating immune complexes, as demonstrated with LCM (Oldstone and Dixon, 1969) or with lactic deshydrogenase-elevating (LDV) (Oldstone and Dixon, 1971) viruses in persistently infected mice. Similar effects were observed in mink persistently infected with Aleutian mink disease virus (AMD) (Porter et al., 1980).

Persistent viruses would be able to give rise to long-term pathologies. Long-term changes in infected cells could be manifested by a shortening of life-span, important in the non-renewing cell populations in the central nervous system, and by alterations in the quality or rate of production of extracellular products such as collagen, myelin, immunoglobulins and hormones (Mims, 1982). Some diseases have been associated with long-term virus-induced pathologies, e.g.,juvenile diabetes with Coxsackie viruses (Yoon et al., 1979), SSPE with measles virus (Bouteille et al., 1965), PML with papovavirus (Padgett, 1971) in man, MV in sheep (Gudnadottir, 1974) and AMD in mink (Porter et al., 1969).

The low pathogenicity of persistent viruses, compatible with host survival, can induce minimal pathological damage like that seen with cytomegalovirus, polyoma-like viruses (JC and BK) and LCM and LDV viruses in mice (Mims, 1982).

Latent persistent infection can be established by retroviruses such as equine infectious anemia (EIA), and MV viruses or by herpesviruses. The

pathogenesis of these infections is characterized by the integration of proviral genome. Under certain circumstances, integrated virus become lytic and causes recurrences such as those seen with EIA virus (Crawford et al., 1978) and herpesviruses (Klein, 1982). Maedi-Visna disease is defined as a slow virus infection in which gradual neurological and respiratory manifestations were observed. However, the integrated genome present in the less permissive cells may provide a source of new, antigenically different virus (Stroop and Bahringer, 1982).

1.1.3 HOST DEFENSE MECHANISMS IN PERSISTENT VIRAL INFECTIONS

The majority of persistent viruses can invade lymphoreticular tissues (Mims, 1978). Viruses invading lymphoid tissues can disturb normal processes concerning the movement and handling of antigens. The invasion of lymphoreticular tissues allows the virus to evade immune defences. The close interaction between persistent viruses and lymphoreticular tissues can be reflected in various ways, such as generalized immunosuppression induced by unrelated antigens in the initial stages of an infection (Notkins et al., 1970), autoimmune phenomena occuring in Epstein-Barr virus and cytomegalovirus infections (Mims, 1982) and gross lymphoid necrosis in some persistent viral infections (Mims and Tosolini, 1969; Mims and Gould, 1978; and Murphy et al., 1977).

However, interactions between immune system components and persistent virus differ for each host-virus system. Multiple mechanisms of viral persistence seem to be involved in each disease and to contribute to the morbidity of the host.

1.1.3.1 Subacute sclerosing panencephalitis (SSPE)

SSPE is a chronic progressive encephalitis of childhood or adolescence associated with measles virus. The immune system can be involved at various levels in measles virus persistency . The population of virus variants may undergo a selection process due to the normal immune response of the host.

In persistent measles virus infection of hamsters, the host antibody response to the virus seemed to be involved in converting the virus from a cell-free to a cell-associated state (Byington and Johnson, 1973; Johnson and Norrby, 1974). The cell-associated phenotype of these variants was related to the selective loss of M antigen at the time when serum antibody levels were increasing. Incomplete synthesis of M protein was accompanied by a decrease in the antigen density of the viral glycoproteins on the surface of infected cells (Johnson et al., 1981). The viral genome could thus persist in those cells not lysed by immune mechanisms. In addition, persistence of the viral genome can alter normal host-cell functions and result in cellular expression of normally sequestered antigens. Panitch et al. (1980) have detected antibodies to myelin basic protein in the cerebrospinal fluid of patients with SSPE.

1.1.3.2 Murine lymphocytic choriomeningitis (LCM) virus infection

Persistent LCM virus infection is the usual outcome of an infection acquired in utero or during the neonatal period. The regular development of persistence appears to be related to the immaturity of the immune system (Lehmann-Grube, 1971). In LCM virus carrier mice, tolerance was associated with an impairment of the cell-mediated immune response since persistently infected animals showed minimal evidence of cytotoxic T cell activity against the persistent virus (Cole et al., 1973; Dunlop and Blanden, 1977). Borden and Nathanson (1974) have proposed that persistence was related to the ability of the virus to infect lymphoid cells. Infectious centers of virus have been demonstrated in peripheral blood, lymphocytes and in monocytes of spleen lymph nodes and thymus of carrier mice (Popescu et al., 1979). However, the relation between the primary defective cell-mediated immune response and viral infection of lymphocytes is still unclear (Rawls et al., 1981).

1.1.3.3 Murine lactate deshydrogenase-elevating virus (LDV) infection

LDV infection of mice produces widespread lymphoid hyperplasia and splenomegaly. Shortly after LDV infection, mice exhibit a transient decrease in the number of lymphocytes in the thymus, in thymic-dependent areas of the spleen, and in the blood (Snodgrass et al., 1972).

LDV infection has been shown to affect the functional capacity of the immune system by enhancing antibody production and depressing the induction of tolerance, graft-versus-host reactions and phagocytosis. Thus, LDV may induce immunosuppression of the host as evidenced by accelerated tumor growth and potentiation of severity of the protozoan infections (Notkins, 1971).

1.1.3.4 Aleutian mink disease (AMD)

AMD is a chronic, slowly progressive condition characterized by glomerulonephritis, arteritis and a systemic proliferation of plasma cells resulting in a marked hypergammaglobulinemia. The hypergammaglobulinemia may progress to a monoclonal IgG gammopathy which is secondary to the plasma cell proliferation. Although anti-AMD virus antibodies were present in high serum levels, they were non-neutralizing (Porter et al., 1969). Thus, AMD may cause a severe breakdown of normal immune regulation (Stroop and Baringer, 1982).

1.1.3.5 Herpesvirus infections

Human herpesviruses (Herpes simplex (HSV) types 1 and 2 and Epstein-Barr viruses (EBV)) have been the most studied with respect to their ability to produce latency, and to establish a relationship between latency and appearance of disease. Virus latency was established in dorsal or autonomic ganglia (Price et al., 1975). Viral reactivation in vivo has been demonstrated in the presence of antibody, following UV-irradiation or cyclophosphamide treatment in chronically

infected immunocompetent mice (Openshaw et al., 1979). Thus, the host's immune response is crucial for the control of the acute phase of ganglionic infection and plays a role in converting the infection from the acute to the latent phase . More information is required to define the role of the immune system during HSV infections and to establish its importance in the modulation of viral expression.

1.1.3.6 Equine infectious anemia (EIA) and maedi-visna (MV)

EIA and MV are two additional diseases in which viral persistence is a feature. These viruses can infect permissive cells where normal viral maturation occurs and non-permissive cells in which a proviral genome is integrated. Persistence is characterized by episodic production of virus with different antigenic specificities (antigenic drift) under selective antibody pressure (Crawford et al., 1978, Narayan et al., 1977).

1.1.3.7 Other persistent infections

Progressive rubella panencephalitis, resulting in the congenital rubella syndrome, seems to be associated with partial virus tolerance and immune complex formation (Stroop and Baringer, 1982). Theiler's virus infection of mice causes acute encephalomyelitis and limb paralysis after intracranial inoculation (Daniels et al. 1952)., Lysis of infected glial cells could give rise to a primary demyelination followed by a secondary immune-mediated demyelination.

1.1.4 VIRAL PROPERTIES IN PERSISTENT INFECTION

1.1.4.1 Selection of virus mutants

Most viruses have been involved in the establishment of persistent infections in various types of cells. Virus evolved in persistently infected cell cultures often differs from parental virus in: 1) virulence markers, including

cytopathogenicity, growth rate and plaque type in permissive cells, and pathogenicity for experimental animals; 2) host range in cell culture; 3) properties such as thermal stability; and 4) thermosensitivity at various temperatures. Thermosensitive (ts) small-plaque mutants evolve in many cells persistently infected with herpesviruses (Hinze and Walker, 1961), paramyxoviruses (Preble and Youngner, 1972), rhabdoviruses (Mudd et al., 1973), togaviruses (Simizu and Takayama, 1969), and arenaviruses (Hotchin et al., 1971). The evolution of ts mutants may also occur during in vivo persistent infection with foot and mouth disease (Straver and van Bekkum, 1972) and SSPE measles viruses in natural hosts (Hodes, 1979). The association between attenuation, small-plaque morphology and thermosensitivity has been often noted, but these changes were due to independent mutations (Simizu and Takayama, 1971; Straver and van Bekkum, 1972; Preble and Youngner, 1973). A relationship between small-plaque phenotype and lack of virulence for both animals and cell cultures has also been demonstrated with measles virus (Rapp, 1964) and Newcastle disease virus (NDV) (Schloer and Hanson, 1968; Reeve and Poste, 1971). In addition, unlike virulent strains of NDV cannot replicate at high temperature (Jones and Hanson, 1976).

The ts phenotype possesses a selective advantage in viral persistence since virus mutants could be replicated at semirestrictive temperatures which allow perpetuation of virus without rapid cell killing. However, a combination of thermosensitivity, attenuation and small-plaque morphology is not a constant feature of persistent infection since 1) some cell cultures persistently infected with measles virus did not generate ts mutants (Wild and Dugré, 1978) and 2) persistent infection initiated with ts mutants of reovirus resulted in the evolution of ts revertants (Fields et al., 1978).

Virus recovered from persistently infected cell cultures can differ from parental virus in properties related to structural proteins. Choppin et al. (1975) suggested that in persistent paramyxovirus infections showing an intracellular

accumulation of defective nucleocapsids, the virus glycoprotein precursor was not cleaved by proteolytic enzymes from host cells.

1.1.4.2 Defective-interfering particles

Defective-interfering (DI) particles are defined as virus particles which lack a portion of the genome, contain normal virus structural proteins, replicate only with the aid of infectious standard helper virus and interfere specifically with the replication of homologous standard helper virus (Huang and Baltimore, 1970). DI particles of DI-like particles are produced by most groups of RNA viruses, e.g., orthomyxoviruses (VonMagnus, 1954), paramyxoviruses (Thorne and Dermott, 1976), rhabdoviruses (Huang, 1973), bunyaviruses (Obijeski et al., 1976), picornaviruses (Cole et al., 1971, McClure et al., 1980), togaviruses (Schlesinger et al., 1972) and reoviruses (Nonoyama et al., 1970). To date, there have been no reports of DI particle production by any member of the coronavirus group.

Several features of persistent infections may be related to the possible involvement of DI particles : 1) all cells in the carrier culture, or at least a large majority of these, may contain viral antigens; 2) carrier cultures are resistant to challenge by homologous virus but support normal replication of heterologous virus; and 3)the virus shed by the carrier culture shows a highly reduced infectivity to particle ratio and shows a strong interference with infectious virus. Another important difference between persistent infections involving DI particles and those not involving DI particles is the ease with which the latter are cured by antibody treatment. In contrast, the DI particle-mediated carrier state allows slow noncytocidal replication of virus within cells in the presence of antiviral antibody (Holland et al., 1976b). DI particles are mainly involved in persistent infections induced by LCM virus (Lehmann-Grube et al., 1969; Stanek et al., 1972), measles virus (Wild and Dugré, 1978), Sendai virus (Kumura et al., 1975; Nishiyama et al. 1976), vesicular stomatitis virus (Wagner et al., 1963) and rabies virus (Kawai et al. 1975).

DI particles clearly can be generated and replicated in vivo and affect the outcome of infection (Von Magnus, 1951; Kingsbury et al., 1970; Holland and Villarreal, 1975; Mims, 1956; Popescu and Lehmann-Grube, 1977). DI particles provided protection from a normally fatal dose of virus. The prophylactic protection of mice achieved with DI particles required replicating and interfering ability and was due to true homologous interference (Jones and Holland, 1980). In addition, DI-induced effects such as interferon induction (Marcus and Sekellick, 1977) and antigenic stimulation (Crick and Brown, 1977) might also play a role in protection.

The involvement of DI particles in persistent infections with DNA viruses has recently been established (Norkin, 1979; Henry et al., 1979; Stinski et al., 1979). The role of DI particles in the persistence of DNA viruses is understandable since viral DNA integration into cellular chromosomes offers a better mechanism for virus persistence.

1.1.4.3 Antigenic drift

Alterations allowing the virus to escape the virus neutralization by antibodies can perpetuate the viral infection in an immunologically competent host. This selection of antigenic variants under immune pressure has been described with herpes simplex virus type 1 (Ashe and Scherp, 1965), MV virus (Narayan et al., 1977) and EIA virus (Kono et al., 1973). Payne and Baublis (1973) have showed that viruses isolated obtained from patients with SSPE were different from viruses from acute measles disease.

1.1.4.4 Interaction between the selection of virus mutants and the interferon system

Sekellick and Marcus (1978, 1979) have proposed that initiation of persistent infection in cell cultures competent for the interferon production could

involve DI particles or virus mutants with the ability to increase interferon synthesis in infected cells. This hypothesis was supported by the demonstration of DI particles of vesicular stomatitis virus (VSV) which are able to induce interferon synthesis in vitro and by the inhibitory role of anti-interferon serum in L cells persistently infected with VSV (Nishiyama, 1977). However, the DI particles of VSV amplified from persistently infected L cells by serial undiluted passages were not more efficient inducers of interferon than DI particles from parental VSV (Frey et al., 1979). In contrast, persistent viral infection can be established in cell lines e.g., (Vero or BHK 21)which are defective interferon producers .

1.1.4.5 Interference with wild-type virus replication by ts mutants

The dominance of the replication of ts mutants over wild-type viruses at permissive temperature has been reported for NDV (Preble and Youngner, 1973), Sendai virus (Kamura et al., 1976), Sindbis virus (Stollar et al., 1974), VSV (Youngner and Quagliana, 1976) and reovirus (Chakraborty et al., 1979).

The spontaneous selection and maintenance of ts mutants in persistently infected cell lines may be explained by the dominance of the ts virus replication (Youngner and Preble, 1982). However, the mechanism governing the dominance of ts mutant replication is not well defined.

1.1.4.6. Integration of the viral genome

Viral information may persist when the viral genome is integrated into the host cell chromosome, modulating a shift from the dynamic state to the static state (latent infection). Integration of the viral genome in less permissive cells has been observed with EIA virus (Bishop, 1978), MV virus (House and Varmus, 1973) and herpesviruses (Puga et al., 1978; Bastian et al., 1972; Miller et al., 1973). Mechanisms involved in the establishment of the latent state are not well

elucidated. However, some researchers suggest that the maintenance of persistent latent infection may be associated with a transcriptional block (Galloway et al., 1979; Bishop, 1978; Haase et al., 1978). HSV thymidine kinase expression and host immune response (Sokawa et al., 1980; Tenser et al., 1979) may be required for the establishment of latent ganglionic infection.

1.1.5 PERSISTENT INFECTION IN LYMPHOBLASTOID CELL LINES

Lymphoblastoid cell lines cultured in vitro provide a convenient system for studying virus-host cell interactions. In spite of the number of viruses able to replicate in lymphoid cells, only a few in vitro persistent infections in lymphoid cell lines have been described. Persistent infections with attenuated neurovirulent or SSPE strains of measles virus have been established in human lymphoblastoid cell lines (Barry et al., 1976; Minagawa et al., 1976; Ju et al., 1978). Cells persistently infected with Schwarz (attenuated measles vaccine virus) strain contained nucleocapsid structures in both the nucleus and the cytoplasm and produced low viral titers, but the cap-formation of measles antigens on the cell membrane was rarely observed. In contrast, a neurovirulent TYCSA strain induced a persistent infection characterized by rare nucleocapsid structures in the nucleus, higher virus titers and cap-formation of measles antigens on the cell membrane (Minagawa et al., 1976). The mechanisms involved in virus persistence were not indentified. However, in studies of thermolability and Ag production at non permissive temperatures Ju et al. (1978) have demonstrated that a human lymphoblastoid cell line persistently infected with Edmonston measles strain induced an heteregeneous population of ts virus mutants. Factors such as interferon, DI particles and extracellular virus did not appear to be important in maintaining the persistent carrier state.

Persistent infections herpes simplex virus and cytomegalovirus were established in lymphoblastoid cell lines (Roumillat et al., 1980; Hammer et al.,

1981; Cummings et al., 1981; Renaldo et al., 1978, Robey et al., 1976; St-Jeor and Weisser, 1977). A dynamic-state infection was characterized by the continuous production of extracellular virus. Persistently infected cells grew as well as uninfected cells, except during occasional periods of crisis (Cummings et al. 1981). Persistently infected cultures were "cured" by treatment with HSV antiserum or by exposure to high temperature but were resistant to reinfection, suggesting that these treatments modulated a shift from the dynamic state to the latent infection. However, virus production was stimulated by PHA treatment (Cummings et al. 1981; Hammer et al. 1981). Antigenic changes in HSV or DI particles were not detected in persistently infected lymphoid cells. An increase in the number of ts variants was observed by Cummings and co-workers (1981), whereas only small-plaque variants were obtained after three months of persistence (Roumillat et al., 1980).

Persistent infections by VSV were restricted to B-cell lines since T-cell lines only supported lytic infection. Restriction of VSV replication in B cells was characterized by a reduction of VSV-RNA transcription and an inability of VSV to inhibit host cell protein synthesis (Creager et al., 1981; Nowakowski et al., 1973).

Polioviruses can induce persistent infection in human lymphoblastoid cell lines (Wallace, 1969; Carp, 1981). Cell viability of infected and mock-infected cells was similar. Persistently infected cell cultures were cured by antiserum treatment, and virus shed by lymphoid cells were thermosensitive and produces smaller plaques.

1.2 THE CORONAVIRUSES

1.2.1 BIOLOGY OF CORONAVIRUSES

The coronaviruses were first recognized and morphologically defined as a group by Tyrrell and co-workers (1968, 1975, 1978). They are defined as pleomorphic or rounded particles with a diameter of 60-220 nm., surrounded by a fringe or layer of typical club-shaped spikes. Coronaviruses have a genome consisting of single-stranded polyadenylated RNA of positive polarity. Viruses are released by internal budding into cytoplasmic vesicles derived from the endoplasmic reticulum. These viruses are widespread in nature and are associated with various diseases.

Avian bronchitis virus (IBV), human coronavirus (HCV) and Parker's rat coronavirus induce respiratory diseases. Bovine, canine and turkey coronaviruses, as well as porcine transmissible gastroenteritis (TGE) virus are associated with gastrointestinal disorders. In addition, porcine hemagglutinating encephalomyelitis virus (HEV) is responsible for vomiting, wasting syndrome and encephalomyelitis in pigs. Murine hepatitis viruses (MHV) can cause various diseases such as encephalomyelitis, hepatitis and diarrheae. Curiously, feline infectious peritonitis (FIP) virus induces peritonitis and granulomatous inflammations in multiple organs, and adenitis is seen with rat sialodacryoadenitis virus. Unclassified members (coronavirus-like) are also responsible for gastrointestinal disorders such as foal and human enteric, parrot and porcine coronaviruses (Wege et al., 1982).

Antigenic relationships between the different coronaviruses are based on results obtained by various serological methods. The avian and the mammalian coronaviruses each appear to fall into two distinct and unrelated groups. Few coronaviruses, such as IBV and MHV, possess more than one serotype. The antigenicity of coronaviruses is related to three major antigens (Hajer and Storz, 1978; Yaseen and Johnson-Lussenburg, 1981). Surface glycoproteins of nonavian coronaviruses are responsible for the induction of neutralizing, complement-fixing

and hemagglutination-inhibiting antibodies (Garwes et al., 1978; McNaughton et al., 1981; Schmidt and Kenny, 1981).

Studies of nucleic acid homologies have been performed by two methods. Hybridization with MHV-specific cDNA showed that a close relationship existed between murine strains MHV-A59, MHV-3 and JHM whereas no homology was detected between the murine and the human 229E coronaviruses (Weiss and Leibowitz, 1981). Another approach using the technique of T_1 oligofingerprinting has demonstrated genomic variations in MHV strains according to the neurovirulence (Lai and Stholman, 1981a; Weiss and Leibowitz, 1981; Wege et al., 1981). These variations did not seem to correlate with the serological relationships of these viruses.

1.2.2 PATHOGENESIS OF MOUSE CORONAVIRUSES EXCEPT MHV-3

The first murine coronavirus isolated from a spontaneously paralyzed mouse was the MHV-JHM strain (Cheever et al. 1949). Subsequently, other strains were isolated from the various organs of mice with different diseases. The diseases resulting from MHV infection are hepatitis, encephalomyelitis and enteritis. However, it is impossible to classify virus strains by the target organ since several organs can be affected and the type of disease is related to the age and genetic background of the host. The virulent strains MHV_2 and MHV_3 and the less virulent MHV_1, MHV_5 and MHV_{A59} can cause hepatitis in newborns and adult mice, MHV_5 induces enteritis in newborns.

The first evidence of an association of host genes with resistance to MHV infection was reported for MHV_2: adult C3H mice were fully resistant to the infection, but PRI mice showed a fulminant hepatitis with high mortality (Bang and Warwick, 1960). Procedures affecting T-cell functions such as thymec tomy (Sheets et al., 1978) and cortisone treatment (Gallily et al., 1964) abolished the

resistance to MHV_2 infection. However, lymphocyte stimulation by concanavalin A induced resistance in susceptible mice (Weiser and Bang, 1977). The in vivo sensitivity to MHV_2 was reflected in cultured peritoneal macrophages which supported virus replication (Bang and Warwick, 1960; Taguchi et al., 1976). The host cell resistance to viral replication was not caused by the lack of specific receptors since cultured macrophages from resistant and susceptible strains adsorbed virus equally well (Shif and Bang, 1970 a, b). Hepatitis caused by MHV_N appeared only when mice were immunosuppressed (Hirano et al., 1979). Viruses isolated from nude mice (MHV_{NuV}, NuA and Nu66) induced chronic hepatitis in athymic mice (Hirano et al., 1975; Tamura et al., 1975; Tamura et al., 1976). Interestingly, Tamura and co-workers (1977, 1980) found in infected athymic nu/nu mice humoral immune response to a thymus-dependent antigen normally not detectable in these animals. Thus, the normal phagocytic functions of macrophages, T-cell functions and humoral immunity were required for protection.

Murine coronaviruses can cause encephalitis in suckling and adult mice (Hirano et al., 1981). The JHM strain is a neurotropic virus causing acute and chronic demyelinating diseases (Cheever et al., 1949). Oligodendrocytes are the main target cells for JHM (Lampert, 1973), but Fleury et al., (1980) have found that the virus can infect neuronal, ependymal and endothelial cells from the young mice. Infected mice showing no clinical manifestation during the first 2 weeks or recovering from the acute disease can develop a chronic demyelinating disease (Herndon et al. 1975). However, virus can be recovered from the liver, brain and spinal cord only during the first days postinfection (Stohlman and Weiner, 1981). Immunosupression shortly after infection enhanced the severity of the disease (Weiner, 1973) but did not affect the outcome of the chronic disease (Stohlman and Weiner, 1981). Ts mutants have been isolated from JHM-infected cell cultures, and some of them appeared less virulent than wild virus. Virulence was related to

the permissivity of the cells since the wild-type virus replicated in both neuronal cells and oligodendrocytes, whereas a ts mutant selectively replicated in oligodendrocytes of the spinal cord (Knobler et al., 1981a,b). Resistance to JHM-induced encephalomyelitis was under the control of at least two host recessive genes, not strongly associated with the H-2 complex (Stohlman and Frelinger, 1978). The development of resistance was correlated with the maturation of macrophages (Stohlman et al., 1980; Stohlman and Prelinger, 1981). In addition, a correlation was observed between JHM-infected macrophages in cultures from resistant and susceptible strains and the outcome of the disease in vivo (Knobler et al., 1981b). However, a mature immune system was not sufficient to protect suckling mice against infection since protection could be transferred with immune spleen cells only and not with normal nonimmune cells (Pickel et al., 1981).

Several enteropathogenic strains of MHV have been isolated: lethal intestinal virus for infant mice (LIVIM) (Kraft, 1962); MHV_{DVIM} (Sato et al., 1976) and MHV_D (Ishida et al., 1978). These viruses were responsible for acute intestinal disease with a high mortality rate during the first 3 weeks of life. The mothers of affected litters were clinically healthy but necrotic foci were found in the liver.

In conclusion, these results suggest that the development of acute, subacute or chronic MHV infections is related to the virulence of the virus and host factors such as age, immune status and genetic background. The pathogenic properties of a viral strain are supported by viral gene sequence. In order to define viral RNA sequences involved in pathogenicity, Lai and co-workers have compared the genomes of several MHV strains and variants by oligonucleotide fingerprinting (Lai and Stohlman, 1981 a, b; Lai et al., 1981). In comparing the virulent JHM large-plaque variant and a less virulent small-plaque variant, they found one unique oligonucleotide sequence missing in each variant. Similar results were obtained when hepatotropic MHV_3 was compared with the less pathogenic $MHV\text{-}_{A59}$.

However, the respective mRNAs and proteins of these oligonucleotides have not yet been identified.

1.2.3 STRUCTURE OF CORONAVIRUSES

The morphology of coronavirions has been described as pleomorphic, although generally spherical particles with a corona of widely spaced club-shaped surface projections. The virus envelope consists of a distinct pair of electron-dense shells and, in negatively stained preparation, an inner tongue-shaped membrane was visible (Bingham and Almeida, 1977, Lamontagne et al., 1980). Internal components were not visualized in negatively stained preparations of intact virions (Tyrrell et al. 1978), but may be visible in thin strand (Apostolov et al., 1970). The ribonucleoprotein has been seen as a long thin strand 1-2 nm in diameter (Davies et al. 1981) or as a helical form condensed into coiled structures of varying diameter (McNaughton et al., 1978).

The coronavirus genome is a positive single-stranded molecule of RNA containing about 18,000 nucleotides (Robb and Bond, 1979). T_1 oligonucleotide mapping indicated that no extensive sequence reiteration occurred in the coronavirus genome (Lomniczi and Kennedy, 1977, Lai and Stohlman 1981 a, b) and that the 3' end of the genomic RNA was polyadenylated (Yogo et al., 1977; McNaughton and Madge, 1978).

The nucleocapsid protein possesses a molecular weight of 50,000-60,000 and is non-glycosylated (Siddell et al. 1982). A cyclic AMP-independent protein kinase was associated with the virion, but it is not yet known if the enzyme is virally coded or a sequestered host cell enzyme (Siddell et al., 1981a).

The virion possesses a lipid envelope containing matrix and peplomer proteins. All coronavirions have a glycoprotein of 20,000-30,000 mol. wt. A small glycosylated portion of the molecule was peripheral to the lipid membrane (Sturman, 1977), whereas a second strongly hydrophobic domain was thought to

correspond to a portion of the molecule integrated into the lipid membrane (Sturman, 1981). A stable complex between this protein and viral RNA was formed in vitro suggesting a third domain in the protein which was internal to the lipid membrane and was responsible for the interaction with viral nucleocapsids. (Sturman et al., 1980; Sturman, 1981). The glycosylation of this matrix protein was not prevented by tunicamycin treatment, and virions released from tunicamycin-treated cells were noninfectious and lacked peplomer proteins (Sturman, 1981, Rottier et al., 1981b). In addition, a minor polypeptide of 14,000-14,500 mol. wt. has only been described for IBV (Stern et al., 1981) and MHV_{A59} (Rottier et al., 1981b). Peplomer proteins are glycoproteins of mol. wt. 80,000-200,000 with one or two major species deriving from a single primary translation product but modified by post-translational cleavage (Sturman and Holmes, 1977). Glycosylation of the MHV peplomer protein was inhibited by tunicamycin. The lack of reabsorption and cell fusion observed in tunicamycin-treated cells suggests that the peplomer protein plays a role in the reception of virions on cell surfaces and in the induction of cell fusion (Sturman et al., 1981). Finally, the viral envelope contained lipids in proportions approximately corresponding to those in the cell. When grown in different cell types, the viral membrane reflected the lipid content of the host cells suggesting that the lipids of the virion were derived from the host cell (Pike and Garwes, 1977).

1.2.4 CORONAVIRUS REPLICATION

Coronavirus infection is initiated by one-hit kinetics, the subsequent phase of 2-4 h is followed by a period of approximately 6 h during which virus is released into the medium. The one-step growth curve at 37°C was completed within about 10-12 h (Siddel et al., 1982).

Cytopathic changes accompanying coronavirus infections were described as cellular vacuolation leading to cell disintegration and syncytium formation (Tyrrell

et al., 1978). However, no studies were performed on host cell DNA, RNA or protein synthesis in infected cells. Viral replication occurred exclusively in the cytoplasm of infected cells without the involvement of any nuclear function (McIntosh, 1974; Robb and Bond, 1979a; Wege et al., 1981b; Wilhelmsen et al., 1981; Brayton et al., 1981). In contrast, Evans et al., (1980) have reported that the growth of IBV was prevented in enucleated, irradiated or α-amanitine treated BHK-21 cells.

Coronavirions were quickly attached over the whole cell surface but were rapidly carried away from the cell periphery by an energy-requiring process (Patterson and McNaughton,1981). Viropexis and envelope-membrane fusion have been described as the mechanisms of penetration (Patterson and Bingham, 1976; Doughri et al., 1976). Virus-specific RNA was detectable 4 h after infection and was distributed in six or seven major cytoplasmic single-stranded RNA segments (Spaan et al., 1981; Wege et al., 1981c; Lai et al., 1981). A virus-specific RNA polymerase which has been isolated only from TGE virus-infected cells showed low activity (Dennis and Brian, 1981; Mahy et al., 1983). The virus-specific polypeptides were encoded in separate subgenomic mRNAs and were translated independently (Siddell, 1983).

The most readily detected immunoprecipitable polypeptide in coronavirus-infected cells (3-4 h post infection) was nonglycosylated with mol. wt 50,000-60,000. This protein was identified as the intracellular form of the virion nucleocapsid protein (Bond et al., 1979; Rottier et al., 1981; Siddell et al., 1981). Intracellular polypeptides with a similar but slightly lower molecular weight than the nucleocapsid protein have been described but appeared to be degradation products of the nucleocapsid protein (Stern et al., 1981). A second nonglycosylated polypeptide (M protein) of 20,000-30,000 mol. wt was synthesized and post-translationally glycosylated before incorporation into the virion (Holmes et al.,

1981; Rottier et al., 1981). The coronavirus matrix protein was localized in tight clusters in the perinuclear area of infected cells (Doller and Holmes, 1980; Holmes et al., 1981), in contrast to the cytoplasmic distribution of envelope glycoproteins.

The third major polypeptide corresponded to the intracellular precursor of the virion peplomer protein. Sturman and co-workers (1981) suggested that the polypeptide was synthesized as a 180,000 mol. wt. species which was cotranslationnally glycosylated and subsequently incorporated into the virus after cleavage which yielded two polypeptides of 90,000 mol. wt. but showing different primary structures. The proteolytic cleavage of MHV_{A59} virion peplomer protein can be produced in vitro using trypsin.

Assembly of coronaviruses was restricted to the cytoplasm where progeny virions were formed from membranes of the rough endoplasmic reticulum by a budding process (Massalski et al. 1981, 1982). The virion obtained the lipid membranes from the cell, excluding host cell proteins in the process, and was subsequently transported through the Golgi complex and accumulated into smooth-walled vesicles. Virions were released from the cell by cell lysis or by fusion of virus containing vesicles with the cytoplasmic membrane.

However, host cells may play a fundamental role in regulating the replication and expression of coronaviruses since comparison of JHM virus replication in various cell types revealed an abrupt cessation of virus assembly with the accumulation of core material at different times postinfection according to the host cell (Massalski et al., 1982). In addition, host-induced changes in viral thermosensitivity have been observed with JHM virus (Stohlman, 1979) and MHV_3 (Lucas et al., 1973).

1.2.5 PERSISTENT INFECTIONS

Coronavirus persistent infections may be established in cell cultures, but only few persistent infections have been described. The majority of coronavirus-induced persistent infections were carried out with MHV strain JHM (Stohlman and Weiner, 1978; Robb and Bond, 1979a; Lucas et al., 1977) and A59 (Holmed and Behnke, 1981; Robb and Bond, 1979a) in a variety of continuous cell lines of neural and non neural origin.

Mouse neuroblastoma cells persistently infected with JHM virus showed virus-specific cytopathic effects and produced virus that was not different from wild-type virus in plaquing behavior, thermosensitivity and in vivo pathogenicity. No DI particles or interferon were detected in the culture fluids (Stohlman and Weiser, 1978). Under antibody pressure, non-virus-producing cell clones were obtained and cold-sensitive mutants were rescued from cell clones after fusion with permissive cells by polyethylene glycol (Stohlman et al., 1979). In contrast, Lucas et al., (1978) reported the persistence of MHV_3 and JHM strains in various cell lines. Temperature shift experiments have demonstrated that the restriction of virus production at high temperature was due to a host factor and not to a selection of ts mutants.

A persistent infection by human coronavirus 229 E was established in the L-132 human cell line. Interferon, DI particles or reverse transcriptase could not be detected. Isolated viruses were similar to the parental virus except that viruses shed from persistently infected cells were more cytocidal than the parental virus. The results also suggested that temperature could play a still undetermined role in the establishment and maintenance of persistently infected cell cultures (Chalonner-Larson and Jonhson-Lussenburg, 1981).

1.3 MOUSE HEPATITIS VIRUS TYPE 3 INFECTIONS

1.3.1 PATHOLOGY OF MHV_3 INFECTION.

MHV_3 is one of the most virulent strains of mouse hepatitis virus. However, the severity and the type of infection is related to the mouse strain tested. Most strains show full susceptibility leading to death within a few days. The A/J strain is unique in that it is fully resistant with 100% survival of infected animals. However, other mouse strains are semisusceptible since animals surviving the acute disease develop chronic manifestations with progressive neurological involvement (LePrevost et al., 1975).

1.3.1.1 Acute MHV_3 infection

In some strains of mice, e.g., C57Bl/6, DBA/2, BalbC and NZB, parenteral administration of 10 LD_{50} of MHV_3 always leads to fulminant hepatitis and death. Peritoneal macrophages and liver Kupffer cells are the major sites of virus replication. Infectious viruses are disseminated to all organs including the liver, spleen, lymph nodes, brain and kidney during the viremic phase (Piazza et al., 1967). There were many areas in the liver in which acute necrosis of hepatocytes was associated with cellular infiltration marked by a predominance of polymorphs. The spleen also showed multifocal necrosis of the red pulp which was associated with multinucleate giant cells, unlike the megakaryocytes often seen there. Several small nuclei instead of the large, twisted forms were observed in the latter cells (Virelizier et al., 1975). Mice died in 5 to 10 days with a clinical picture indicative of acute hepatic failure (LePrevost et al. 1975a)

In contrast, full resistance to MHV_3 was observed in the A/J mouse strain even after the administration of large doses of virus ($10^7 LD_{50}$). Histopathological studies showed an absence of lesions. Resistant adult mice always

displayed a good antibody response after virus clearance. However, the full resistance of A/J mice was age-dependent since up to 15 days of age, neonates regularly died even when injected with low doses of virus. Resistance to the viral disease developed suddenly during the third week of life (LePrevost et al., 1975).

1.3.1.2 Persistent MHV_3 infection

In contrast to full susceptibility or resistance, F_1 (susceptible and resistant parents) C3H, AKR and A2G mice showed a genetically determined semisusceptibility leading to virus persistence (Virelizier et al., 1975, LePrevost et al., 1975). Animals surviving the acute disease after intraperitoneal injections developed a chronic disease starting after the 3rd week of infection. This phase was characterized first by incoordination and the development of paralysis. A wasting syndrome occurred before the onset of paralysis. The animals became hunchbacked. The spine and ribs became clearly evident through the skin, and dehydration, sometimes complicated by diarrhea, occurred. Almost all of the animals became paralyzed within 3 to 6 months after infection (LePrevost et al., 1975).

Histopathological examination of the liver from semisusceptible mice 11 to 240 days after infection showed occasional, small foci of a few necrotic liver cells accompanied by sparse cellular infiltration around them. The spleens of some animals contained many polymorphs. Thirty days postinfection, a few mice presented an acute renal cortical infarctus and active chronic vasculitis of capsular vessels with lymphocytes and plasma cells. Six months later, amyloid was found in large amounts in the spleen, liver and kidney and formed homogeneous eosinophilic masses (Virelizier et al., 1975).

Lesions observed in the spinal cord and brain varied according to the mouse strain examined. The neuropathological lesions observed in semisusceptible mice were vasculitis and meningoependymitis; the former predominated in C3H

mice, whereas the latter was seen in the A2G mouse strain. There was no demyelination of extensive neuronal damage other than the rims of spongy changes around well-defined vascular region was demonstrated. In contrast to the other organs, no amyloid was detected in neural tissues (Virelizier et al., 1975).

1.3.2 VIROLOGIC OBSERVATIONS OF MHV_3 INFECTIONS

During the first 4 days of infection, virus was recovered from the liver of resistant as well as susceptible mouse strains. In A/J strain mice, the viral titers were consistently 10^3 LD_{50}/ml, whereas in susceptible DBA/2 mice, titers greater than 10^4 were always found. In the resistant mouse strain, infectious virus was cleared from the liver, brain and serum within 7 days. In contrast, virus continued to replicate in susceptible animals until death (LePrevost et al., 1975).

The chronic period of the disease was characterized by a persistent viral infection since MHV_3 was recovered at very low titers from the brain, liver, spleen and lymph nodes. (LePrevost et al., 1975, Virelizier et al., 1975). Virus was recovered from paralyzed and nonparalyzed mice during the first 50 days following MHV_3 injection. However, no infectious viruses were found in paralyzed animals tested 80 days or more after inoculation. Macrophage culture regularly yielded virus when the cells were collected during the first 3 months of infection but rarely after that time (Leray et al. 1982). In addition, tests conducted 4 days after infection indicated that the clinical evolution was correlated with the virus titer in the serum (LePrevost et al., 1975). Immunofluorescent studies of brain tissue from persistently infected mice indicated that viral antigens were present in ependymal wall and choroid plexus cell populations. No fluorescence was found in neurons or meningeal cells. (Virelizier et al. 1975).

1.3.3 HUMORAL IMMUNITY

Humoral immunity does not seem to play an important role in resistance to MHV_3 since serum from immune A/J mice was relatively ineffective in confering protection to susceptible mice. In spite of the fact that A/J mice can produce antibodies after infection, no in vivo neutralizing activity was found. However, when newborns from immunized A/J mothers were injected during the first week of life, they did not die as rapidly as controls. This result suggested that maternal antibodies can modulate resistance since newborns remained susceptible after the disappearance of maternal antibodies (LePrevost et al., 1975). In addition, sublethal irradiation of immunized adult A/J mice did not abrogate the resistance to a virus challenge (Dupuy et al., 1975).

Chronic persistent viral infection was associated with a low titer of antibody. Kinetic studies indicated that complement-fixing antibodies were first detected 15 days post infection and regularly increased up to the end of the third month of infection. Titers decreased 180 days post infection and reached residual levels. An increase of antibody titers was observed at 60 to 70 days post infection. No correlation was observed between the onset of paralysis and the increase of MHV_3 antibody (Leray et al., 1982), in spite of the fact that bound immunoglobulins associated with viral antigens were found in choroid plexus vessels (Virelizier et al., 1975). In addition, most of the anti-MHV_3 antibody in chronically infected mice was of the IgM class.

In contrast to the development of anti-MHV_3 complement-fixing antibodies in chronically infected mice, there was a progressive decrease in all immunoglobulin types during the first three months, followed by a return to normal values in the surviving animals. Primary and secondary antibody responses were markedly diminished in paralyzed as well as in non-paralyzed mice. The MHV_3 induced

immunodeficiency observed during the chronic disease seemed to be related to virus-induced lympholysis and inhibition of lymphocyte proliferation.

1.3.4 CELL-MEDIATED IMMUNITY

Adult A/J strain mice are resistant to MHV_3 infection, but immunosuppressive treatments can abrogate the resistance. A/J mice exposed to x-irradiation (500 rads) or treated with anti-lymphocyte antiserum became susceptible to MHV_3 and died of an acute disease. Thymectomy and the graft-vs-host reaction also modified the response of resistant animals to virus infection (Dupuy et al., 1975).

Protection of newborn mice against MHV-3 infection requires the transfer of several cell populations originating from adult syngeneic donors: adherent spleen cells, T lymphocytes and a third population present in the non-adherent spleen cell fraction, in peritoneal exudates and in bone marrow cells. This cell population was responsible for resistance to MHV_3 and displayed some similarities to natural killer cells (Tardieu et al., 1980).

Activated lymphocytes can produce interferon that is able to modulate the immune response. High levels of natural killer (NK) cell activity and high titers of interferon were observed in peritoneal exudate cells derived from susceptible mice. However, NK cell activity and interferon titers in the peritoneal exudates were significantly lower in resistant than in susceptible mouse strains (Schindler, 1982). Permanent low levels of interferon were associated with chronic immunodepression (Virelizier et al.,1976). Thus, interferon and NK cells may not be of overwhelming importance in the defense of mice against MHV_3 (Schindler, 1982).

1.3.5 GENETIC STUDIES

Genetic study of acute and chronic MHV_3 diseases indicated that at least two recessive genes are involved in resistance to acute and chronic diseases and showed that the genes involved in both diseases were different. The capacity to resist the development of paralysis was also conferred to heterozygote as well as to homozygote mice by the $H\text{-}2^f$ allele, indicating that resistance to paralysis is H-2-linked (Levy-Leblond, 1979). Similarly, susceptibility to the acute disease has been found to be quantitatively influenced by the H-2 haplotype of semisusceptible mouse strains, as determined by serum levels of cobalt-activated acylase (Sabolovic et al., 1982).

1.3.6 CORRELATIONS BETWEEN IN VIVO AND IN VITRO SENSITIVITIES TO MHV_3 INFECTION

1.3.6.1 Macrophages

Virelizier and Allison (1978) have shown that there was in contrast to macrophages from fully susceptible mouse strains, no virus replication in macrophages from resistant strains, whereas in macrophage cultures from mouse strains in which persistent infections occur, an intermediate susceptibility was observed, as judged by the intensity of cytopathic effect, presence of the cytoplasmic viral antigens and levels of viral replication.

However, the in vitro resistance of macrophages to MHV_3 infection was not confirmed by McNaughton and Patterson (1980), Dupuy and co-workers (1980) and Taguchi and co-workers (1981). They observed that, although infected macrophages from susceptible mouse strains developed cytopathic effects sooner than those from semisusceptible or resistant mouse strains, the cytopathic effects

and viral titers were comparable for all macrophage strains. No difference in MHV_3 uptake was seen between macrophages originating from resistant, semi-susceptible and susceptible animals (Krzystyniak and Dupuy, 1981). However, macrophages obtained from infected resistant mice restricted viral replication as early as one hour postinfection (Dupuy et al ., 1980).

1.3.6.2 Hepatocytes

Hepatocytes constitute the primary target cells for MHV_3 infection. Cultured hepatocytes, isolated from resistant adult animals exhibited resistance to in vitro virus infection, whereas those from susceptible mouse strains were fully permissive for MHV_3 infection. Resistance was MHV_3 specific and was not related to the action of interferon (Arnheiter et al., 1982).

1.3.6.3 Lymphocytes

Lymphocytes were another target cell for MHV_3 infection since immunodepression was associated with lymphbcyte depletion and lympholysis. No information was available on viral infection of lymphocytes except that a similar uptake of radiolabelled MHV_3 was observed in whole spleen cells, purified T lymphocytes and thymocytes, and that viral replication can occur in lymphocytes (Krzystyniak and Dupuy, 1981).

CONCLUSIONS

This literature review has demonstrated that the host age and genetic background, the biological properties of the virus strain, and the dose and route of inoculation are the major factors which determine the outcome of coronavirus infection. For most coronaviruses causing enteric and respiratory diseases, the pathophysiological events leading to clinical symptoms are probably related to

acute cytocidal infection of the target cells and dissemination of the infection limited by a local humoral immune response. In contrast, many coronaviruses may be maintained and spread in the population as inapparent and subclinical infections, although only a percentage of animals develops a chronic disease. The sequence of events leading to chronic disease is still unknown. Viral persistency can be involved in the pathogenesis of acute and chronic diseases. The result depends on the expression of viral genes, the functional impairment of host cells and the interaction between the virus and the host's immune système. At the present time, no information is available on the mechanisms involved in the development and maintenance of persistent infections. However, the use of permanent cell cultures may be very helpful in this respect.

In several murine systems, the host's genetic background is an essential parameter which determines resistance and the outcome of disease. This genetic restriction can be expressed at cellular levels, but the mechanism for this restriction is as yet difficult to define and cannot be generalized to various cell systems.

Persistent MHV_3 infection of various cell systems appears to be an excellent model in which to analyze prolonged virus-host relationships. Immunological studies performed during chronic MHV_3 disease have revealed a pronounced state of immunodeficiency mediated by strong inhibition of lymphocyte proliferation and viral replication in lymphocytes and macrophages. Viral persistency in these cells was associated with progressive destruction of lymphoid organs, impairment of the antigen-processing step and inhibition of lymphocyte proliferation. In order to study the mechanisms involved in the viral persistency in lymphoid cells, we established persistent in vitro infection in mouse lymphoid cell lines. Cellular factors involved in the establishment and the maintenance of a such persistent viral infections were studied: outcome of infected cell populations and

individual cells, mode of transmission of the infection and role of cellular metabolism in the maintenance of the viral infection.

The establishment and maintenance of persistent infections can be affected by viral factors such as DI particles, occurrence of ts mutants, antigenic drift and the interaction between viral mutants and the interferon system. We analyzed physical, biochemical and pathogenic properties of virus progeny from persistently infected lymphoid cell lines.

The role of the host's genetic background in the occurrence of in vivo virus persistency was studied by the establishment and analysis of persistent infection in embryonic fibroblast, macrophages and lymphoid cell cultures originating from various mouse strains showing different in vivo sensitivities to MHV_3 infection.

These experiments helped to partially elucidate the mechanisms involved in the MHV_3 persistency with respect to the genetic status of the host.

CHAPTER 2: PERSISTENT INFECTION WITH MOUSE HEPATITIS VIRUS TYPE 3 IN MOUSE LYMPHOID CELL LINES

2.1 PREFACE

The sensitivity of mice to MHV_3 infection varies according to the strain, age and immune status of the animals (Dupuy et al., 1975; Le Prevost et al., 1975a). In semi-susceptible strains, mice surviving the acute phase of infection develop a chronic disease characterized by the occurence of paralysis, virus persistency and immunodeficiency. The mechanisms involved in in vivo viral persistency are still unknown.

The marked involvement of the lymphoid system in MHV_3-infected animals, the capacity of the virus to replicate in lymphocytes in vitro (Krzystyniak and Dupuy, 1981), the occurrence of lympholysis and the inhibition of antigen-driven lymphocyte proliferation (Leray et al., 1980) led us to develop an in vitro model of MHV_3 persistent infection in mouse lymphoblastoid cell lines.

We have demonstrated that MHV_3 persistent infection can be established in YAC and RDM4 mouse lymphoid cell lines and that the mechanism underlying viral persistency was related to that governing "carrier state" infections.

This article is the first part of a study of factors involved in MHV_3 persistency in lymphoid cell lines.

2.2 ABSTRACT

The sensitivity of mice to mouse hepatitis virus 3 (MHV_3) varies according to the strain, age and immune status of the animals. In semi-susceptible strains, mice surviving the acute phase of infection develop a chronic disease characterized by the occurrence of paralysis, virus persistency and immunodeficiency. Persistent MHV_3 infections established in vitro in YAC and RDM-4 mouse lymphoid cell lines were characterized by virus production, occurrence of cytoplasmic viral antigens and cell lysis. The occurrence of cell "crisis" in YAC cells was manifested by a sharp increase in cell lysis and in number of fluorescent cells and, concomitantly, by a marked decrease in virus titers. A relationship was observed between the percentage of fluorescent cells, cell lysis and virus yield and was modulated by renewal of culture media, change in temperature or inhibition of cellular RNA synthesis. The initial virus dose was important for the maintenance of MHV_3 persistency. Cell cloning and antibody treatment experiments indicated that the type of viral transmission was horizontal and not vertical. These data indicate that the persistent infection induced by MHV_3 in lymphoid cell lines is characterized by a viral "carrier state" in which production of infectious viral particles remains in equilibrium with cell permissivity.

2.3 INTRODUCTION

The sensitivity of mice to mouse hepatitis virus 3 (MHV_3) infection varies according to the strain, age and immune status of the animals (Dupuy et al., 1975; Le Prevost et al., 1975a). Three types of sensitivity were observed: resistance, full susceptibility and semisusceptibility. Semisusceptible mice acquire a chronic disease characterized by the occurrence of paralysis and viral persistency since MHV_3 can be recovered of most animals during the first 3 months postinfection from the brain, liver, spleen and lymph nodes (Le Prevost et al., 1975b). In addition, it was observed that resistance to the development of paralysis in semisusceptible F_1 hybrids was governed by genes of the H-2 complex (Levy-Leblond et al., 1979). During the chronic phase of the disease, a progressive immunodeficiency occurred which was related to lympholysis and the inhibition of antigen-driven lymphocyte proliferation (Leray et al., 1982). The marked involvement of the lymphoid system in MHV_3 infected animals, the presence of MHV_3 in lymphoid cells (Le Prevost et al., 1975b) and the capacity of the virus to replicate in vitro in lymphocytes (Krzystiniak and Dupuy, 1981) led us to develop an in vitro model of MHV_3 infection in lymphoblastoid cell lines. Persistent viral infections induced in vitro with coronaviruses (MHV_3 and JHM strains) have mainly been established in continuous cell lines of neural origin and in mouse myeloblast or rat hepatoma cells (Stohlman and Weiner, 1978; Lucas et al., 1978). The mechanisms involved in such persistent infections are still unknown.

Herein, we show that MHV_3 persistent infections can be carried out in vitro in permanent YAC and RDM-4 lymphoid cell lines and that persistency was related to a "carrier type" mechanism.

2.4 MATERIALS AND METHODS

2.4.1 CELLS

YAC and RDM-4 mouse lymphoid cell lines were obtained from Drs. S. Lemieux and D. Oth (Armand-Frappier Institute, Laval, Quebec). YAC cells (YAC-1 substrain) were derived from a Moloney virus-induced T-cell lymphoma of A/Sn origin and have been propagated as a suspension line in culture for several years (Yogeeswaran et al., 1981). RDM-4 cells originated from a B-cell lymphoma of AKR origin and were maintained by passages in vivo as well as in vitro (Lemonnier et al., 1977). Cells were grown in suspension culture in RPMI 1640 (Flow Laboratories, McLean, Va.) medium containing fetal calf serum at a concentration of 10% for YAC cells and 5% for RDM-4 cells, penicillin (100 U./ml) and streptomycin (100 mg/ml). Subcultures were performed by dilution with fresh culture medium when cell density reached 5×10^5 cells/ml (YAC cells) and 1×10^6 cells/ml (RDM-4 cells). The doubling time of YAC and RDM-4 cells was 18 h and 24 h, respectively. L2 cells, a continuous mouse fibroblast cell line, (provided by Dr. R. Anderson, University of Western Ontario, London, Ontario, Canada) were grown in Eagle minimal essential medium (MEM) with glutamine (2 mM), 5% fetal calf serum and antibiotics. L2 cells were used for the propagation, cloning and titration of MHV_3.

2.4.2 VIRUS

The MHV_3 used was a cloned substrain already described (Dupuy and Rodrigue, 1981). For virus production, L2 cells were infected with 10^4 $TCID_{50}$/ml. Infected cells were frozen when cytopathic effects were observed in 50% of the cellular monolayer. Virus suspensions obtained after two freezing-thawing cycles were clarified by

centrifugation at 1000 g for 30 min. Supernatants, stored at -70°C in small aliquots, were titrated in L2 cells and used as viral inoculum. Virus titer was calculated by the Reed and Muench method (Reed and Muench, 1938) and expressed as 50% of the tissue culture infectious dose ($TCID_{50}$).

2.4.3 ANTI-MHV_3 ANTIBODY

Anti-MHV_3 antibody (Ab) was raised in resistant A/J strain mice after 2 intraperitoneal injections of 1000 LD_{50} of virus given 2 weeks apart. Sera were collected three days after the last injection, pooled, titrated using the complement fixation (CF) test(1:1024) and kept at -70°C. Antisera were decomplemented at 56°C for 30 min before use. For Ab treatment, anti-MHV_3 Ab at a final concentration of 1:25. was added to persistently infected or uninfected cell cultures At each cell passage, the antibody concentration was adjusted to maintain initial values. After anti-MHV_3 Ab treatment, the cells were washed three times in excess of RPMI 1640 medium and were resuspended in fresh medium.

2.4.4 DETERMINATION OF CELL COUNT AND VIABILITY

In all cell cultures, cell counts and viability were regularly determined. Cell viability was assessed by the trypan blue exclusion test (Payment and Pavilanis, 1980).

2.4.5 VIRUS TITRATION

Viral suspensions were diluted serially in 10-fold steps in Earle's MEM. L2 cells cultured in 96-well microtiter plates were infected with 0.025 ml of the viral dilution and virus was allowed to adsorb for 1 h at 37°C. Culture medium without fetal calf serum was added (0.2 ml), and

the plates were incubated for three days at 37°C in 5% CO_2. Viral titers were expressed in $TCID_{50}$/ml. In lymphoid cell cultures, extracellular virus titers were determined in supernatants after centrifugation at 1000 g for 15 min. Sedimented cells, after washings and resuspension at the initial volume, were used for the determination of cell-associated virus titers.

2.4.6 INDIRECT IMMUNOFLUORESCENCE (IF) TEST

Sedimented cells of infected and uninfected control cultures were used for IF after the addition of 0.1 ml of Haygman medium (20 vol. bovine albumin 20%; 60 vol. PBS, pH 7.2; 8 vol. EDTA 5%), pH 6.8. Cells deposited in wells of Teflon glass slides (12 wells; Flow Laboratories, McLean) in a volume of 0.025 ml per well, were air-dried and kept at 4°C until fixation. Cell fixation was performed in a solution of methanol:acetic acid:PBS, (89:1:10), pH 7.2 at 20°C for 30 min. Glass slides were washed in PBS, pH 7.2 for 15 min under agitation and were air-dried. Four units of specific antiserum (0.025 ml/well) were added to the fixed cells in each well. The slides were incubated in a humidified atmosphere at 37°C for 30 min, washed twice in PBS under agitation for 15 min and then air-dried. Four units of either fluorescent anti-mouse IgG or anti-mouse Fab_2 antisera (Cappel Laboratories, Cochranville, Pa.) were added to each well. After further incubation and washings, slides were treated with glycerol-PBS (9:1) and examined under fluorescent microscopy. The intensity of the fluorescence was assessed on a scale ranging from 0 to 4. The percentage of cells with granular cytoplasmic fluorescence of 2 or more was determined for a minimum of 200 cells per sample. Each experiment included 4 controls: 1) similarly treated uninfected control cells, 2) infected cells incubated with fluorescent anti-mouse IgG antiserum alone, 3) a known positive control and 4) a negative serum control.

2.4.7 CELL CLONING

Cloning of infected and uninfected cells was performed by the limit dilution assay (1 cell per 0.2 ml of medium) in 96-well microtitration plates. The plates were centrifuged at 500 g for 10 min. All plates were checked daily and the number of cells in each clone as well as the number of clones were recorded. When the number of cells of clones reached 300, the cell clones were divided among 3 wells, and the plates were further incubated for 2 to 3 days until use.

2.4.8 ACTINOMYCIN D (ACTD) TREATMENT

The toxicity of ACTD (Sigma, St. Louis) was tested at different drug concentrations in normal lymphoid cell cultures. According to the experiments, various doses of ACTD were added when persistently infected and uninfected cells were subcultured.

2.4.9 EXPERIMENTAL DESIGN

In all experiments, studies were carried out in triplicate samples of MHV_3-infected and uninfected cell cultures or cell clones. The 3 identical samples were used for: 1) cell count and viability; 2) IF; and 3) determination of extra-cellular and cell-associated virus titers.

2.5 RESULTS

2.5.1 ESTABLISHMENT OF MHV_3-PERSISTENT INFECTION

Persistent infections were established in YAC and in RDM-4 cells (Fig. 1) and were maintained up to 100 days in some experiments. They were stopped at that time for convenience. In every culture, the number of viable cells was regularly counted and virus persistency was determined

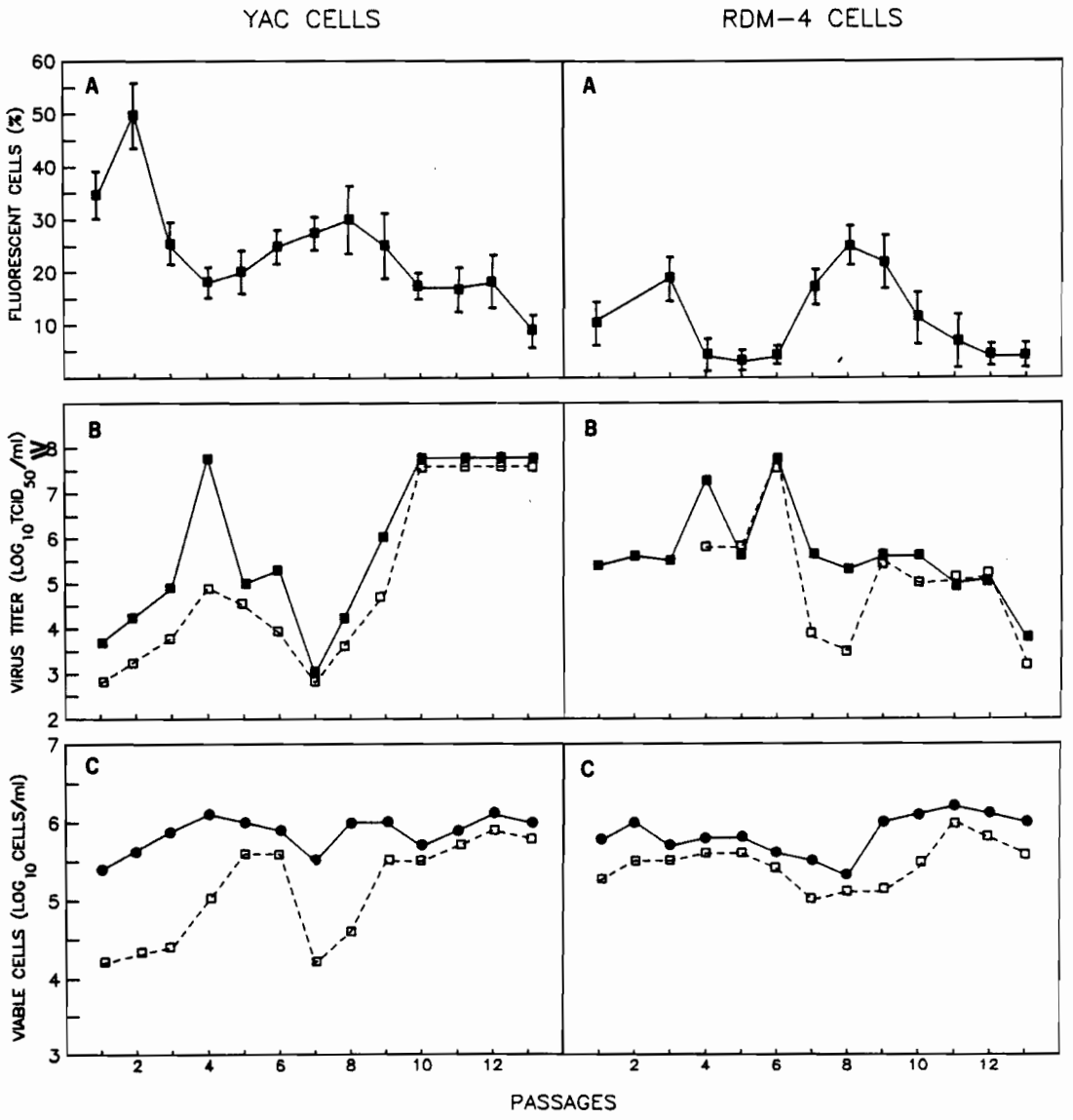


Figure 1: MHV_3 persistent infection induced in YAC and RDM-4 cells. Percentage of fluorescent cells (A); free (■—■) and cell-associated (□- -□) virus production (B); cell count of MHV_3 infected (□- -□) and uninfected (●—●) cell cultures (C).

by virus production and detection of cellular viral antigens by immunofluorescence. No difference in cell number was observed when infected and uninfected RDM-4 cells were compared. A marked difference, however, was observed in YAC cells (Fig. 1C). In addition, a cell "crisis", characterized by a drastic decrease in cell number, occurred in infected cell populations at passages 7 and 15 in this persistent infection (Fig. 1C).

Indirect immunofluorescence examination of infected and control cells revealed that the patterns of fluorescence varied according to the time of infection (Fig. 1A). Large fluorescent syncytia were frequently seen during the first 2 passages postinfection, whereas small unfused fluorescent cells, were observed thereafter.

Infectious virus was produced by both persistently infected cell lines but reached a higher titer in RDM-4 cells. Titers varied with time and no significant difference was seen between free virus and cell-associated virus titers (Fig. 1B) expected at passage 4 in YAC cells and at passages 4, 6 and 7 in RDM-4 cells.

The induction and maintenance of persistent MHV_3 infections in YAC and RDM-4 cells were tested at various m.o.i. In YAC cells, a persistent infection occurred only at m.o.i. greater than 1, whereas all m.o.i. induced viral persistency in RDM-4 cells (Fig. 2). Persistent MHV_3 infections in YAC cells have also been induced with 10 on 100 m.o.i. (results not shown).

In order to study the effect of fresh medium on the development of persistent infection, culture medium was changed every other day starting at the time of infection of YAC and RDM-4 cells with 1 m.o.i. As shown in Fig. 3, in infected YAC cells, this procedure resulted in marked increases in cell lysis, in fluorescent cells and in virus titers. No significant variations were observed in RDM-4 cells.

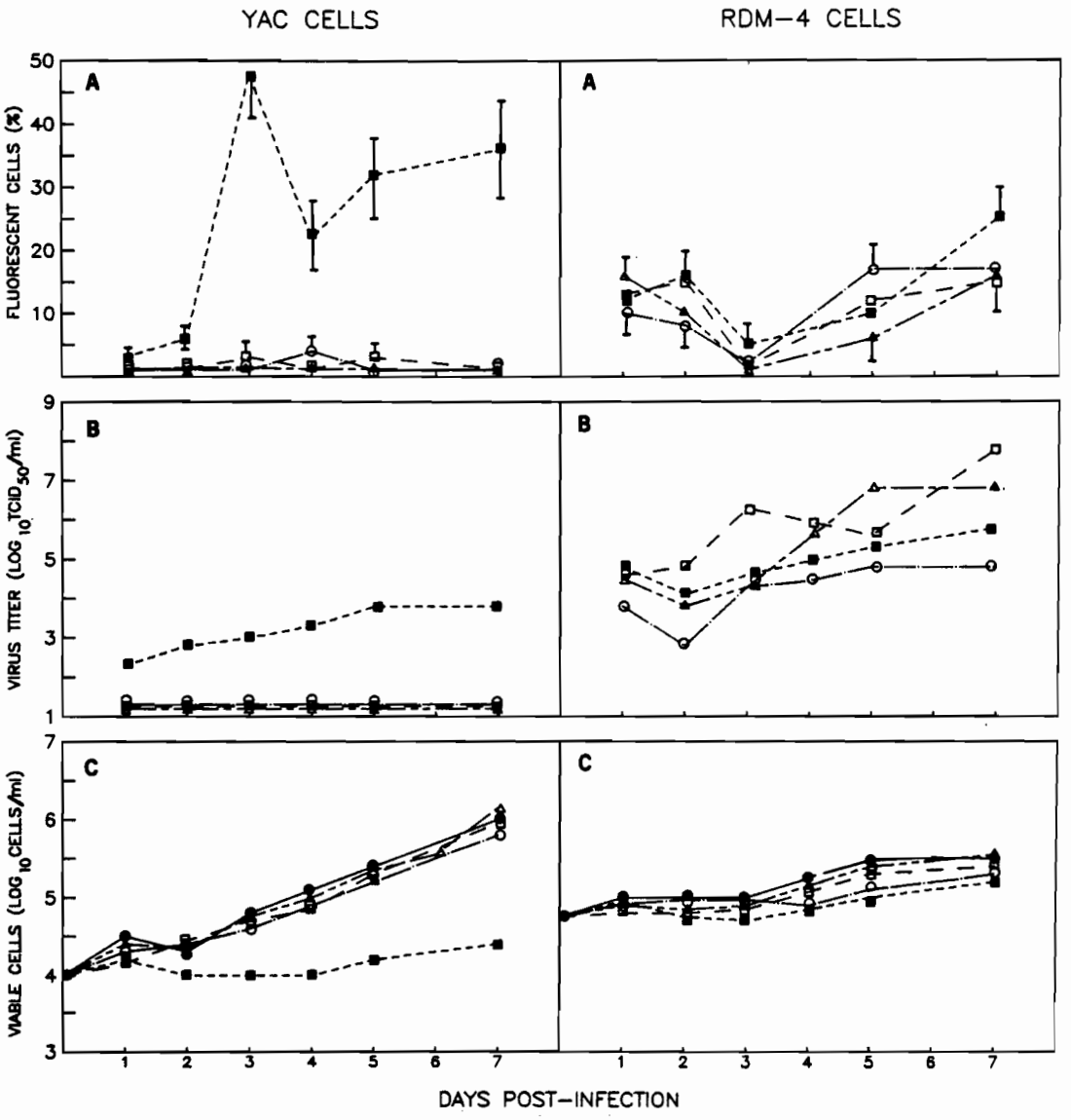


Figure 2: Effect of the dose of virus on maintenance of MHV_3 persistent infection in YAC and RDM-4 cells. Cells cultures were infected at various m.o.i: 1 (■–■); 0.1 (□–□); 0.01 (○–○) and 0.001 (△–△). Uninfected control cells (●–●).

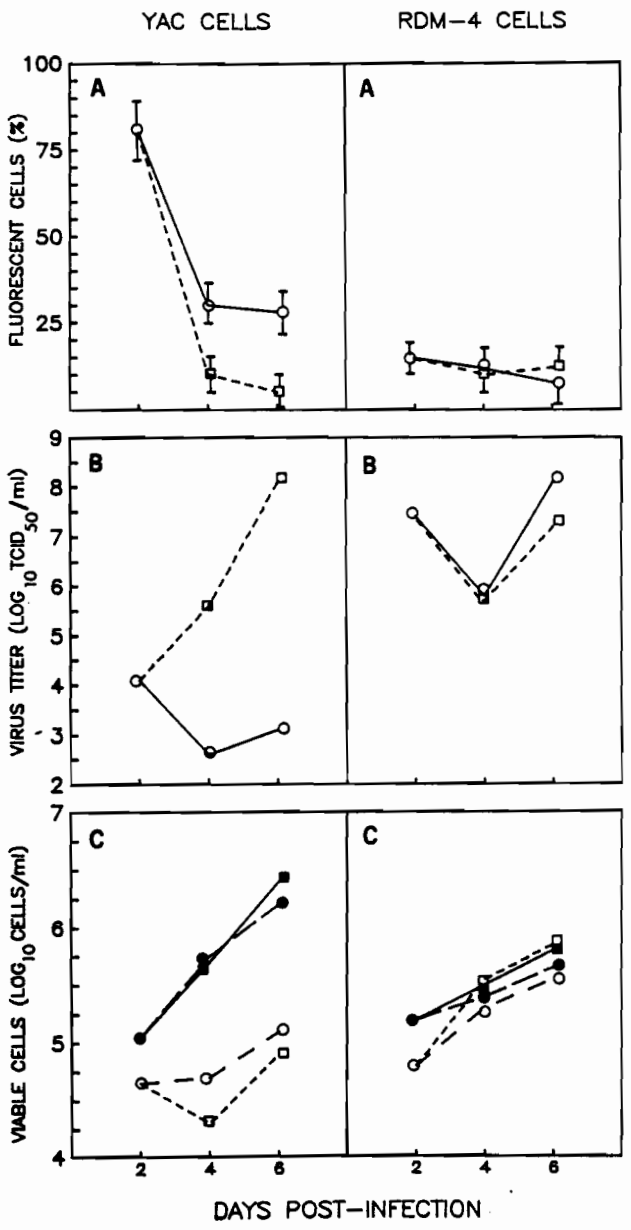


Figure 3: Effect of fresh medium on the development of MHV_3 persistent infection in YAC and RDM4 cells. Culture medium was changed (□-□) or not (○—○) every other day. MHV_3 infected (□ ○) or uninfected (■ ●) cell cultures.

2.5.2 CELL CLONING

Cell cloning of persistently infected and non-infected YAC and RDM-4 cells was performed using the limit dilution method. Cloning efficiency was estimated as the ratio of number of growing clone to number of expected clones. The cloning efficiency of infected (I) versus uninfected control (C) cells, was expressed as an I/C ratio. The results obtained in the cell cloning experiments are shown in Table 1. In the first 2 experiments, the I/C ratios for YAC cells were 0.77 and 0.72. In experiment 3, a normal ratio of 1.01 was related to an abortive infection since YAC cells had been inoculated with a low m.o.i. (0.1). Virus did not persist in any of the clones tested, as evidenced by the absence of virus production (0/272), of immunofluorescent cells (0/160) and of resistance to superinfection (0/155). In 2 experiments performed with RDM-4 cells (experiments 4 and 5), the I/C ratios were 0.94 and 1.08. Similarly, neither production of virus (0/168) nor resistance to superinfection (0/101) was observed.

2.5.3 EFFECT OF ANTI-MHV$_3$ ANTIBODY

In order to evaluate the role of extracellular transmission of infection in virus persistency in YAC cells, anti -MHV$_3$ Ab at a final concentration of 1:25 was added during 4 successive passages. As shown in Fig. 4, the number of cells in infected cell populations progressively reached a level similar to that of uninfected populations, while the number of fluorescent cells progressively decreased. Free or cell-associated viruses were not recovered after the first passage. Cell cultures were maintained during 9 subsequent passages after extensive washings and culture in antibody-free medium. Incubation with antibody resulted in the

TABLE 1. Cell cloning of MHV_3 infected and uninfected lymphoid cell line cultures

Experiment	Cell population origin	Cell population IF[1]	Cell Cloning: Cells	Cell Cloning: No. of clones/ No. of expected clones	Cell Cloning: %	Cell Cloning: Ratio I/C[2]	No. positive clones/No. tested clones: Virus production[3]	No. positive clones/No. tested clones: IF[1]	No. positive clones/No. tested clones: Resistance to super infection[4]
1	YAC	N.T	infected	265/445	60	.77	0/106	N.T	0/8
			control	75/96	78				
2	YAC	20%	infected	70/740	29	.72	0/70	0/70	0/66
			control	90/233	40				
3	YAC	0%	infected	175/231	75	1.01	0/96	0/90	0/81
			control	169/227	74				
4	RDM-4	N.T	infected	212/400	53	.94	0/72	N.T	0/9
			control	54/96	56				
5	RDM-4	11%	infected	167/234	71	1.08	0/96	N.T	0/92
			control	64/96	66				

(1) Indirect immunofluorescence test.
(2) Ratio between the percentage of clones obtained in infected cultures (I) and that of control, uninfected cultures (C).
(3) Detected by cytopathic effects on L2 cell cultures.
(4) Tested by infection of cell clones with MHV_3 (1 m.o.i.) for 48 hrs. at 37°C. Infectious virus production was detected by evaluation of cytopathic effects on L2 cell cultures.

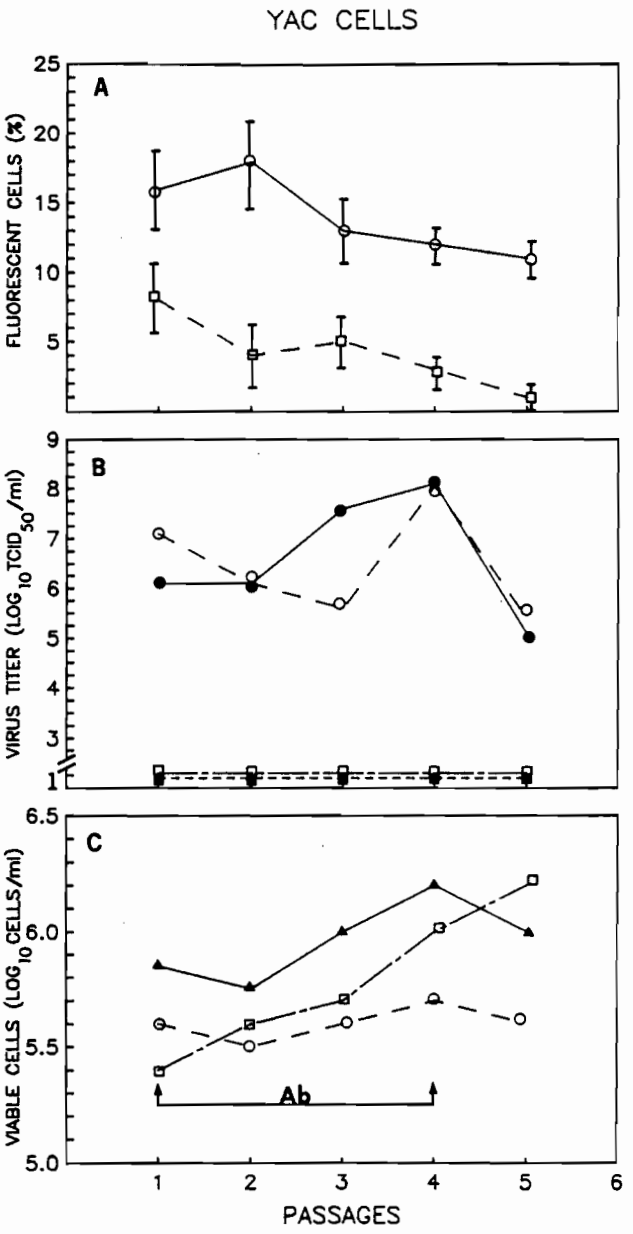


Figure 4: Anti-MHV_3 Ab treatment.

MHV_3 infected YAC cells were cultured with (□ ■) or without (○ ●) antibody. Percentage of fluorescent cells (A); free (□ ○) and cell-associated (■ ●) virus production (B); cell count of MHV_3 infected (□ ○) and uninfected (▲) cell cultures (C).

disappearance of virus since no evidence of viral infection was detected by infectious virus production, IF or resistance to superinfection. In addition, as determined by immunoblotting analysis, no antigenic modulation was induced in infected cells using antiserum treatment (results not shown).

2.5.4 EFFECT OF TEMPERATURE

When YAC cell cultures were incubated at 33°C for 2 successive passages, the number of viable cells in infected cultures became similar to that in control cultures. A switch of temperature from 33°C during the first passage to 37°C during the second passage resulted in a marked increase in cell lysis. Although such temperature changes did not result in variations in virus titers, they caused marked differences in the number of fluorescent cells. During 2 successive passages at 33°C, the number of fluorescent cells regularly increased from 15% to 75%. A switch of temperature from 33°C during the first passage to 37°C during the second passage produced a rapid decrease (from 45% to 25%) in fluorescent cells, which remained at 25% during 2 subsequent passages. Similarly, infected cells cultured only at 37°C during the same period of time displayed a constant number of fluorescent cells (around 20-25%).

2.5.5 EFFECT OF ACTINOMYCIN D

In order to study the role of cellular RNA synthesis in the control of viral persistency, low doses (0.025 and 0.050 ug/ml) of ACTD were added to YAC cells at 2 different stages of culture. No modification of fluorescent cell number but increase of viral titers were observed when ACTD was added at a stage associated with high numbers of fluorescent cells and low virus titers (Fig. 5, stage 1). However, ACTD added at a stage of low fluorescent cell number and high virus titer resulted in a rapid increase in the number of fluorescent cells and a marked decrease in virus

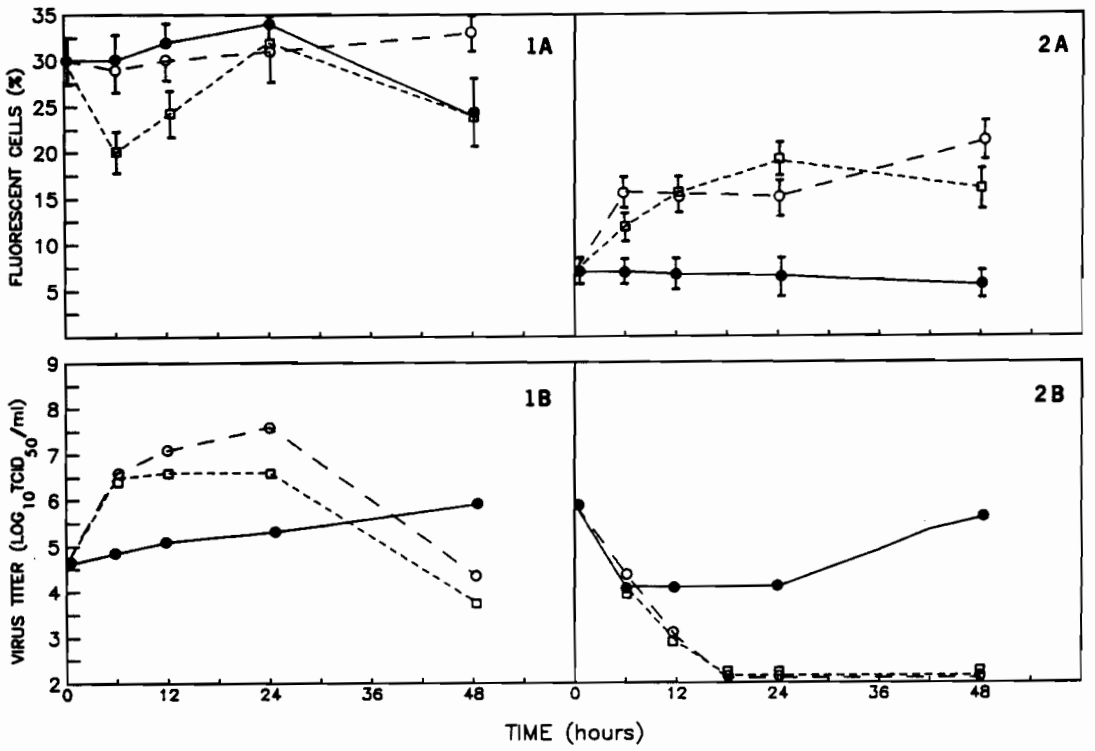


Figure 5: Effect of actinomycin D on MHV_3 persistent infections in YAC cells at 2 different stages of cultures (stage 1: high number of fluorescent cells; stage 2: low number of fluorescence cells). Cells were incubated without (●—●) or with 0.025 ug/ml (□- -□) or 0.050 ug/ml (○—○) of ACTD. Results are expressed as percentage of fluorescent cells (A) and total virus titers (B).

2.6 DISCUSSION

In semisusceptible strains, mice surviving the acute disease develop a chronic disease characterized by the occurrence of paralysis, virus persistency and immunodeficiency. Immunologic study of chronically infected animals revealed a marked impairment of B and T cell number and function (Dupuy et al., 1975). Several lines of evidence point to the direct role of MHV3 persistency in the immune depression: the marked decrease in lymphocyte numbers during the chronic disease (Leray et al., 1982), the failure of MHV3-infected lymphocytes to undergo blast transformation in vitro upon stimulation with mitogens or allogeneic cells (unpublished data) and the permissiveness of lymphocytes with respect to MHV3 replication (Krzystyniak and Dupuy, 1981). Thus, it was important to see whether MHV3 persistency could be established in vitro in lymphocyte cultures and and whether such a model could be utilized for studying MHV3-cell interactions and for better understanding the mechanism of MHV3 persistency.

MHV3 persistency could be readily established in vitro in the 2 lymphoid cell lines tested: YAC cells, which is a line of T cell origin, and RDM-4 cells which derive from a B cell lymphoma. The dose of virus was important for the initiation and maintenance of MHV3 persistency, and the m.o.i. required for infection were always higher in YAC cells than in RDM-4 cells (Fig.2). Such YAC cell cultures may require an high concentration of viruses to infect a low number of permissive cells and to induce a persistent infection. Persistent MHV3infection was characterized in vitro by several features, e.g. virus production, occurrence of cytoplasmic viral antigens and cell lysis. Although some differences were observed between YAC and RDM-4 cells, a general relationship was observed between these 3 factors. High virus titers were associated with a decrease in the number of

fluorescent cells and in cell lysis. Conversly, low virus titers were associated with a high number of fluorescent cells and increased lysis. Such a relationship suggests sequential events where virus production would be followed by accumulation of viral antigens and cell death. Massalski et al. (1982) have demonstrated an accumulation of core material after an abrupt cessation of virus assembly in L2 cells infected by the JHM strain of murine coronavirus. Preliminary electron microscopy studies of infected YAC cell cultures revealed low viral production in morphologically intact cells and the presence of clustered cores in cytoplasm of vacuolized cells or in cells undergoing lysis. Infected RDM-4 cells showed an apparent exclusion of clustered cores by polarization of viral antigens in the cytoplasm. These data indicate that only a small number of infectious virus-producing cells can be found during the persistent infection at any given time. Similar observations were noted by Roumillat et al. (1979, 1980), Robey et al. (1976) and Graze and Rayston (1975) with lymphoblastoid cell lines persistently infected by various herpes viruses.

The relationship between virus production and cell lysis was particularly remarkable in YAC cells where "crisis" occurred. Such "crisis" were manifested by a sharp increase in cell lysis and in fluorescent cell numbers and, concomitantly, by a marked decrease in virus titers. Similar cell "crisis" have already been demonstrated in mouse L cells persistently infected with VSV (Ranseus and Friedman, 1978; Youngner et al., 1976), in human lymphoblastoid cells infected with parvoviruses (Bass and Hetrick, 1978) or with HSV (Cummings et al., 1981). As with RDM-4, certain human B lymphoblastoid cell lines infected with HSV, however, did not present such critical periods (Leinbach and Summers, 1979; Robey et al., 1976 and Roumillat et al., 1980). A cell crisis may be triggered by several factors,

e.g., decreased interferon production (Sekellick and Marcus, 1979), enhanced virulence of the virus (Kawai and Matsunoto, 1977; Thacore and Youngner, 1969) or a sudden decrease in the number of defective-interfering virus particles (Holland et al., 1976). Such factors, however, were not found in MHV3-infected cell cultures (see chapter 3).

The relationship observed between the number of fluorescent cells, cell lysis and virus yield could be modulated by various factors, such as renewal of culture medium, change in temperature or inhibition of cellular RNA synthesis. Regular changes of culture medium resulted in increased cell lysis, whereas a low incubation temperature or actinomycin D treatment led to an accumulation of fluorescent cells. These factors caused important changes in the cellular metabolism. It was found that expansion and modulation of virus receptors may determine the outcome of certain viral infections in lymphoid cells or cell lines (Morishima et al., 1982). The binding of encephalomyocarditis virus to receptors of lymphoblastoid cells peaked during the exponential growth phase. In addition, there was a good correlation between the number of virus receptors and the production of viral particles in these cell lines (Morishima et al, 1982). The rapid growth of YAC cells due to fresh medium changes could, therefore, lead to increased expression of viral receptors, as well as to a rise in virus production. Similarly, modifications of cell metabolism such as those induced by low temperature or actinomycin D treatment may have resulted in variations in the viral cycle since inhibition of cellular metabolism causes an acceleration of viral infection. Temperature shift experiments have already been performed during persistent infections with HSV (Cunnings et al., 1981), polio viruses (Carp, 1981), VSV (Preble and Youngner, 1975; Youngner et al., 1976) and measles (Ju et al., 1978). Lucas et al. (1978) have

demonstrated a virus-cell relationship with restriction of MHV_3 production at high temperatures. The mechanism involved in such an interaction, however, is still unclear.

The results obtained with anti-MHV_3 Ab treatment and cell cloning in two sets of experiments indicated that, in persistent MHV_3 infection of YAC and RDM-4 cells, the type of viral transmission was horizontal and not vertical. Infected cells seemed to be cured after treatment with anti-MHV_3 Ab since virus was not recovered during subsequent culture in antiserum-free medium and cells did not resist challenge with MHV_3. Antiviral antiserum treatment is known to induce antigenic modulation and to affect viral replication (Fujinami and Oldstone, 1980). Electrophoretic blot procedure carried out in MHV_3 persistently infected YAC cells, in the presence or absence of anti-MHV_3 Ab, did not show any significant differences in viral proteins (results not shown). Results obtained with anti-MHV_3 Ab treatment are similar to those previously described for other viruses (Cummings et al., 1981; Hanner et al., 1981; Roumillat et al., 1980 and Stohlman and Weiner, 1978) and strongly point to a horizontal transmission as the route of viral persistency in YAC and RDM-4 cell cultures. This is further evidenced by the absence of viral information in cell clones, indicating that clone-producing cells either underwent abortive infection without any effect on clonal growth or were never infected. These data indicate, therefore, that persistent infection induced by MHV_3 in lymphoid cell lines is characterized by a viral "carrier state" in which the production of infectious viral particles remains in equilibrium with cell permissivity.

The ability of MHV_3 to induce in vitro persistent infections in lymphoid cell lines suggests that a similar mechanism may be involved in

vivo. Carrier-type transmission of infection produce atrophy of lymphoid organs during the chronic phase of the disease and progressive immunodepression (Leray et al., 1982). Conversely, high anti-MHV_3 antibody titers should block transmission of infection and cure the animals. During the chronic disease, a second and higher rise in anti-MHV_3 Ab levels occurs during the third month of infection. Although MHV_3 can easily be recovered from organs of infected mice during the first trimester of infection, all usual methods of virus recovery failed after that time, (Leray et al., 1982). Work is now in progress to determine whether or not MHV_3 infection can be completely abrogated in semisusceptible animals, and the use of an in vitro model of viral persistency will help us to analyze the mechanisms involved in delayed or blocked viral elimination.

CHAPTER 3: LOSS OF *IN VIVO* PATHOGENICITY OF MOUSE HEPATITIS VIRUS TYPE 3 PRODUCED IN PERSISTENTLY INFECTED MOUSE LYMPHOID CELLS

3.1 PREFACE

In the precedent article, we have showed that MHV_3 persistent infection established in YAC and RDM4 cell lines was related to a "carrier state" type of infection.

During persistent infections, different patterns of virus evolution have been described. Such variations were related to the production of defective-interfering particles, antigenic drift, selection of virus mutants or interaction with interferon system (Youngner and Preble, 1981).

In our study, the thermosensitivity, biochemical and in vivo pathogenic properties of YAC-derived MHV_3 were analyzed and compared to those of parental MHV_3. Interferon and defective-interfering viral particles were examined.

A rapid loss of in vivo pathogenicity of YAC-derived MHV_3 appeared during viral persistency; however, none of the factors mentioned above was involved in MHV_3 persistency.

This work is the second part of a study of the factors involved in MHV_3 persistency in lymphoid cell lines.

The contribution of Dr. Krzystyniak in this work concerned the biochemical analysis of MHV_3 proteins by electrophoretic blot procedure.

3.2 ABSTRACT

In semisusceptible mouse strains, mouse hepatitis virus type 3 (MHV_3) induces a persistent viral infection associated with immunodepression and atrophy of lymphoid organs. The biological and biochemical properties of MHV_3 variants derived from persistently infected YAC lymphoid cells were characterized. Interferon or defective-interfering viral particles were not generated during persistent infection of YAC cells infection. The thermosensitivity and thermolability of cloned viruses originating from persistently infected YAC cells, as well as parental virus suspensions, were studied. A similar heterogeneity was observed when YAC-derived cloned substrains (YAC-MHV_3) were compared to parental-derived cloned viruses, indicating that no selection of temperature-sensitive mutants was induced in persistently infected YAC cells. However, the capacity of MHV_3 to induce a lethal acute disease when injected into susceptible mice was lost very rapidly. The absence of pathogenicity was related to the induction of a subclinical infection which elicited defense mechanisms. YAC-MHV_3 variants showed this non-pathogenic property in contrast to pathogenic cloned viruses derived from parental MHV_3. Biochemical analysis of cloned viruses, carried out by SDS-PAGE blotting procedure, did not indicate a link between in vivo pathogenicity and viral proteins. These data suggest, therefore, that MHV_3 replication in lymphoid cell lines leads to induction or selection of variants which maintain pathogenicity in vitro but display reduced pathogenic effects in vivo.

3.3 INTRODUCTION

Mouse hepatitis virus type 3 (MHV_3) can induce various types of infection according to mouse strain, age and immune status of the host (Le Prevost et al., 1975). A chronic disease, characterized by the occurrence of paralysis in semisusceptible mouse strains, is associated with the atrophy of lymphoid organs, immunodepression (Leray et al., 1982) and the presence of virus in lymphocytes (Krzystyniak and Dupuy, 1981). In an attempt to investigate whether there is a causal relationship between virus persistency and lymphocytes, an in vitro study of MHV_3 replication in lymphocyte cell lines was undertaken. Viral persistency was established in YAC and RDM-4 cell lines and was related to a "carrier state" infection (see chapter 2).

The establishment and maintenance of persistent infections, are affected by several viral factors, e.g.,: defective-interfering particles, the occurrence of thermosensitive mutants, antigenic drift and the interaction between viral mutants and the interferon system (Youngner and Preble, 1981).

In the present article, we studied the thermosensitivity, biochemical and pathogenic properties of MHV_3 variants derived from persistently infected YAC cells. We found that such variants lost their capacity to induce an acute lethal disease when injected into susceptible mice.

3.4 MATERIALS AND METHODS

3.4.1 CELLS

The YAC lymphoid cell line was obtained from Dr. S. Lemieux (Armand-Frappier Institute, Laval, Ouebec). YAC cells (YAC-1 substrain) were derived from a Moloney virus-induced T-cell lymphoma of A/Sn origin and were propagated as a suspension line in culture for several years

(Yogeswaren et al., 1981). Cells were grown in RPMI 1640 medium (Flow Laboratories, McLean) containing 10% fetal calf serum, penicillin (100 U./ml) and streptomycin (100 ug/ml). Subcultures were performed by dilution with fresh culture medium when cell density reached 5 x 10^5 cells/ml. The doubling time of YAC cells was 18 h. L2 cells, a continuous mouse fibroblast cell line, (provided by Dr. R. Anderson, University of Western Ontario, Ontario, Canada) were grown in MEM with glutamine (2mM), 5% fetal calf serum and antibiotics. L2 cells were used for the propagation, cloning and titration of MHV_3.

3.4.2 VIRUS

The parental MHV_3 used was a cloned substrain already described. For virus production, L2 cells were infected at a multiplicity of infection (m.o.i) of 0.01. Infected cells were frozen when cytopathic effects were observed in 50% of the cellular monolayer. Virus suspensions obtained after two freezing-thawing cycles were clarified by centrifugation at 1 000 g for 30 min. Supernatants, stored at -70°C in small aliquots, were titrated in L2 cells and used as viral inoculum. The MHV_3 derived from a persistently infected YAC cell culture (45 days postinfection) was used as YAC-MHV_3 virus. Virus titer was calculated by the Reed and Muench method (Reed and Muench, 1938) and expressed as 50% of the tissue culture infectious dose ($TCID_{50}$).

3.4.3 VIRUS TITRATION

Viral suspensions were diluted serially in 10-fold steps in Earle's MEM. L2 cells growing in 96-well microtiter plates were infected with 0.025 ml of viral dilutions and virus was allowed to absorb for 1 h at 37°C.

Culture medium without fetal calf serum was added (0.2 ml) and plates were incubated for three days at 37°C in 5% CO_2. Viral titers were expressed as $TCID_{50}$/ml.

3.4.4 DEFECTIVE-INTERFERING PARTICLE ASSAYS

In vivo and in vitro tests were used. A volume of 0.1 ml of 10-fold dilutions of virus suspension was injected intraperitoneally (i.p.) into each of 3 adult $C_{57}Bl/6$ mice (susceptible to MHV_3) per dilution. The number of dead animals per group was recorded. For the in vitro interference assay, different concentrations (10^2 to 10^4 $TCID_{50}$) of the parental virus were mixed with 10^4 $TCID_{50}$ of a virus suspension obtained from persistently infected cell cultures (35 days postinfection). Two cell monolayers in microtiter plates were infected with 0.025 ml of the mixture, incubated at 37°C for 48 hours and scored as in the in vitro titration assays. Each titration was done in triplicate.

3.4.5 VIRUS CLONING

Supernatants of L2 cell cultures infected with parental MHV_3 and of MHV_3-persistently infected YAC cells (YAC-MHV_3) were used as starting materials for the preparation of viral clones. Virus cloning was performed on L2 cells using the limit dilution assay.

3.4.6 THERMOSENSITIVITY OF CLONED VIRUS

Determination of thermosensitivity was carried out in L2 cells in microtitration plates, infected with cloned viruses (0.01 m.o.i.) and maintained in culture at 33°C, 37°C or 39.5°C for various lengths of time. Each titration was done in triplicate. A virus yield ratio was calculated

according to the difference in virus titers obtained when cells were cultured at 2 different temperatures for 18 or 24 hours.

3.4.7 THERMOLABILITY OF CLONED VIRUS

Thermolability was determined by the rate of reduction of $TCID_{50}$/ml after incubation of virus samples at 37°C, 45°C and 50°C. Undiluted virus stocks were incubated in a water bath at the 3 different temperatures, aliquots were sampled at various times and frozen at -70°C. Residual infectivity was determined by virus titration in L2 cells.

3.4.8 INTERFERON ASSAY

Supernatants of persistently infected cell cultures were treated by UV-irradiation (30 min) or heat (50°C for 15 min) in order to destroy viral infectivity prior to testing. The interferon assay was carry out according to published method (Dahl and Degré, 1972).

3.4.9 PERITONEAL EXUDATE CELLS (PEC)

PEC were obtained after washings of the peritoneal cavity with 8 ml of RPMI 1640 medium containing 1% heparin (Allen & Hanbury, Toronto). PEC were seeded in microtitration plates (5×10^5 cells/well) and cultured at 37°C under 5% CO_2 for 3 days.

3.4.10 SDS-PAGE BLOTTING PROCEDURE

Uncloned and cloned virus substrains were analyzed by the SDS-PAGE blotting procedure (Toupin and Gordon, 1979). Briefly, cells were solubilized in 2% SDS, precipitated with trichloroacetic acid and dissolved in electrophoresis sample buffer (0.1 mM dithiothreitol, 2% SDS, 0.08 M

Tris-HCl, pH 6.8 and 10% glycerol). Samples were then boiled for during 2 min and cooled, and SDS-PAGE was performed in 12.5% acrylamide and 0.8% bis-acrylamide. Specific protein bands of viral antigens were visualized, after electrophoretic transfer to nitrocellulose paper, by immunoperoxidase staining. Nitrocellulose blots were incubated with anti-MHV_3 antibody obtained from immunized A/J mice and, after several washings, with peroxidase-conjugated anti-mouse IgG (Boehringer-Mannheim). Enzymatic coloration was performed in the presence of 4-chloro-1-naphtol and H_2O_2 (Sigma, St. Louis), for 10 min at room temperature. Finally, the papers were washed in distilled water and dried.

3.4.11 IN VIVO PATHOGENICITY ASSAY

In vivo pathogenicity was determined by i.p. injection of 0.1 ml of viral suspensions into susceptible C57Bl/6 newborn and/or adult mice. Each group consisted of 5-10 mice and the number of animals which survived during the first 2 weeks after injection was recorded.

3.5 RESULTS

3.5.1 THERMOSENSITIVITY

The search for thermosensitive mutants was carried out in cloned virus substrains originating from either infected L2 cells or from YAC cells a persistently infected with MHV_3 for 45 days. As shown in Fig. 6, viral populations derived from L2 or YAC cells cultured at 33°C, 37°C and 39.5°C, exhibited heterogenous pattern of thermosensitivity. Mean titers of cloned virus substrains derived from YAC cells and cultured at the 3 different temperatures were similar to those obtained with cloned viruses isolated from L2 cells and cultured at the same temperatures. For each

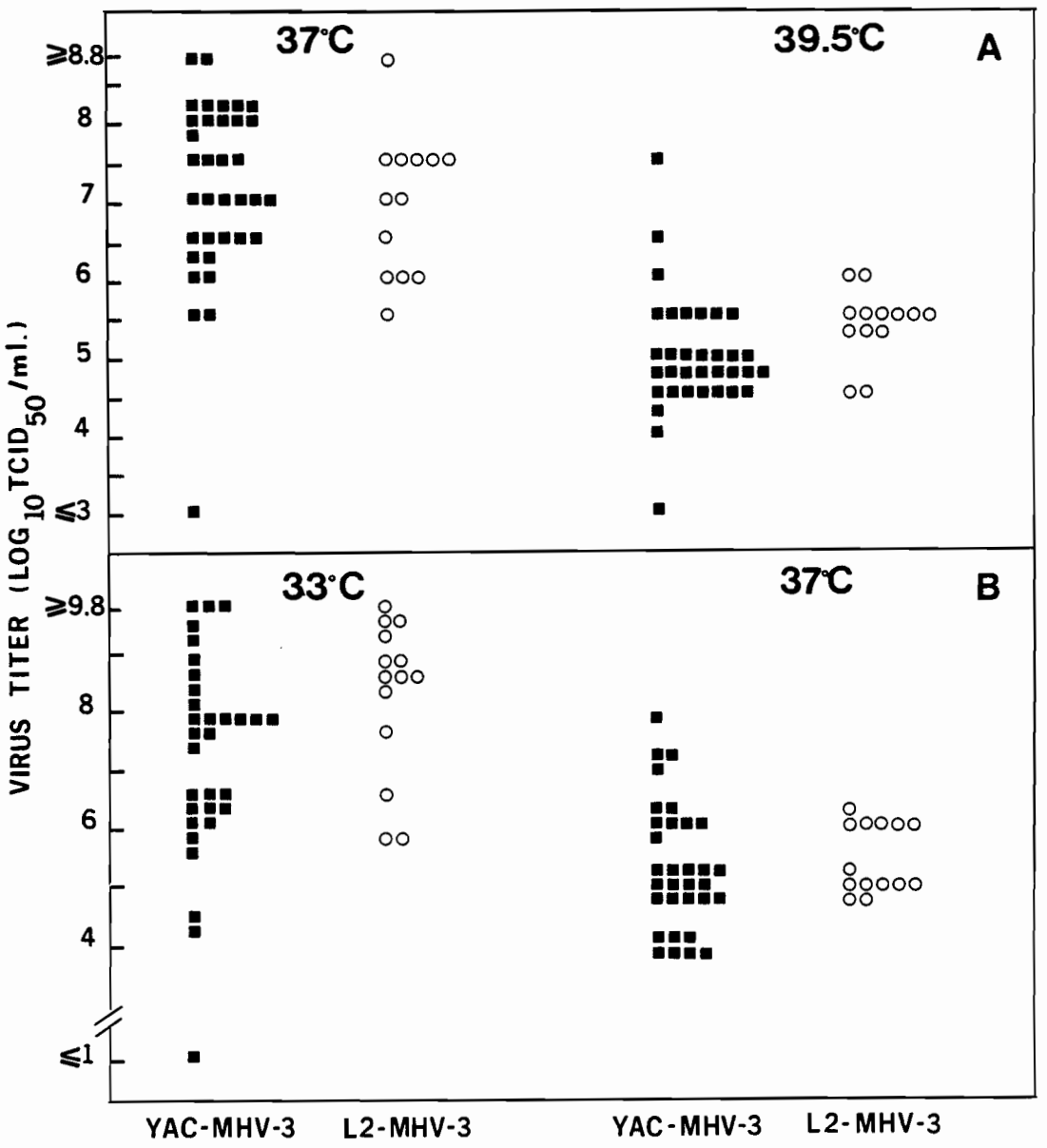


Figure 6: Thermosensitivity of cloned MHV_3 derived from infected L2 cells (O) and from persistently infected YAC cells (■). Viral titrations were performed after incubation of virus clones at 37°C for 18 hrs (A) and 24 hrs (B).

cloned virus, a yield ratio was calculated according to the difference of virus titers observed for 2 temperatures tested. As shown in Table 2, a similar heterogeneity in the yield ratios was observed when YAC-derived cloned substrains were compared to L2-derived cloned viruses.

Kinetics of heat inactivation of ten isolates obtained from persistently infected YAC cells was compared to that of the parental virus (Fig. 7). Parental MHV_3 was completely inactivated at 45°C and 50°C in 20 and 10 minutes, respectively. In addition, a 1.5 log 10 reduction of viral titer was obtained after 3 hr incubation at 37°C. Eight cloned viruses originating from persistently infected YAC cells exhibited similar thermolability. When compared to the parental virus, however, one viral isolate appeared to be more thermolabile at 37°C and 45°C and another, more thermostable at 37°C.

3.5.2 INTERFERON

Interferon was not detected in none of 6 undiluted culture supernatants of MHV_3 persistently infected YAC cells at various times of the cultures.

3.5.3 DEFECTIVE INTERFERING-PARTICLES (DI) IN VITRO

Since persistent infections induced by several RNA viruses are mediated by DI particles (Holland and Villareal, 1974, Holland et al., 1976 and Kawas et al., 1975), search for such a mechanism was investigated in MHV_3 persistently infected YAC cell cultures.

Similar virus titers were obtained when L2 cells were infected either with various m.o.i. of parental MHV_3 alone or when the latter were mixed with 10^4 $TCID_{50}$ of YAC- MHV_3. YAC-MHV_3 was obtained from YAC cell cultures 35 days postinfection.

TABLE 2. **Replication at different temperatures of viral clones isolated from MHV$_3$ infected L2 and YAC cells**

Yield Ratio[1] (log 10)	33°C / 37°C		37°C / 39.5°C		33°C / 39.5°C	
	YAC-MHV$_3$ No. clones (%)	L2-MHV$_3$ No. clones (%)	YAC-MHV$_3$ No. clones (%)	L2-MHV$_3$ No. clones (%)	YAC-MHV$_3$ No. clones (%)	L2-MHV$_3$ No. clones (%)
-1	3 (25)	0 (0)	1 (3)	0	0	0
0	1 (25)	2 (13.3)	2 (6)	2 (18)	2 (6)	1 (9)
1	10 (25)	1 (6.7)	9 (28)	5 (45)	1 (3)	0 (0)
2	7 (17.5)	3 (20)	9 (28)	3 (27)	3 (10)	0 (0)
3	6 (15)	3 (20)	8 (25)	1 (9)	3 (10)	1 (9)
4	7 (17.5)	5 (33.3)	3 (9)	0 (0)	7 (23)	1 (9)
5	5 (12.5)	1 (6.7)	0 (0)	0 (0)	4 (13)	5 (45)
6	1 (2.5)	0 (0)	0 (0)	0 (0)	8 (28)	2 (18)
7	0 (0)	0 (0)	0 (0)	0 (0)	4 (13)	1 (9)
Total No. of clones tested	40	15	32	11	31	11

1. Yield ratio was calculated to difference in virus liters obtained when cells were cultured at various temperatures for 18 hours (37°C-39°C) or 24 hours (33°C-37°C).

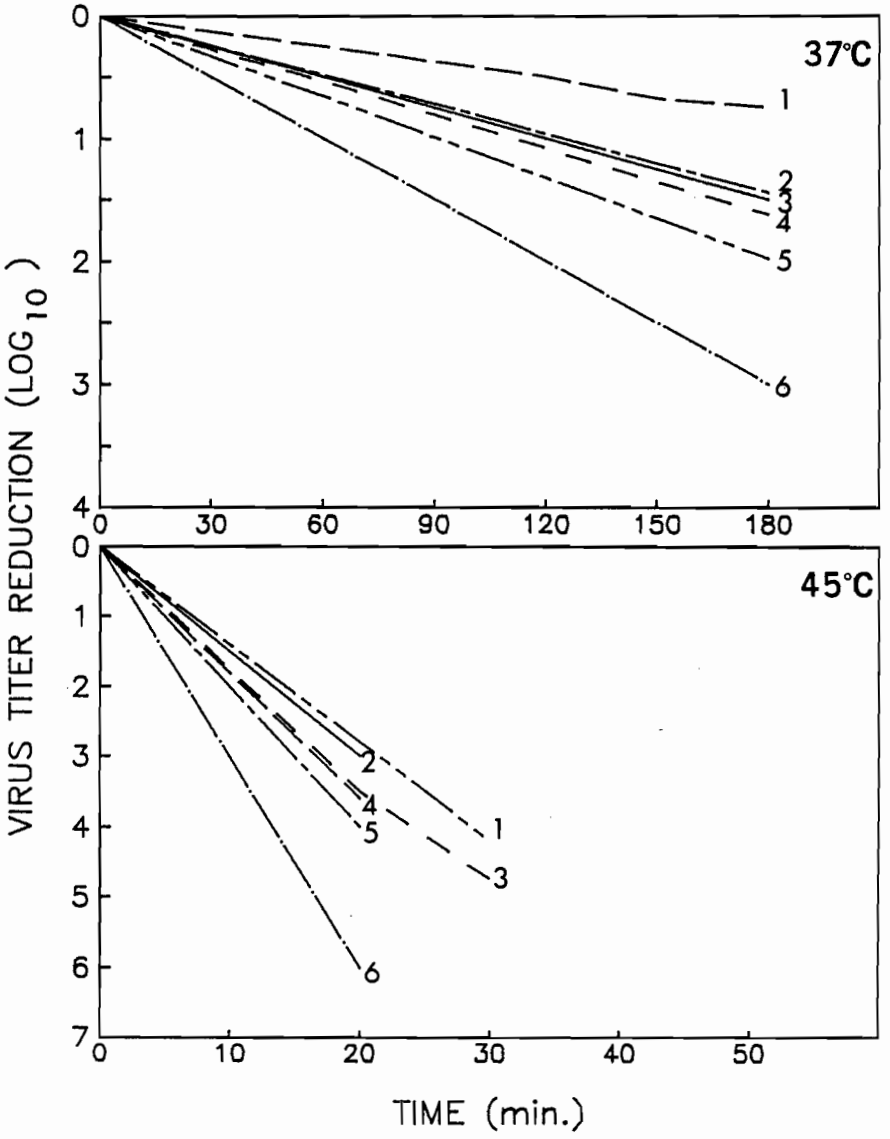


Figure 7: Thermolability of uncloned MHV_3 derived from L2 and YAC cells and from cloned YAC-MHV_3 at 2 different temperatures. At 37°C: L2-MHV_3 (3), YAC-MHV_3 (2), cloned YAC-MHV_3 (1,4,5,6). At 45°C: L2-MHV_3 (2), YAC-MHV_3 (3), cloned YAC-MHV_3 (1,4,5,6).

3.5.4 BIOCHEMICAL ANALYSIS

SDS-PAGE blotting procedure was performed on MHV_3-infected L2 and YAC cell cultures. L2 cells had been infected 18 hours previously either with parental MHV_3 or with YAC-derived cloned MHV_3. Controls included uninfected and Sindbis virus-infected L2 cells. As shown in Fig. 8, all 5 major viral components, i.e., gp 180, gp 90, p. 56, p. 50 and gp 24, surface glycoproteins (gp 180, gp 90) nucleocapsid (p. 56, p. 50) and matrix glycoprotein (gp 24) were present in all viral substrains tested. Although viral components may be present at various concentrations, no major differences were observed between YAC cell-derived viruses and the MHV_3 parental strain. Differences seen in protein bands from viral clones were related to degradation products of viral proteins.

3.5.5 IN VIVO PATHOGENICITY

The capacity of YAC cell-derived MHV_3 substrains to induce an acute disease upon injection into susceptible mice was tested. YAC cells were inoculated with 1 m.o.i. of parental MHV_3. *In vitro* virus titers and *in vivo* pathogenicity were determined in culture supernatants at various times postinfection. As shown in Table 3, although viral replication was regularly expressed by YAC cells during 45 days of culture with a yield between 10^4 and 10^7 TCID 50/ml, the property of *in vivo* pathogenicity rapidly disappeared. Eight days after infection, YAC cell culture supernatants had lost their ability to induce an acute disease when injected into susceptible C57Bl/6 mice. Since the disappearance of *in vivo* pathogenicity may be related to an accumulation of defective-interfering particles (Holland *et al.*, 1976), nonpathogenic (NP) MHV_3 was diluted (10^{-1} to 10^{-6}/ml) and *in vivo* pathogenicity was tested. No recovery of pathogenicity was obtained following NP virus dilutions (results not shown).

In an attempt to determine whether *in vivo* injection of NP MHV_3

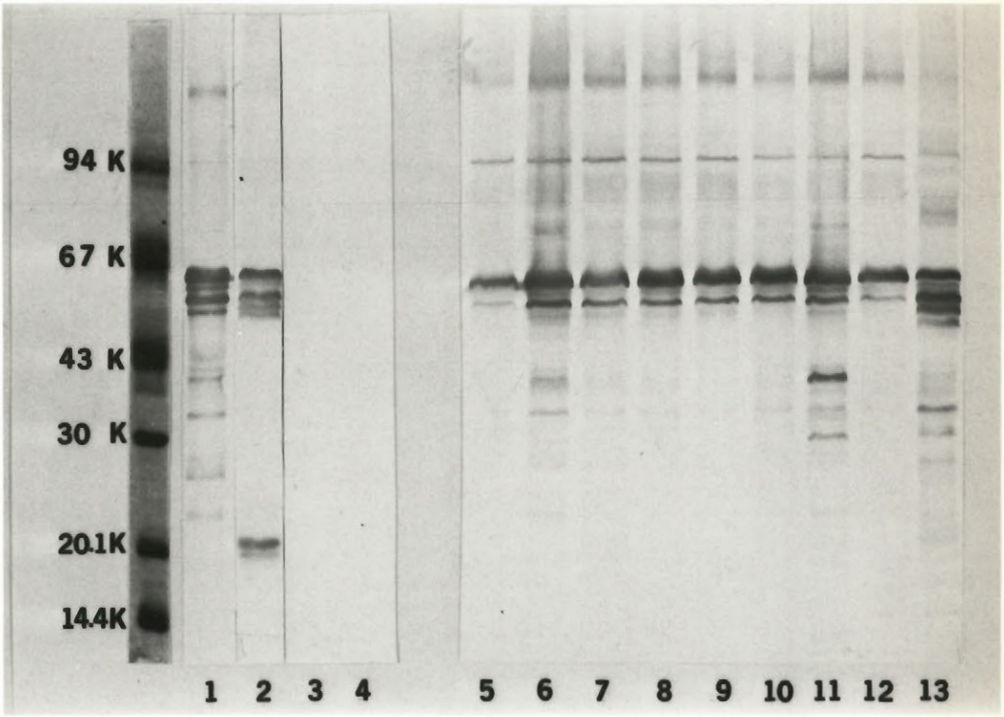


Figure 8: Biochemical analysis by SDS-PAGE blotting procedure of MHV_3 infected L2 cells (1), MHV_3 infected YAC cells (2), uninfected L2 cells (3), Sindbis infected L2 cells (4) and L2 cells infected with cloned YAC-MHV_3 (5 to 13).

TABLE 3: In vivo and in vitro pathogenicity of YAC cell derived MHV$_3$

YAC-MHV$_3$[1]	Pathogenicity[2]		
Days post-infection	In vivo		In vitro
	No. survival/No. tested	%	$TCID_{50}$/ml
0	0/12	0	$10^{5.1}$
1	2/6	33	$10^{3.1}$
2	0/6	0	$10^{6.1}$
4	0/6	0	$10^{7.8}$
7	2/6	33	$10^{7.8}$
8	6/6	100	$10^{6.2}$
11	6/6	100	$10^{5.1}$
15	6/6	100	$10^{6.1}$
20	12/12	100	$10^{6.6}$
25	12/12	100	$10^{4.1}$
45	12/12	100	$10^{6.1}$

1. YAC cell cultures were infected with 1 m.o.i. of MHV-3. At various time intervals post-infection, culture supernatants were tested for virus titer and in vivo pathogenicity.

2. 0.1 ml of culture supernatants were injected i.p. in $C_{57}BL/6$ adult mice.

liver and peritoneal macrophages of infected C57Bl/6 mice were examined for the presence of virus. As shown in Fig. 9, a persistent viral infection developed in C57Bl/6 mice infected with NP MHV_3, as evidenced by focus formation in PEC and by the detection of low titers of virus in the liver. Subsequent experiments revealed that virus can be recovered from the peritoneal macrophages, liver and brain of some animals infected with NP virus for several months postinfection (data not shown).

The in vivo pathogenicity of uncloned and cloned MHV_3 derived from YAC or L2 cells was determined. As shown in Table 4, uncloned and cloned YAC-MHV_3 were partially pathogenic for newborn but not for adult C57Bl/6 mice, whereas full pathogenicity was observed with uncloned and cloned L2-MHV_3. In addition, the injection of NP MHV_3 into susceptible mice led to viral persistency and paralysis in some animals. In order to determine whether NP MHV_3 could be used as an immunogenic agent, groups of 5 to 10 C_{57}Bl/6 mice were injected with NP MHV_3 on day 0, and challenged with wild-type MHV_3 at various internals. When animals were challenged with-type wild MHV_3 between postinfection days 1 and 4, 20 out of 20 died, whereas all mice challenged from day 5 on ward survived (40/40).

3.6 DISCUSSION

The chronic disease induced by MHV_3 in semisusceptible mouse strains is characterized by the occurrence of paralysis and virus persistency (Leray et al., 1982). Extremely low titers of virus were recovered from the liver, brain, spleen and lymph nodes. Since it was previously observed that MHV_3 could replicate in lymphocytes (Krzystyniak and Dupuy, 1981, Leray et al., 1982), persistent infections were carried out in

TABLE 4: In vivo and in vitro pathogenicity of YAC and L2 cell-derived MHV_3

Origin of cell-derived MHV_3 (No. of clones)	No. of passages (days) In vitro	In vivo pathogenicity (1) No. survival/No. tested (%)		In vitro Virus titer $TCID_{50}$/mL
		Newborns	Adults	
YAC-MHV_3 uncloned	-	5/20 (25)	19/19 (100)	$10^{5.5}$
YAC-MHV_3 cloned (21)	2 (2)	11/135 (8)	21/21 (100)	$10^{6.5}$
L2-MHV_3 uncloned	-	N.D.	0/15 (0)	$10^{5.0}$
L2-MHV_3 cloned (14)	2 (2)	N.D.	0/42 (0)	$10^{6.0}$

1. 0.1 ml of culture supernatants were injected i.p. in each C57BL/6 mice.

vitro in mouse lymphoid cell lines in order to studying virus-lymphocyte interactions. We found that persistency was maintained as a "carrier state" infection, a mechanism requiring an equilibrium between cellular permissivity and viral cytopathogenicity (see chapter 2). During persistent infections, different patterns of virus evolution have been described where cytolytic viral infections were converted to more temperate host-virus interactions. Such variations could be related to the production of defective-interfering particles, antigenic drift, selection of mutants or interaction with the interferon system (Youngner and Preble, 1981). The selection of temperature-sensitive (ts) mutants has also been suggested as an important mechanism responsible for the establishment and the maintenance of persistence (Youngner and Preble, 1981). Persistent infections induced in lymphoid human cell lines by measles or herpes virus (Cummings et al., 1981 and Ju et al., 1978), for example, have been shown to promote the selection of ts mutants. Most RNA viruses generate ts mutants in persistently infected cell cultures. Moreover, ts mutants also appear during persistent viral infections in vivo (Youngner and Preble, 1981).

We found that parental MHV_3 produced viral populations that were heterogeneous with respect to thermosensitivity. Viral heterogeneity was maintained in persistently infected YAC cell cultures, and no selection of ts mutants appeared to be induced. Similar negative results were obtained with neuroblastoma cells persistently infected with JHM virus (Stohlman and Weiner, 1978). In addition, Lucas et al. (1978) have demonstrated that the rapid inhibition of MHV_3 and JHM synthesis resulting from a shift to high temperature was not associated with the appearance of ts mutants but was related to a host factor. However, cold-sensitive mutants of the JHM strain have been isolated from cloned, persistently infected Nj cells, in the

presence of antibodies, after fusion with permissive cells (Stohlman et al., 1979).

Sekellick and Marcus have proposed that initiation and maintenance of persistent infections in cell cultures competent for the interferon system may be related to the development of DI particles or virus mutants with an increased capacity of cells to induce interferon (Sekellick and Marcus, 1979). Neither interferon nor DI particles were detected with used methods in MHV_3 persistently infected YAC cell cultures. Similar negative results were obtained in persistent infections induced in vitro with other coronaviruses in animal (Stohlman and Weiner, 1978) and in human cell lines (Chaloner-Larson and Johnson-Lussenburg, 1981).

Virus evolved from persistently infected lymphoid cell cultures can differ from parental virus with respect to virulence markers such as in vivo pathogenicity. In persistent infections, the selective advantage of virus mutants of lower pathogenicity is apparent since it allows host survival (Youngner and Preble 1981). MHV_3 variants produced in persistently infected YAC cell cultures differed from the parental virus in their lack of pathogenicity when injected into susceptible mouse strain. Similar results were observed in MHV_3 persistent infection carried out in RDM-4 mouse lymphoid cells (data not shown). The disappearance of the in vivo pathogenicity of YAC-MHV_3 occurred rapidly and was detected in all viral clones tested. The loss of pathogenicity was related not to the state of viral persistency per se, but rather to the origin of the replicating cells, as this was observed in lymphoid cells but not in fibroblasts (results not shown). A formation of pseudotype virus between endogenous retrovirus and MHV_3 was not evidenced by neutralization tests (results not shown).

Virus factors involved in viral pathogenicity are not well characterized. Envelope glycoproteins and hemagglutinin, or hemagglutinin-neura-

minidase glycoproteins are responsible for the adsorption and penetration into cells, for myxoviruses and paramyxoviruses, respectively (Klenk, 1980). Proteolytic cleavage of glycoproteins is required for the expression of biological activity. Glycoproteins of pathogenic virus strains are cleaved in a wide spectrum of hostcells, whereas those of nonpathogenic strains are activated in only a few host systems (Klenk, 1980). The pathogenesis of mouse infection with reovirus involves three outer capsid proteins (Fulds and Greene, 1983). The attachment and fusion activities of mouse coronaviruses (JHM strain) have been associated with viral glycoproteins (Collins et al, 1981). The sequencing by oligonucleotide mapping of mouse coronaviruses exhibiting various pathogenicities suggested that genetic sequences associated with viral pathogenicity were present in mRNA species 1 and 3, which corresponded to genes coding for the major envelope glycoprotein and for RNA polymerase (Lay et al., 1981). Biochemical analysis carried out with the SDS-PAGE blotting technique on L2 and YAC cell-derived MHV_3 variants did not allow to link in vivo pathogenicity with viral proteins since all major viral proteins were present in nonpathogenic variants (Fig.8). When this procedure was applied to cloned MHV_3 variants, it revealed great heterogeneity in the protein bands. No marked differences in viral proteins, however, were observed between cloned and uncloned YAC-derived MHV_3 variants. A relationship between viral attenuation, small plaque morphology and temperature sensitivity has been observed in various persistent infections. These changes seem to be related to independent mutations (Youngner and Preble, 1981). A relationship between thermosensitivity and in vivo pathogenicity was not found in YAC cell-derived MHV_3. These data indicate, therefore, that MHV_3 replication in lymphoid cells leads to induction or selection of variants

which maintain in vitro pathogenicity but display reduced in vivo pathogenic effects. Such variants seem to be responsible for the development of a subclinical infection in susceptible mice. They display antigenic properties as they can elicit defense mechanisms which enable infected animals to survive a challenge with virulent MHV_3. Thus, MHV_3 evolved from persistenty infected lymphoid cell lines became attenuated.

Persistent MHV_3 infection in lymphoid cell lines represents an interesting model for studying virus-lymphocyte interactions as well as cellular mechanisms involved in the loss of pathogenicity. The in vivo significance, however, of the latter phenomenon has to be established, and its existence in normal lymphocytes would be an observation of major importance.

CHAPTER 4: RESISTANCE TO MHV3 INFECTION EXPRESSED IN VITRO IN PERITONEAL EXUDATE CELLS AND IN LYMPHOCYTES

4.1 PREFACE

In the first articles, we found that MHV_3 persistency can be maintained in lymphoid cell line cultures by a "carrier state" mechanism in which infectious virus production was in equilibrium with the renewal of permissive cells. In such a system, the in vivo pathogenicity of virus progeny was rapidly modified.

The in vivo significance of these results has been explored using macrophages and lymphoid cell populations derived from mouse strains exhibiting various sensitivities to MHV_3 infection. A "carrier state" infection of these cells was obtained by short-term virus progeny passages in newly collected peritoneal exudates cells, adherent and nonadherent spleen cells and with thymocytes originating from various mouse strains.

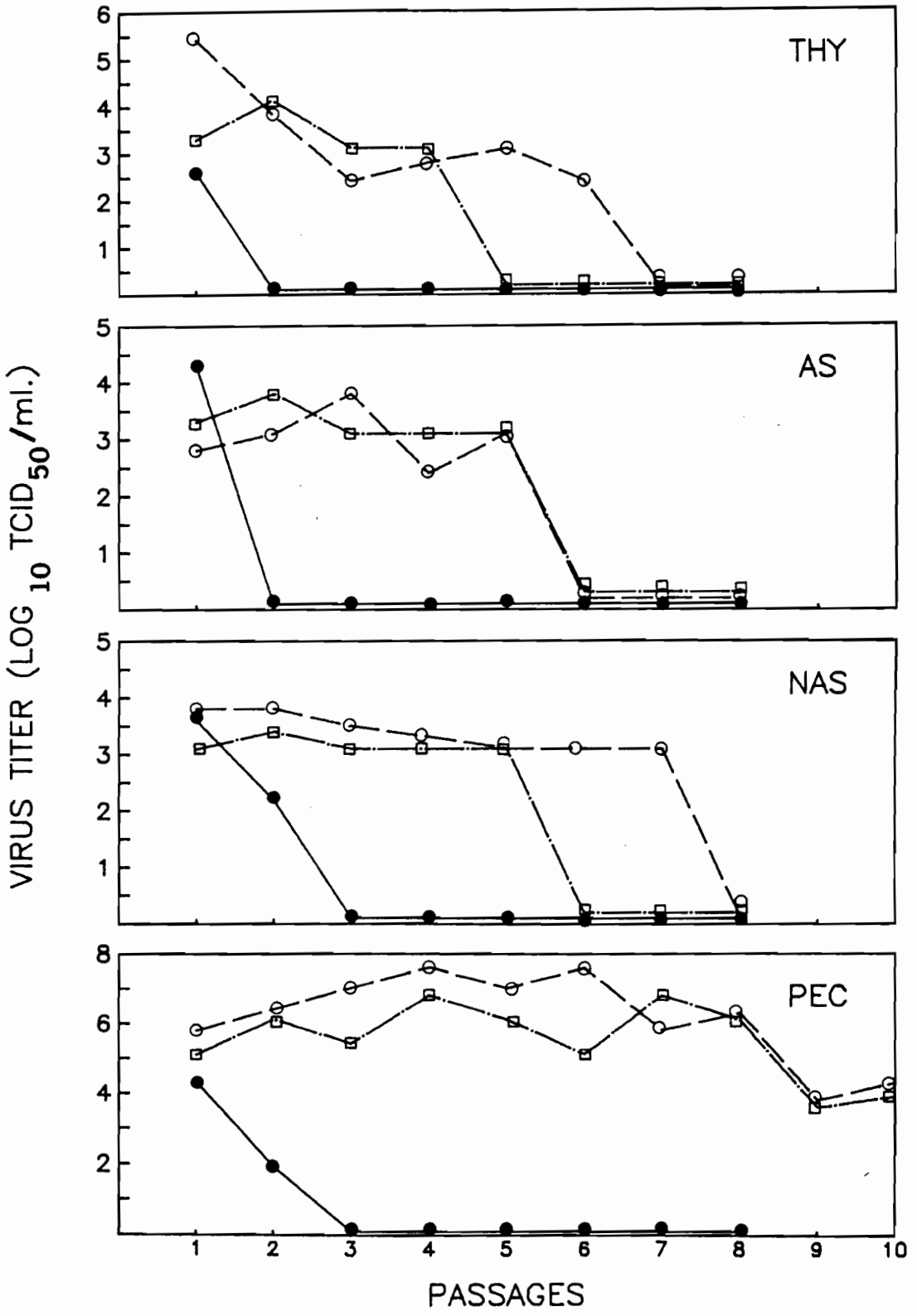
We found a correlation between phenotypic expression of in vivo sensitivity and restriction of viral infectivity occurring at various times postinfection according to the cell population.

Previous work suggested that a natural resistance mechanism plays an important role in the outcome of infection induced by MHV_3 (LePrévost et. al., 1975). Resistance to acute (fulminant hepatitis leading to death) or to chronic (paralyses and viral persistency) disease is under the influence of at least 2 major genes or a gene complex, which are different for each disease. In addition, resistance to paralyses is H-2 linked (Levy-Leblond et. al., 1979). The expression sites of these genes are still unknown. Virus persistency can be maintained in lymphoid cell line cultures by a "carrier state" mechanism in which infectious virus production is in equilibrium with the renewal of permissive cells. In such a system, the in vivo pathogenicity of virus progeny was rapidly modified (see chapter 3). In order to study the in vivo significance of this virus-host interaction, we carried out MHV_3 "carrier state" infections in peritoneal exudate and in lymphoid cell populations derived from mouse strains exhibiting various sensitivities to MHV_3. According to the cell population studied, we found a correlation between phenotypic expression of in vivo sensitivity and restriction of viral infection occurring at various times postinfection.

Resistant A/J, semisusceptible (C57BL/6 x A/J)F_1 hybrids ($B6AF_1$) congeneic A/Sn, A.BY, A.SW, A.CA, and fully susceptible (C57BL/6) mouse strains were used. A "carrier state" infection was carried out using short-term virus progeny passages in newly collected peritoneal exudate cells (PEC), adherent (AS) and nonadherent (NAS) spleen cells and thymocytes (THY) originating from various mouse strains. Cell cultures were infected with 0.01 m.o.i. of a cloned substrain of MHV_3 (Dupuy and Rodrigue, 1981). Supernatants were collected every two days after infection and used as viral inoculum (1:10) for the infection of newly collected cells originating from mice of the same strain.

As shown in Fig. 10, no infectious virus was detected in PEC from A/J mice after the second passage. Conversly, PEC from C57BL/6 and $B6AF_1$ animals supported virus replication for more than 10 successive passages. CPE characterized by focus formation, cell lysis, and immunofluorescence were only observed when

Figure 10: MHV_3 replication in peritoneal exudate cells (PEC), adherent (AS) and nonadherent (NAS) spleen cells and thymocytes (THY) originating from resistant A/J (●), semisusceptible (C57Bl/6xA/J) F_1 (◘) and fully susceptible C57Bl/6 (o)mice . PEC were obtained after washings of the peritoneal cavity with 8 ml of RPMI 1640 medium containing 1% heparin (Allen and Hanbury, Toronto). PEC were seeded in microtitration plates (5×10^5 cells/well) with RPMI 1640 containing 10% fetal calf serum, penicillin (100U./ml) and streptomycin (100mg/ml). Other cell populations were obtained according to a previously described methods (Tardieu et. al.,, 1980). Cell cultures were infected with 0.01 m.o.i. of a cloned substrain of MHV_3[3]. Supernatants were then collected every two days and used as viral inoculum (1:10) for the infection of newly collected cells originating from mice of the same strain. Infected cell cultures were incubated at 37°C in 5% CO_2. Cytopathic effects, characterized by focus formation and cell lysis, were recorded daily. Virus titrations were performed on L2 cells growing in 96-well microtiter plates. The cells were infected with 0.025 ml of viral dilutions and virus was allowed to absorb for 1 h at 37°C. Culture medium without fetal calf serum was added (0.2 ml) and plates were incubated for three days at 37°C in 5%CO_2 . Virus titers were calculated by the Reed and Muench (1938) method and expressed as 50% of the tissue culture infectious dose ($TCID_{50}$). All experiments were made in triplicate.


THY
AS
NAS
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VIRUS TITER (LOG 10 TCID50/ml.)
PASSAGES
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infectious viruses were produced. The results obtained with infected THY and NAS cells varied according to the origin of the cells. MHV_3 -infected NAS cells from A/J mice restricted virus infectivity after 2 passages whereas it took 7 passages, for NAS cells derived from susceptible C57Bl/6 mice to display a similar restriction. Infected NAS cells originating from $B6AF_1$ mice exhibited an intermediate behavior. Similar results were observed with the thymocytes. Restriction of virus infection occurred after 1 passage in AS cells from A/J and after 5 passages in AS cells from C57BL/6 and $B6AF_1$ mice.

Four semisusceptible congeneic strains differing only at the H-2 complex were studied: A/Sn, A.BY, A.SW and A.CA. Viral restriction was not observed in PEC from any congeneic strains tested and occurred after 2 or 3 passages in NAS and THY cells. This behavior was not different from that observed in PEC from $B6AF_1$ indicating that H-2 associated genes do not play any role the intrinsic cellular resistance to MHV_3 infection.

It is noteworthy that, except in A/J mice where all cell populations tested could restrict viral replication after 1 or 2 passages, macrophages and lymphocytes from susceptible and semisusceptible mouse strains differed in their ability to restrict viral infection. No restriction occurred in PEC, even after 10 passages, whereas restriction was seen in spleen cells and in thymocytes after 5 to 7 passages. This suggests that cellular restriction of MHV_3 infection may be under the influence of various genes and that the factors involved are different for macrophages and for lymphocytes.

Interferon production and the induction of DI viral particles could be related to the loss of <u>in vitro</u> virus infectivity after passages in macrophages or lymphocytes (Huang and Baltimore, 1970; Ho and Enders, 1959). Interferon synthesis was tested in supernatants of cell cultures using a previously described method (Dahl and Degré, 1972) and presence of DI particles was established by making dilutions (10^{-1} to 10^{-4})

of the last passage containing infectious viral particles inoculating newly collected cells and titrating at various times postinfection. Neither interferon nor DI virus were detected in the supernatants of cell cultures.

The restriction of virus infectivity observed in vitro in macrophages and lymphocytes may play an important role in the outcome of the in vivo infection. In mouse strains susceptible or resistant to acute disease, the liver is the main target of MHV_3 and the privileged site of virus replication, (Piazza et. al., 1967). In susceptible mice, the virus reaches high titers and causes severe hepatitis, whereas in resistant mouse strains, virus titers remain low and cellular lesions in the liver are scarce. A correlation was observed between the evolution of clinical disease and the virus titer in serum tested 4 days postinfection: animals with high virus titers died of an acute disease, whereas mice with low titers survived the acute infection and subsequently developed paralyses (LePrévost et. al., 1975). In addition, resistant A/J mice can eliminate the virus within 7 days, postinfection whereas MHV_3 could be recovered for several months from the brain, liver, spleen, lymphnodes and PEC of semisusceptible animals affected by a chronic disease. In the latter mice, a progressive immunodeficiency was observed during viral persistency and was related to virus-induced lympholysis and inhibition of lymphocyte proliferation (Leray et. al., 1982). Macrophages, hepatocytes and lymphocytes appear, therefore, to be the main target cells for MHV_3.

The present results suggest that the resistance to MHV_3 infection observed in A/J mice may be the result of a modulation of virus infectivity or replication by peritoneal exudate and lymphoid cells. In addition, the resistance to virus infection displayed by other target cells such as hepatocytes (Arnheiter et. al., 1982) and fibroblasts (see chapter 5) should minimize pathological damage and therefore assure the survival of infected mice and the development of an adequate immune response leading to total elimination of the virus. In contrast, susceptible mice infected with

the virus can neither restrict viral replication nor resist virus-induced cellular injuries. Dissemination of the infection thus leads to extensive pathological lesions and death. The intermediate behaviour displayed by infected cells originating from semisusceptible mouse strains seems to be related to an incomplete restriction of viral replication which may lead to virus persistency, cell lysis and subsequent immunodepression. In hypothesis, macrophages would be a reservoir of infectious viruses as they cannot restrict virus replication, and the survival of infected animals with less extensive pathological lesions would allow continuous MHV_3 replication in meningeal and choroid plexus cells leading to damage of subjacent nervous tissues. Further work is needed to define the genetically - related host mechanisms involved in virus-cell interactions.

CHAPTER 5: NATURAL RESISTANCE OF MICE TO MHV3 INFECTION EXPRESSED IN EMBRYONIC FIBROBLAST CELL CULTURES

5.1 PREFACE

In the preceding article, we described a mechanism of restriction of viral infectivity operating in macrophages and lymphocytes. Preliminary genetic analysis showed that H-2 associated genes did not play a role in the expression of such a mechanism. In order to investigate the mechanism involved in natural resistance, viral infection was carried out in cultures of embryonic fibroblast cells originating from various mouse strains.

No significant differences in viral production or interferon synthesis were observed in fibroblast cell cultures derived from various mouse strains. However, cytopathic effects, characterized by cell lysis, were related to in vivo phenotypes. These results indicate that the resistance of fibroblast cells is expressed in virus-induced cell lysis and not in viral replication.

5.2 ABSTRACT

Different strains of mice display various types of sensitivity to mouse hepatitis virus 3 (MHV_3) infection : resistance, semisusceptibility (paralysis) and full susceptibility (acute lethal hepatitis). In order to study the mechanism of inborn resistance, viral infection was carried out in primary cultures of embryonic fibroblasts originating from various mouse strains. Virus-induced cytopathic effects and cell membrane antigens, as well as virus replication and interferon synthesis, were studied. Persistent infection was induced in 4/6 primary embryonic fibroblast cultures and in 6/6 secondary cultures. A high yield of virus was obtained, as determined by viral titers and cell membrane antigen detection. No significant differences in viral production or in interferon synthesis were observed between fibroblast cultures derived from the various mouse strains tested. Cytopathic effects characterized by cell lysis were related, however, to in vivo phenotypes. In addition, resistance to virus-induced cell lysis was abrogated by actinomycin D treatment. These results indicate that the intrinsic resistance of fibroblast cells is expressed not in viral replication but in virus-induced cell lysis. This type of cellular control mechanism may be an important factor in in vivo resistance.

5.3 INTRODUCTION

Resistance to mouse hepatitis virus 3 infection is mainly dependent on genetic and immune factors (Levy-Leblond et al., 1979; Dupuy et al., 1975; Le Prévost et al., 1975a). Besides full susceptibility or resistance, some strains of mice exhibit semisusceptibility which results in early death or, in the surviving animals, in the development of a chronic disease with neurologic manifestations and virus persistency (Le Prévost et al., 1975b). The mechanisms involved in viral persistence have not yet been elucidated.

The genetic sensitivity of mouse strains to MHV_3 infection in vivo has been related to the degree of virus replication observed in vitro in hepatocytes (Arnheiter et al., 1982) and in macrophages (Virelizier and Allison, 1976; Dupuy et al., 1980) derived from adult mice. Genetic analysis indicates that acute and chronic diseases are under the influence of at least 2 major genes, or a gene complex, which are different for each disease and that, in addition, resistance to paralyses is H-2 linked (Levy-Leblond et al., 1979).

The use of embryonic cell cultures may provide a fruitful model for the study of natural resistance mechanisms under genetic control. This work reports host cell genetic control of MHV_3-induced cell lysis during persistent infections established in embryonic fibroblasts derived from various mouse strains.

5.4 MATERIALS AND METHODS

5.4.1 MICE

Inbred mice were purchased from the Jackson Laboratory, Bar Harbor, Me. Resistant (A/J), semisusceptible (C3H, SJL and (C57BL/6 x A/J) F_1) and fully susceptible (C57BL/6 and BALB/c) mouse strains were used. The animals were kept under conventional conditions. In the text, C57BL/6 and (C57BL/6 x A/J) F_1 will be described as B6 and $B6AF_1$, respectively.

5.4.2 CELLS

Embryonic fibroblast cells were obtained from 15- to 18- day-old mouse embryos. Head, members and tail were removed under sterile conditions and embryos were minced and washed twice in PBS. Trypsinizations were performed 2 or 3 times for 30 min. The cells were centrifuged and cells were washed twice in RPMI 1640 medium with antibiotics (Flow Laboratories, McLean). Cell viability was verified by the trypan blue exclusion dye test. Cells were resuspended at a concentration of 500 000 cells/ml in RPMI medium with 10% fetal calf serum, penicillin (1 00 U/ml) and streptomycin (100 μg/ml). Secondary cultures were obtained after trypsinization of confluent primary cultures, 5 to 7 days after explantation.

L2 cells, a continuous mouse fibroblast cell line, (provided by Dr. R. Anderson, University of Western Ontario, Ontario, Canada) were grown in Eagle minimal essential medium with glutamine (2 ml), 5% fetal calf serum and antibiotics. L2 cells were used for the production and titration of MHV_3.

5.4.3 VIRUS

MHV_3 was a cloned substrain already described (Dupuy and Rodrigue, 1981). For virus production, L2 cells were infected at a multiplicity of infection (m.o.i.) of 0.01. Infected cells were frozen when cytopathic effects were observed in 50% of the cellular monolayer. Virus suspensions obtained after two freezing-thawing cycles were clarified by centrifugation at 1000 g for 30 min. Supernatants, stored at -70°C in small aliquots, were titrated in L2 cells and used as viral inoculum. Virus titers were calculated by the Reed and Muench method (1938) and expressed as 50% of the tissue culture infectious dose ($TCID_{50}$). Embryonic fibroblast cells were infected with 0.01 to 0.1 m.o.i. of MHV_3. Virus titers, cytopathic effects and immunofluorescence were recorded at various times post infection.

5.4.4 VIRUS TITRATION

Viral suspensions were diluted serially in 10-fold steps in Earle's MEM. L2 cells cultured in 96- well microtiter plates were infected with 0.025 ml of viral dilutions and virus was allowed to absorb for 1 h at 37°C. Culture medium without fetal calf serum was added (0.2 ml) and the plates were incubated for three days at 37°C in 50% CO_2. Viral titrations were made in triplicate and standard error was less than 1 log.

5.4.5 CYTOPATHIC EFFECTS (CPE)

CPE were recorded daily, assessed on a scale varying from 0 to 4+ and expressed in percentage (1+ = 5 to 25%; 2+ = 25 to 50%; 3+ = 50 to 75% and 4+ = 75 to 100%).

5.4.6 INDIRECT IMMUNOFLUORESCENCE TEST

Anti-MHV_3 antibody (Ab) was raised in resistant A/J strain mice after 2 intraperitoneal injections of 1 000 LD_{50} of virus given 2 weeks apart (Le Prévost et al., 1975a). Sera were collected three days after the last injection, pooled, titrated by complement fixation (CF; 1:1024) and kept at -70° in small aliquots. Antisera were decomplemented at 56°C for 30 min before use.

Infected and uninfected cells were cultured in wells of Teflon glass slides (12 wells; Flow Laboratories, McLean). Cell fixation was performed in cold acetone for 30 min. Glass slides were washed in PBS (pH 7.2) for 15 min under agitation and were air-dried. Four units of fluorescent anti-mouse IgG or anti-mouse Fab_2 antisera (Cappel Laboratories, Cochranville) were added to each well. After further incubation and washing, the slides were treated with glycerol-PBS (9:1) and examined under the fluorescent microscope. The intensity of fluorescence was assessed as described. The percentage of cells with granular cytoplasmic fluorescence of 2 + or more was determined using a minimum of 200 cells per sample. Each experiment included 3 controls: 1) similarly treated uninfected control cells; 2) infected cells incubated with fluorescent anti-mouse IgG antiserum alone; and 3) known positive controls.

5.4.7 INTERFERON ASSAY

Supernatants of persistently infected cell cultures were treated with UV-irradiation (35 min.) or heat (50°C ; 15 min) in order to destroy viral infectivity prior to testing. The interferon assay was carried out according to published method (Dahl and Degré, 1972). Mouse α-interferon (Calbio

chem, La Jolla) and anti-mouse -A- interferon (NIAID, Bethesda) at respective concentrations of 2,400 I.U. and 6,000 I.U. were used in persistently infected cell culture.

5.4.8 IN VIVO PATHOGENICITY

In vivo pathogenicity was determined by intraperitoneal (i.p.) injection of 0.1 ml of viral suspension into susceptible adult C57BL/6 mice. Each group contained at least 6 mice and the number of animals which survived during the first 2 weeks after infection was recorded .

5.4.9 ACTINOMYCIN D TREATMENT

Actinomycin D at a final concentration of 0.05 μg/ml (Sigma, St. Louis) was added to infected and uninfected cell cultures .

5.5 RESULTS

5.5.1 MHV_3 INFECTION IN PRIMARY AND SECONDARY EMBRYONIC FIBROBLAST CELL CULTURES

Embryonic fibroblast cells were infected with MHV_3 during either primary or secondary cultures. MHV_3 infection was followed by sequential determinations of cytopathic effects (CPE), cell membrane antigens and virus titers. For each mouse strain tested, three cell cultures were infected with MHV_3 and similar results were obtained.

In primary cultures (Fig. 11), high virus titers were observed thrayhout A/J and B_6AF_1 cell cultures from day 2 postinfection. In B_6 cells, virus titers were high at days 2 to 4 postinfection but decreased regularly thereafter, becoming negative by day 10. MHV_3-induced CPE

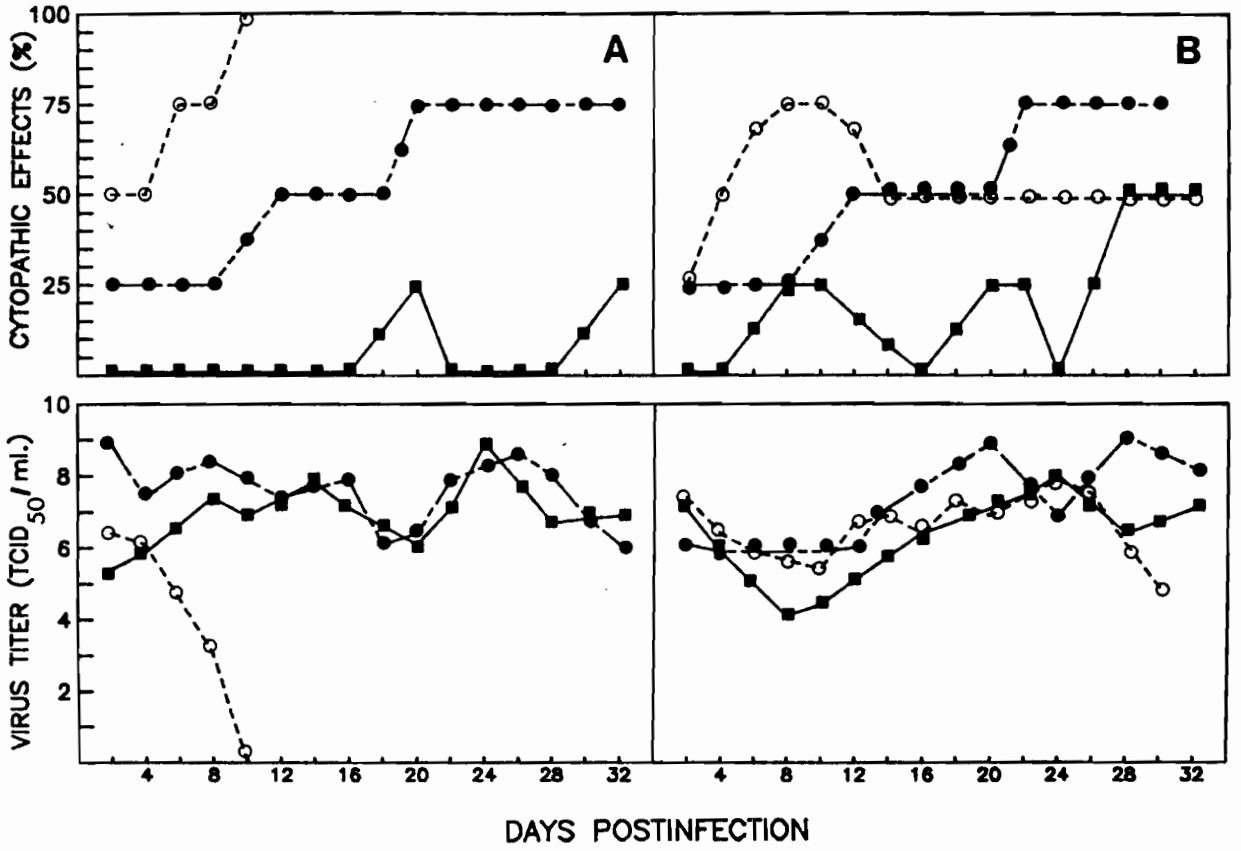

Fig.11: MHV_3 infection into primary (A) and secondary (B) cell cultures of embryonic fibroblasts originating from resistant A/J (■—■), semisusceptible $B6AF_1$ (●··●) and susceptible B6 (○··○) mouse strains;.

exhibited striking differences according to mouse strains. No CPE were seen in fibroblast cultures derived from the resistant A/J strain, whereas cells from susceptible B_6 mice expressed a high percentage of CPE by day 2 postinfection and were entirely lysed by day 10. Fibroblast cultures derived from B_6AF_1 hybrids displayed an intermediate behaviour as CPE, already present by day 2 postinfection, regularly increased thereafter without reaching total cell lysis.

When cells were infected at the time of secondary culture, virus persistency with high titers was established in all 3 cell lines. In addition, CPE differences according to strain origin were less remarkable than in primary cultures. Although A/J derived cells remained resistant to MHV_3-induced cell lysis, B_6 fibroblasts displayed a percentage of CPE similar to that expressed in F_1 cells, thus allowing B_6 cells to develop a persistent infection. In embryonic fibroblast cultures, MHV_3-induced CPE were characterized by occurrence of granular cells, cell rounding and ballonning preceeding cell lysis, without any development of foci or syncytium formation.

Virus infection was also carried out in primary and secondary embryonic fibroblast cultures derived from BALB/c (susceptible) and from SJL and C_3H (semisusceptible) mouse strains. The results were similar to those reported above with slight differences, i.e.; total cell lysis occurred in primary BALB/c cell cultures; by day 2 postinfection whereas only minimal (25%) CPE were exhibited by SJL and C_3H-derived cells during the primary cell culture. All secondary cell cultures supported persistent viral infections (Fig. 2).

Infected and uninfected embryonic fibroblast cell cultures were evaluated for immunofluorescence 24 hours after trypsinization and the

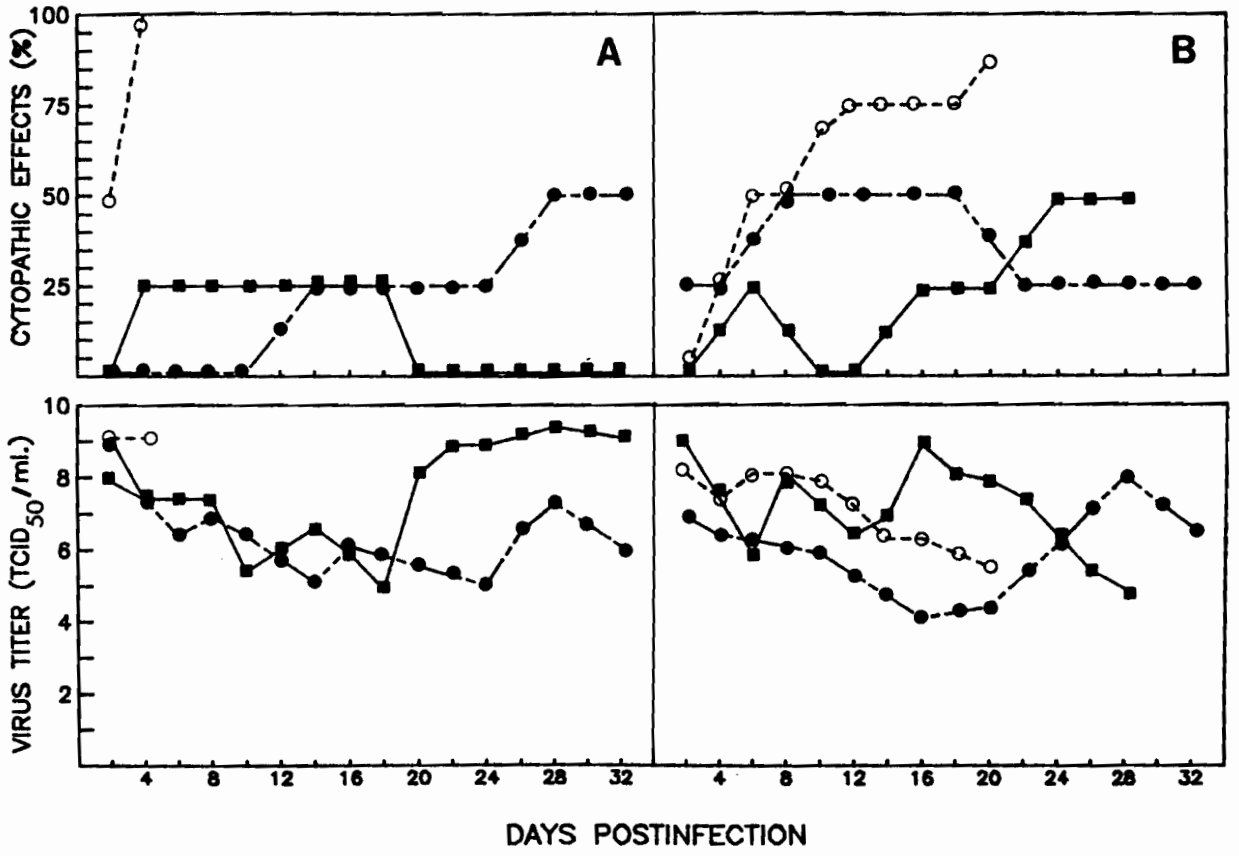

Fig.12: MHV_3 infection into primary (A) and secondary (B) cell cultures of embryonic fibroblast originating from semisusceptible SJL (●—●) and C3H (■—■) and from susceptible Balb/c (○—○) mouse strains.

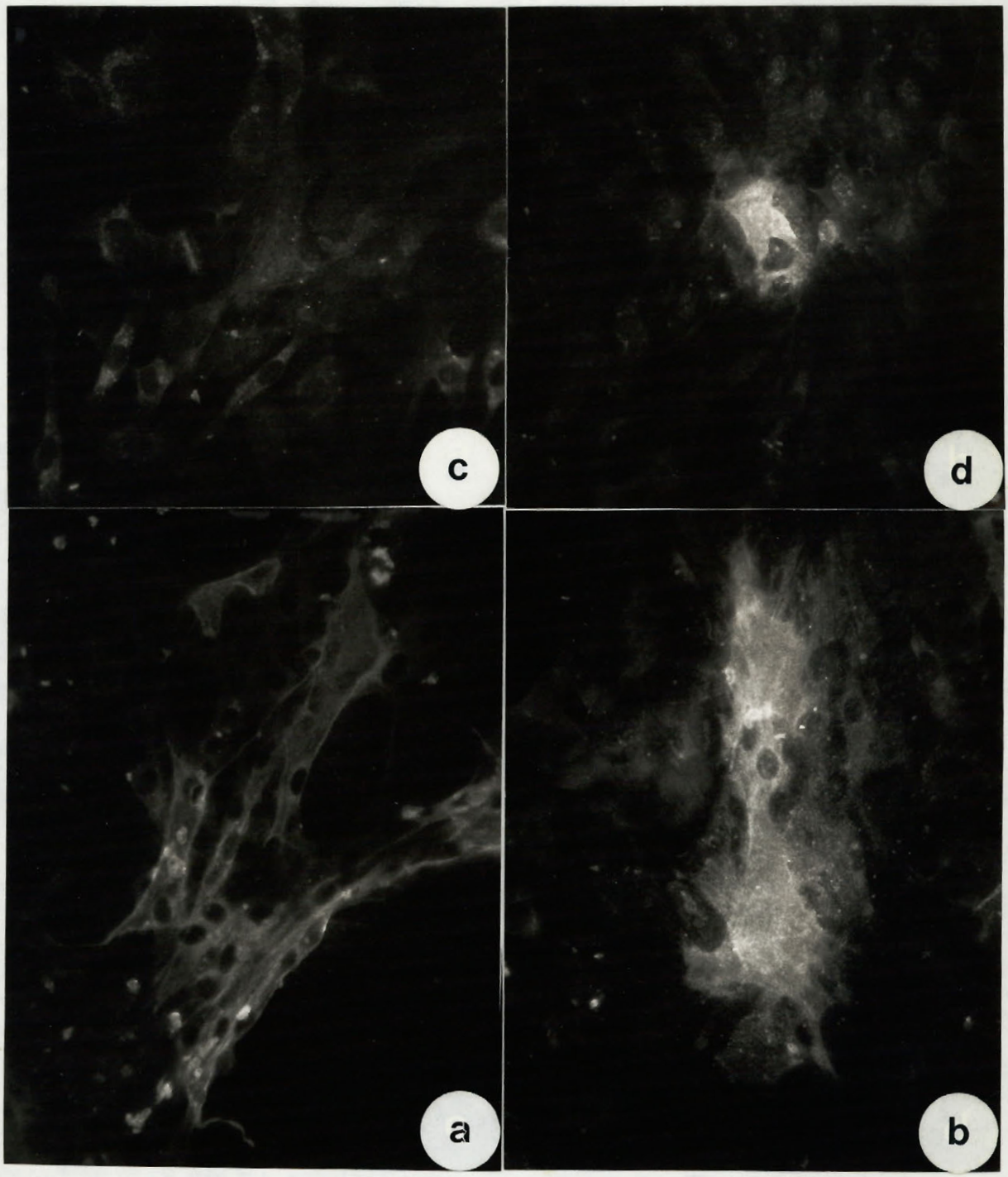

Figure 13: Immunofluorescence patterns of persistently MHV_3 infected embryonic fibroblast cell cultures originating from Balb/c (a) and C3H (b,c,d), displaying fluorescent cells scattered (a) or clustered (b) over monolayers, with cytoplasmic (c) or perinuclear (d) fluorescence.

number of fluorescent cells was correlated with CPE. A low percentage (5 to 15%) of fluorescent cells was associated with CPE (25%). Conversly, a higher number of fluorescent cells (22% to 82%) was observed with increased CPE (50 to 75%). Various patterns of cell fluorescence were observed (Fig. 3). Fluorescent cells could be scattered, spread over monolayers (Fig. 3a) or clustered (Fig. 3b). Virus-specific fluorescence was only observed in cytoplasm and not in the nucleus. Homogeneous and intense fluorescence was observed in cell cultures displaying marked CPE (Fig 3a, b), whereas weaker granular cytoplasmic (Fig. 3c) or nuclear (Fig. 3d) fluorescence was observed in cell cultures without CPE or with minimal CPE. No fluorescence was found in uninfected cells.

5.5.2 IN VIVO PATHOGENICITY

Previous work showed that MHV_3 persistency established in mouse lymphoid cell lines may rapidly affect the in vivo pathogenicity of virus progeny (see chapter 3). In order to test whether persistency itself or host-cell factors were responsible for this viral modification, the in vivo pathogenicity of MHV_3 derived from persistently infected embryonic fibroblasts obtained from various mouse strains was tested. No loss of in vivo pathogenicity was observed when virus produced in persistently infected (8 to 38 days p.i.) B6,A/J and $B6AF_1$ embryonic fibroblast cultures was injected into susceptible B6 mice.

5.5.3 INTERFERON AND ACTINOMYCIN D (ACTD) TREATMENTS

The search for interferon production was carried out at-5 day intervals using supernatants of embryonic fibroblast cell cultures persistently infected with MHV_3. No interferon was detected. The

TABLE 5: Actinomycin D treatment of MHV_3 persistently infected embryonic fibroblast cell cultures originating from the resistant A/J mouse strain.

Cell culture	Actinomycin D treatment(1)	Virus titers (TCID 50/ml) 24 hr.	Virus titers (TCID 50/ml) 48 hr	Cytopathic effects (%) 24 hr	Cytopathic effects (%) 48 hr
MHV_3 persistently infected	-	$10^{4.6} \pm 0.3$	$10^{6.1} \pm 0.1^2$	0	0^2
MHV_3 persistently infected	+	$10^{5.0} \pm 0.9$	$10^{4.7} \pm 0.1$	0	75
uninfected	-	0	0	0	0
uninfected	+	0	0	0	0

1. Actinomycin D was added to cell cultures at a final concentration of 0.05 µg/ml.
2. Experiments were made in triplicate.

addition of mouse α-interferon or anti-α-interferon to persistently infected cell cultures did not affect viral persistency or CPE.

The role of host cell metabolism in the resistance to virus induced cell lysis was studied in embryonic fibroblasts derived from the A/J mouse strain. After ACTD treatment, no significant modifications were observed in viral titers, but resistance to virus-induced CPE was abrogated (Table 5).

5.6 DISCUSSION

Embryonic fibroblast cultures provide a useful model for the study of inborn resistance mechanisms. Our results indicate that the genetically determined sensitivity of mice to MHV_3 infection is reflected in embryonic fibroblasts in terms of the establishment of persistent infections and resistance to virus-induced cell lysis.

No significant differences in viral replication were observed between embryonic fibroblast cultures originating from various mouse strains. This result is in agreement with previous studies by Dupuy et al.,(1980) and by McNaughton and Patterson (1980), which no correlation was observed between the in vivo pathogenicity of MHV_3 and its ability to replicate in macrophage cultures. In addition, no difference in MHV_3 uptake was seen between macrophages originating from resistant, semisusceptible and susceptible mouse strains (Krzystyniak and Dupuy , 1982). These data, however, are at variance with those of Virelizier and Allison (1976), who observed that macrophages from resistant A/J mice restricted MHV_3 replication in contrast to macrophages originating from susceptible or semisusceptible mouse strains. A partial resistance to MHV_3 replication in

isolated hepatocytes derived from resistant mouse strains was expressed as a reduction in virus titers relative to the high titers observed in cells originating from susceptible strains (Arnheiter et al., 1982).

Our work, however, demonstrated a clear correlation between in vivo sensitivity and virus-induced cell lysis, as evidenced by the cytopathic effects observed in primary cultures of infected embryonic fibroblasts derived from resistant, semisusceptible and susceptible mouse strains. Using immunofluorescence, it was shown that the number of MHV_3-infected embryonic fibroblasts expressing viral antigen was related to cytopathic effects. The various patterns of fluorescence visualized in infected embryonic fibroblasts may possibly reflect various steps of MHV_3 replication. Granular perinuclear fluorescence has been associated with the E_1 glycoprotein (gp 25) and homogeneous cytoplasmic fluorescence with the E_2 viral glycoprotein (gp 180) (Arnheiter et al., 1982). In vivo pathogenicity and cell membrane fusion properties of coronaviruses were related to the E_2 glycoprotein (Collins et al., 1981). No loss of in vivo pathogenicity was detected in virus progeny of persistently infected embryonic fibroblast cells. No foci or syncytia, however, was observed in those cell cultures. Resistance to virus-induced cell lysis was abrogated by actinomycin D treatment, suggesting that the cell ular control of viral infection requires synthesis of new host cell mRNA. The induction of viral cytopathic effects may be related to modifications of lysosomal membranes by the insertion of viral proteins (Allison, 1967), to a depletion of surface membrane resources following the emergence of enveloped virus without compensatory membrane production (Quigley et al., 1972) or to differences in synthesis and processing of viral proteins.

Host cell control of viral infections in fibroblast cell cultures has been associated with interferon (Harnett and Shellam, 1982) or with

DI viral particle production (Darnell and Koprowski, 1974). However, these such mechanisms do not seem to be involved in MHV_3-infected embryonic fibroblast cultures since neither interferon nor DI particles (unpublished data) were found.

MHV_3 *in vivo* pathogenicity depends on various factors: cellular and humoral immunity (Dupuy *et al.*, 1975; Le Prévost *et al.*, 1975), natural resistance mechanisms such as NK cells (Le Prévost *et al.*, 1975), and intrinsic cellular resistance (Arnheiter *et al.*, 1982). Le Prévost *et al.* (1975) have observed a correlation between the outcome of the infection and the virus titer in the serum 4 days postinfection. This suggests that natural resistance plays an important role in controlling the replication and spread of infection. No correlation, however, was observed between NK cell activity or serum interferon titers and the sensitivity of various mouse strains to MHV_3 *in vivo* (Schindler *et al.*, 1982).

Our work indicates that embryonic fibroblast cells originating from various mouse strains support MHV_3 replication and that the development of partial cytopathic effects allows the establishment of persistent infections. In addition, genetically determined sensitivity to MHV_3 infection in these cells is related to virus-induced cell lysis. Further work on enzymatic or structural host cell factors should allow us to better define the mechanisms involved in intrinsic cellular resistance acting under genetic control.

CONCLUSION

Our work indicates that persistent infections induced by MHV_3 can be established in lymphoid cell lines and are characterized by a viral "carrier state" in which production of infectious viral particles remains in equilibrium with cell permissivity. The ability of MHV_3 to induce persistent infections in lymphoid cell lines in vitro suggests that a similar mechanism may be involved in vivo. Carrier-type transmission of infection would cause, atrophy of lymphoid organs during the chronic phase of the disease and progressive immunodepression (Leray et al., 1982). Conversely, high anti-MHV_3 antibody titers should block transmission of infection and cure the animals. During the chronic disease, a second and higher rise of anti-MHV_3 Ab levels occurs during the third month of infection. Although MHV_3 can easily be recovered from the organs of infected mice during the first trimester of infection, all usual methods of virus recovery failed after that time (LePrevost et al., 1975b; Leray et al., 1982).

Our results suggest that MHV_3 replication in lymphoid cell lines leads to the induction or selection of variants which maintain pathogenicity in vitro but display reduced pathogenic effects in vivo . In addition, these variants are responsible for the development of a subclinical infections in susceptible mice. Genetically determined resistance to MHV_3 infections was expressed in macrophages, lymphoid cells, embryonic fibroblasts and hepatocytes. These natural cellular resistance mechanisms should play an important role in controlling the dissemination of infectious viruses and in determining the outcome of the disease.

MHV_3 infection of the resistant A/J mouse strain may be restricted by modulation of virus infectivity in peritoneal exudate cells and in lymphoid cells. In addition, resistance to virus-induced cell lysis displayed by other target cells, such as embryonic fibroblast cells, or decreased virus production, as observed in hepatocytes (Arnheiter et al., 1982), should minimize pathological damage and enable the survival of infected mice. Such natural cellular resistance mechanisms

are not sufficient to eliminate infectious viruses but may allow the establishment of adequate cellular and humoral immune responses. In contrast, susceptible mice infected with the virus can neither restrict viral replication nor resist the cellular injury caused by the virus. Thus, dissemination of the infection leads to extensive pathological lesions and death.

The intermediate behaviour of infected cells originating from semisusceptible mouse strains may involve a partial restriction of virus replication in lymphoid cells, leading to virus persistency, cell lysis and subsequent immunodepression. Macrophages constitute a reservoir of infectious viruses as they cannot restrict viral replication. Lymphoid cell proliferation in response to viral antigenic stimulation may continuously provide new permissive cells for MHV_3 infection. Less extensive pathological damage may allow infected animals to survive longer. Viruses, however, continue to be replicated and can infect meningeal and choroid plexus cells in the brain. In most of the surviving animals, inflammatory responses in the brain may cause damage to subjacent nervous tissues before the re-establishment of normal immune response mechanisms (Leray et al., 1982).

Persistent MHV_3 infections in lymphoid cells and in embryonic fibroblast cells provide interesting models for the studying virus-host cell interactions and cellular mechanisms involved either in the resistance to virus-induced cell lysis or in the modification of in vitro and in vivo pathogenicity. Such mechanisms may reveal a new facet of natural resistance to viral infections.

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