STUDIES ON MECHANISMS OF GENETIC RESISTANCE TO A LETHAL VIRUS INFECTION

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ABSTRACT

Pichinde virus, a member of the arenaviridiae, causes a fatal disease when injected intraperitoneally into the inbred MHA strain of hamsters, but not in other strains. The fatal infection is associated with an inability to limit virus replication, and death appears to be a consequence of the virus-induced cytopathic effect within the reticuloendothelial system. The purpose of the current studies was to determine whether susceptibility to the lethal Pichinde virus infection was genetically acquired, and to obtain an understanding of the basis for this susceptibility.

Studies on survival and the ability to limit viremia following Pichinde virus infection in F_1 and back-cross progeny gave the results expected if a single autosomal dominant gene or linked genes were responsible for each of these phenotypes.

Initial studies on the basis for the susceptibility of MHA hamsters to fatal Pichinde virus infections were designed to test the hypothesis that this strain was unable to mount a cell-mediated immune response against the virus. However, MHA hamsters were able to limit Pichinde virus replication and they survived the infection when the virus was inoculated by the footpad route. Furthermore, footpad-immunized MHA hamsters survived a normally lethal intraperitoneal challenge of Pichinde virus. These observations suggested that susceptible MHA hamsters were able to produce a protective immune response when the virus was given by this route.

Since cells of the reticuloendothelial system appeared to be a major target for Pichinde virus replication in vivo, a search for a

target cell difference within the spleens of susceptible and resistant hamsters was undertaken. No difference in the ability of various spleen cell fractions from susceptible or resistant hamsters to support Pichinde virus growth in vitro could be demonstrated. However, the spleens of MHA hamsters which had been infected with Pichinde virus in vivo contained 10-fold more virus-producing cells at three days after infection than did spleens of resistant hamsters. The majority of the virus-producing cells in MHA hamster spleens were associated with the non-adherent fraction which sedimented at a rate typical of lymphocytes. In contrast, spleen cells from the resistant LSH strain of hamsters appeared to be deficient in this population. These observations support the hypothesis that the susceptible strain of hamsters had a spleen target cell for Pichinde virus replication which the resistant strain lacked.

Interestingly, the putative target cell was observed to co-purify with a cell population which mediated in vitro cytotoxicity against syngeneic or allogeneic tumour target cells. The cytotoxic effector cell was shown to be a non-adherent, non-phagocytic, small- to medium-sized cell which lacked detectable surface immunoglobulin. The cytotoxic activity was labile at 37°C, but was not abrogated by pretreatment with ammonium chloride. Thus, this hamster effector cell resembled the natural killer (NK) lymphocyte which has been described in several species. Susceptible MHA hamsters exhibited high levels of endogenous NK activity, and this cytotoxicity was further augmented by Pichinde virus infection. In contrast, the resistant LSH strain showed lower levels of endogenous cytotoxicity, and Pichinde virus infection did not induce the same magnitude of increase in activity. These results support the hypothesis that susceptible MHA hamsters have an additional splenic target cell for

Pichinde virus replication which the resistant strain lacks, and are consistant with the possibility that this target cell is in fact the NK cell.

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LIST OF ABBREVIATIONS

αMEM	alpha formulation of Eagles' Minimal essential medium supplemented as described in Materials and Methods
ADCC	antibody-dependant cellular cytotoxicity
B lymphocyte	bone marrow-derived lymphocyte
BHK	baby hamster kidney cells
BSA	bovine serum albumin
CF	complement fixation, complement-fixing
CPM	counts per minute
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminotetraacetic acid
F-15 MEM	F-15 formulation of Eagles' Minimal essential medium supplemented as described in Materials and Methods
FBS	fetal bovine serum
FOCMA	feline oncornavirus-associated cell membrane antigen
HBSS	Hanks' balanced salts solution supplemented as described in Materials and Methods
HEPES	N-2-hydroxyethyl piperazine-N-2'-ethane sulfonic acid
HSV	herpes simplex virus
IP	intraperitoneal(ly)
Ir	immune response genes located within the major histocompatibility complex
LAD	a cell line derived from tumours induced by injection of adenovirus type 12 into neonatal LSH hamsters
LCMV	lymphocytic choriomeningitis virus
LLV	lymphoid leukosis virus

LIST OF ABBREVIATIONS (cont'd)

LSH	an inbred strain of Syrian hamsters originally bred at the London School of Hygiene
LVG	an outbred, closed colony of Syrian hamsters maintained in Lakeview, N.J. (Lakeview Golden)
M cell	marrow-dependant cell
MAD	a cell line derived from a tumour induced by injection of adenovirus type 12 into neonatal MHA hamsters
MHA	an inbred albino strain of Syrian hamsters originally bred at Mill Hill (Mill Hill Albino)
MHC	major histocompatibility complex
MHV	mouse hepatitis virus
MOI	multiplicity of infection
MULV	murine leukemia virus
n	number
NK lymphocyte	natural killer lymphocyte
PBS	phosphate-buffered saline
pfu	plaque-forming unit(s)
PMN	polymorphonuclear leukocyte
poly I:C	polyinosinic: cytidilic acid
RNA	ribonucleic acid
SD	standard deviation
SEM	standard error of the mean
SHE	a continuous line of fibroblasts derived from Syrian hamster embryos
SR	spontaneous release
SFFV	spleen focus-forming virus
T lymphocyte	thymus-derived lymphocyte

LIST OF ABBREVIATIONS (cont'd)

VBD	veronal buffered diluent
VSV	vesicular stomatitis virus
CAFE	carbonyl iron

CHAPTER 1

INTRODUCTION

Viruses may represent the ultimate form of parasitism: they depend absolutely on the host to provide the cellular machinery involved in replication of the viral nucleic acid and synthesis of viral proteins, and thus are parasites at a molecular level. At least part of the damage to the host arises as a consequence of this fact.

Viruses which infect animals commonly enter the host's body via the gastrointestinal tract, lungs or blood, and begin to replicate in the cells surrounding the portal of entry. After adsorbing to the cell membrane, the virion penetrates into the host cell where the protein coat is removed. Cellular metabolic processes then begin to function under the direction of the viral nucleic acid. Synthesis of viral enzymes or host enzymes which are required for replication of the virus is carried out, and then copies of the viral nucleic acid are made and packaged into newly made protein coats. The progeny virions are then released into body fluids of the host and are collected in lymphatic tissues where a second cycle of infection may occur.

Important in the pathogenesis of virus infections is the ability of cells in the lymphatic tissues to recognize the viral antigens as foreign and initiate the effector functions involved in clearing the virus particles. If the virions are not cleared by the lymphatic tissues, they may then pass into the lymph and from there into the blood. The virus particles are thus carried to other potential target cells in other organs.

Because the infecting virus converts the cell's mechanisms for producing macromolecules to its own use, the synthesis of host nucleic

acids and proteins which are necessary for normal cell growth may be prevented or altered. This can lead to damage or even death of the host cell. Death can also be caused by lysis as the progeny virions leave the host. These direct cytopathic effects can have serious consequences when the target cell is necessary for the organism's well-being. An alternative outcome of the infection is that the cell may become persistantly infected with the virus or undergo transformation, in which case the viral nucleic acid remains associated with the cell for a long period of time without replicating. This association may be manifested as disease years after the original exposure to the virus.

An agent such as a virus which can kill its host before the host attains reproductive age must impose strong selective forces on the organism. Indeed, animals have evolved a complex series of mechanisms for preventing or limiting viral infections (reviewed by Fenner, 1973). When an animal is confronted with a replicating antigen such as a virus, early mechanisms of defense could be very important in limiting the agent's growth. The production of interferon by virus-infected cells may inhibit the replication of the virus in neighbouring cells. Activation of the alternate pathway of complement can result in direct lysis of virus particles or can enhance phagocytosis by macrophages. Virions alone are also engulfed by macrophages, within which many viruses are unable to replicate. The production of defective virions has been postulated to interfere with the replication of infectious virus progeny. Recently it has been demonstrated that the activity of natural killer lymphocytes is enhanced early after virus infections; these cells may have a role in clearing intracellular virus by lysing infected cells.

These non-specific defenses may continue to function until the virus is completely cleared from the host.

Animals usually begin to produce specific defense mechanisms against the virus within four days of infection. Bone marrow-derived (B) lymphocytes differentiate into plasma cells which secrete virus-specific antibodies. Antibodies can reduce virus numbers by neutralization of their infectivity, or by opsonization, which enhances phagocytosis by various effector cells. Antigen-antibody complexes can activate the classical pathway of complement, which can lyse either the infected cell or the virion itself. Antibodies can also "arm" a variety of effector cells, which then specifically lyse virus-infected target cells.

The second form of specific immunity is mediated by thymus-derived (T) lymphocytes. Two subclasses of T cells, T helper cells and T suppressor cells, are involved in regulating the nature and magnitude of the immune response, and interact with macrophages, B lymphocytes and other T lymphocytes to generate specific effector functions. Cytotoxic T lymphocytes specifically lyse virus-infected target cells which share the same histocompatibility antigens as the effector T lymphocytes. A variety of lymphokines may be secreted by sensitized T lymphocytes in response to contact with the viral antigen, leading to infiltration by mononulear cells and polymorphonuclear leukocytes and other manifestations of inflammation or delayed hypersensitivity. Thus, the host has an arsenal of defense mechanisms against virus infections.

Pichinde virus, an arenavirus, causes a lethal disease in one strain of Syrian hamsters, MHA, but not in other strains. The fatal outcome is associated with an inability to limit virus replication, and

death appears to be due to a virally-induced necrosis within the reticuloendothelial system, (Buchmeier and Rawls, 1977; Murphy et al., 1977; Rawls and Leung, 1979). The elucidation of the mechanism(s) by which the resistant strain is able to limit Pichinde virus growth and subsequently survive the infection has been the subject of these studies. The results presented in this thesis suggest that MHA hamsters may possess a spleen target cell for Pichinde virus replication which the resistant strain lacks. The data are consistant with the hypothesis that this target cell may be the hamster natural killer (NK) lymphocyte.

1.1 Strain Differences in the Susceptibility of Syrian Hamsters to Lethal Pichinde Virus Infections

Pichinde virus is a member of the arenaviridiae, a group of viruses that is distinguished morphologically by the presence of ribosome-like particles within the core (reviewed by Rawls and Leung, 1979). Arenaviruses are enveloped, round or oval-shaped viruses with an average diameter of 110-130 nm. (Rowe et al., 1970 a, b). The nucleic acid is ribonucleic acid (RNA); of the five species which have been identified in Pichinde virus, only two, the 31S (L) and 22S (S) segments, are thought to be virus specific. Each of the virion RNA fragments appears to have unique genetic information (Vezza et al., 1977a) and probably does nothave a messenger function (Leung et al., 1977). The three other species, of sizes 28S, 18S and 4-6S, are probably derived from host ribosomal RNA and transfer RNA (Carter et al., 1973; Peterson, 1973). The virion has at least four structural polypeptides. The major protein, VP-1, has a molecular weight of 66,000 daltons and is thought to be a ribonucleoprotein. It appears to

be responsible for the induction of complement-fixing antibodies (Rawls and Buchmeier, 1975; Buchmeier et al., 1977) and contains antigenic determinants which cross-react serologically with proteins from other members of the arenavirus group (Buchmeier and Oldstone, 1977). Two glycoproteins can be demonstrated in Pichinde virions; VP-2 has a molecular weight of 64,000 daltons, whereas VP-3 is approximately 34,000 daltons. Both these glycoproteins are associated with the envelope (SenGupta and Rawls, 1979; Vessa et al., 1977). A fourth non-glycosylated polypeptide, VP-4, with a molecular weight of 12,000 daltons, has been described (Ramos et al., 1972; Buchmeier et al., 1977; SenGupta and Rawls, 1979). This protein appears to be internal and has considerable antigenic activity (SenGupta and Rawls, 1979).

Pichinde virus was isolated from the cricetine rodent, Oryzomys albigularis, in the Pichinde valley of Colombia, South America (Trapido and Sanmartin , 1971). Like other arenaviruses, Pichinde virus produces a persistant infection in its natural host. Animals which have been infected with arenaviruses at a very early age shed virus in the urine and saliva throughout their lives and may manifest no clinical signs of infection (Johnson et al., 1973; Webb et al., 1975). In addition to these naturally occurring persistant infections, several arenaviruses cause severe disease in humans; these viruses include the agents responsible for lymphocytic choriomeningitis (LCMV), Argentine hemorrhagic fever (Junin virus), Bolivean hemorrhagic fever (Machupo virus) and Lassa fever (Lassa virus). Typical features of the pathology in patients suffering from fatal arenavirus infections include generalized hemorrhaging into the tissues, focal hepatic necrosis and

hyperplasia of reticular cells (Elsner et al., 1973; Child et al., 1967; Winn and Walker, 1975).

Junin and Machupo viruses are two members of the so-called Tacaribe complex, the members of which are serologically related. This group includes Tacaribe, Amapari, Parana, Tamiani, Latino and Pichinde viruses. It has been observed that Pichinde virus induces a lethal infection in one strain of Syrian hamsters, (Mesocricetus auratus) but not in other strains (Buchmeier and Rawls, 1977). It does not, however, cause a clinically significant infection of humans (Buchmeier et al., 1974). Thus, the pathogenesis of this infection is amenable to study and permits the comparison of the disease in two strains of animals which differ in their susceptibility to the infection.

Both the LVG and MHA strains of hamster are susceptible to a lethal infection with Pichinde virus when injected early in life (Buchmeier, 1976; Buchmeier and Rawls, 1977). The LVG strain rapidly acquires resistance to the infection; by 8 days of age the mortality is usually between 0% and 10%. In contrast, adult MHA hamsters are markedly susceptible. The fatal infection is accompanied by high levels of viremia. At 8 days of infection, virus titres in the blood of infected MHA hamsters peaked at approximately 10^8 plaque-forming units (pfu) per ml compared to less than 10^3 pfu/ml in resistant LVG animals.

The pathogenesis of Pichinde virus infection in these two strains of hamsters has been studied by immunofluorescense and by light and electron microscopy (Murphy et al., 1977). Lymphoid organs of infected animals underwent pathological alterations. In the spleens of MHA hamsters, the infection was initiated in the marginal zones and the

periarteriolar lymphatic sheath. Extensive necrosis of both the white and red pulp was exhibited by day 7; destruction was essentially complete by day 10. A mononuclear cell appeared to be the major target for virus growth. Some infiltration by granulocytes, but not by mononuclear cells, was evident. In contrast, the small amount of viral antigen in spleens of LVG animals at 4 days after infection was cleared by day 10 and a granulocytic infiltration marked the end of histological alterations in the spleen. Lymph node cells of both strains showed prominant germinal centre development, and a few foci of viral antigen were found in reticular cells. In addition, lymph nodes of MHA hamsters showed evidence of macrophage involvement, while lymph node cells of LVG animals did not. In the bone marrow of MHA animals, antigen was present in approximately 1 in 10,000 cells, and in approximately 10% of the megakargocytes; no antigen was found in LVG marrow. The thymus showed no evidence of infection.

Viral antigen was first demonstrable in the liver of MHA hamsters within 4 days of infection. Focal necrosis of the Kupffer cells and hepatocytes was extensive at 10 days, involving 20-30% of the cells and the necrosis paralleled virus antigen content. Much cellular debris was present at the site of the lesions and in nearby sinusoids. At this time, no inflammatory cell infiltration in the lesions was evident. In LVG hamsters, virus antigen was detectable at 4 days of infection, but at no time afterwards. Moreover, focal necrosis was limited to less than 1% of the cells and was very localized.

The blood of infected animals showed a granulocytosis that was more extensive in MHA than in LVG animals. In addition, a slight

reduction in the numbers of small and large lymphocytes was apparent. Viral antigen was present in kidneys of adult MHA hamsters at 4 days after infection; moderate pathological changes were evident in the medulla late in infection. Antigen was also demonstrable in the brains of infected MHA animals. However no viral antigen or histological changes were evident in the brains of LVG hamster. Other organs showed variable and modest degrees of infection.

In summary, Pichinde virus exhibited a tropism for cells of the reticuloendothelial system in Syrian hamsters; the extent and severity of the necrosis was extremely pronounced in the susceptible MHA strain. The lack of a mononuclear cell infiltrate suggests that the lesions were not attributable to a host immune function, but rather were due to a direct viral cytopathic effect. The histologic analysis of Pichinde virus pathogenesis resembles the findings associated with Lassa fever and Argentine and Bolivean hemorrhagic fevers in humans (Winn and Walker, 1975; Elsner et al., 1973; Child et al., 1967).

Preliminary studies on the basis for the dichotomy in the response of LVG and MHA hamsters to Pichinde virus have been performed. Primary kidney and peritoneal exudate cells from LVG hamsters supported Pichinde virus replication in vitro as well as did cells derived from the susceptible MHA strain. This suggested that no innate difference existed in the capacity of these cells to support virus replication in vitro. Of course, this is not necessarily the case in vivo. In fact, the titres of virus in various organs after Pichinde virus infection in vivo generally paralleled levels of viremia. This suggests that events occurring in vivo may well differ from the situation in vitro.

Studies on the production of antibodies directed against Pichinde virus antigens have been done (Buchmeier and Rawls, 1977). The kinetics of production of complement-fixing antibodies were similar in both the LVG and MHA strains, and the peak titres of antibody were comparable. This antibody was thought to detect antigenic determinants in the ribonucleoprotein of the virion. Sera were also assayed for the presence of antibodies directed against antigen present on the surface of infected cells by the ability of the sera to block the binding of ^{125}I-labelled anti-Pichinde virus immunoglobulin. Again, no major difference in the kinetics of titres of antibody were detected in the response of LVG or MHA hamsters to this antigen. Thus, the humoral immune response in Pichinde virus infection as assessed by antibody production appeared to be intact in both susceptible and resistant hamsters.

It has been noted that cyclophosphamide treatment of Pichinde-virus-infected LVG hamsters abrogated their age-acquired resistance to the disease. This observation has led to the suggestion that cell-mediated immunity may have a role in limiting Pichinde virus replication (Buchmeier and Rawls, 1977). Furthermore, evidence suggesting a protective role for immunity is derived from the observation that newborns of resistant strains are susceptible to lethal Pichinde virus infection, the assumption being that the immaturity of immune responsiveness accounts for susceptibility. The studies by Murphy et al., (1977) on the pathogenesis of the viral disease have implicated the viral cytopathic effect as the primary cause of cellular necrosis. One explanation for the susceptibility of MHA hamsters to Pichinde virus

infection, then, is that this strain lacks an adequate cell-mediated immune response to Pichinde virus.

1.2 Factors Underlying the Genetically Determined Susceptibility to Viral Infections

That susceptibility to a virus infection can be genetically inherited may seem obvious. Many instances are known in which viruses are restricted to a narrow host range. Factors such as body temperature or the presence of suitable target cells in a particular species determine whether or not that species is susceptible to a virus. However, many cases in which strains within a species show differences in susceptibility to a virus infection are known and have been documented extensively in the literature (reviewed by Pincus and Snyder, 1973; Bang, 1978). Studies of these cases have provided information concerning the mechanisms by which virus replication may be restricted in genetically resistant animal strains. Some examples which illustrate the ways in which a host can prevent virus growth or spread are presented in the following brief review.

A virus must have access to a target cell if it is to replicate. In chickens, the presence of a particular "tv" allele results in the expression of a receptor on the cell surface which facilitates the adsorption of the avian RNA tumour viruses (Vogt and Ishizaki, 1965). Susceptibility to each of the five subgroups of avian RNA tumour viruses is controlled by a "tv" gene specific for that group (Pani, 1976). Cells which do not express a receptor for viruses of a given subgroup are resistant to infection *in vitro*. However, if the cell is infected with pseudotypes of nucleic acid derived from the excluded virus wrapped in

protein coats derived from viruses for which the cell has a receptor, then the resistance of the cell to the virus replication is overcome (Crittendon, 1968). This suggests that once the avian tumour virus has adsorbed to and penetrated the resistant chick cell, no further block on replication is present.

Marek's disease virus (MDV), a herpesvirus, causes a lymphomatous and neuropathic disease in certain strains of chickens. It has been suggested that resistance to MDV is controlled by at least two genes, one of which is at the Ly-4 locus. The presence of the susceptibility allele results in the expression of a T lymphocyte antigen which may act as a receptor for virus adsorption to target T lymphocytes; the absence of the gene product is thus associated with resistance to infection (Gallatin and Longenecker, 1979)

Many animal strains possess genetic information which leads to the restriction of virus replication once the virus has penetrated into the cell. The genetic resistance of mice to Friend leukemia virus disease is under complex controls. The virus stock actually consists of two components, spleen focus-forming virus (SFFV) which is defective for replication, and lymphoid leukosis virus (LLV), which acts as a helper virus (Lilly and Pincus, 1973). The susceptibility of mice to the LLV component is under the control of a single autosomal gene, Fv-1, which has two alleles, n and b. Cells which express the $Fv\text{-}1^n$ allele are able to restrict the growth of b-tropic murine leukemia viruses and conversely, n-tropic viruses grow only poorly in cells synthesizing the $Fv\text{-}1^b$ product. The mechanism of restriction is not fully understood; however, studies with pseudotypes of vesicular stoma-

titis virus (VSV) nucleic acid wrapped in MuLV coats demonstrated that adsorption and penetration of incompatible viruses did occur (Huang et al., 1973). Jolicoeur and Baltimore (1976) have suggested that the Fv-1 gene product may restrict the integration of the pro-viral DNA into host chromosomal DNA, an event that is required for virus replication.

Another example in which host cells are able to abort virus infection is provided by the genetically determined resistance of mice to infection with St. Louis encephalitis virus (Webster, 1937). Crosses of the resistant strain with the susceptible strain yielded F_1 progeny with a survival rate similar to the resistant parent. When the F_1 progeny were backcrossed to the parental strains, the results indicated that resistance was controlled by a single dominant gene. Cultures of brain cells from susceptible mice supported virus replication in vitro to high titers; in contrast, cells from resistant mice supported virus growth only poorly.

Yellow fever virus, like St. Louis encephalitis virus, is a flavivirus. Mice of either the Swiss or PRI strains were susceptible to a lethal infection with yellow fever when young, but the PRI strain rapidly acquired resistance to the virus. This age-acquired resistance was a dominant trait controlled by a single gene (Sabin, 1952, quoted by Bang, 1978). Similarily, the resistance of mice to Russian spring-summer encephalitis virus and louping ill virus, two other flaviviruses, is also controlled by a single dominant autosomal gene.

Some comprehension of the nature of the genetic resistance to these flaviviruses was gained from studies on a fifth member of the

group, West Nile virus. Primary cultures of spleen cells and peritoneal macrophages from susceptible mice yielded high titres of virus after infection with the virus in vitro, while peritoneal exudate cells derived from resistant strains produced almost no virus after several days in culture. Lung cultures displayed this dichotomous response as well, but primary kidney cells did not (Goodman and Koprowski, 1962). Thus, these studies implicated macrophages as the crucial cell in determining susceptibility to the fatal infection. The reduced ability of cells derived from resistant mice to support viral growth in vitro suggests that a similar event may occur in vivo, resulting in low levels of virus.

Another example of a genetically determined cellular susceptibility to a virus is the age-acquired resistance of certain mouse strains to infection with mouse hepatitis virus, MHV-2. In this case, susceptibility is dominant in F_1 progeny of (susceptible X resistant) parents, and is controlled by a single gene (Bang and Warwick, 1960; Kantoch et al., 1964; Weiser and Bang, 1976). The in vivo susceptibility of the mice correlated with the ability of macrophages derived from susceptible but not resistant strains to support virus replication in vitro (Weiser et al., 1976). However, it has been observed that thymus-derived (T) lymphocytes from adult resistant strains are also required to protect young mice. These results imply that a macrophage-T lymphocyte interaction may be important in vivo in rendering the macrophages resistant to the virus infection. Again, the exact mechanism by which adult macrophages from resistant strains suppress MHV-2 replication is not clear. No difference in adsorption has been demonstrated. It has

been hypothesized that a block in virus development occurs after penetration (Shif and Bang, 1970).

Inbred strains of mice which differ in their susceptibility to influenza virus type A have also been described. Resistance is controlled by an autosomal dominant gene (Lindenmann, 1962). Primary cultures of kidney cells from susceptible or resistant mice supported virus replication in vitro equally well; however, macrophages from resistant mice appeared to be resistant to the virus, while macrophages obtained from susceptible strains supported virus replication in vitro (Lindenmann et al., 1978). The mechanism(s) by which macrophages restrict virus replication have not been defined.

The above examples of genetically acquired resistance to a virus infection depend on the ability of the host to prevent a virus from entering or replicating in the target cell. If the host cannot block virus replication within susceptible target cells, then its survival could depend on mechanisms which limit spread of the virus progeny to secondary targets. Immune defense mechanisms, which have already been discussed, reach their full potential some time after the animal is born. The immaturity of the immune response early in life frequently is responsible for the increased susceptibility of young animals to virus infections. For example, the age-acquired genetic resistance of mice to infection with ectromelia virus reflects age-acquired differences in the immune response to viral antigens (Schell, 1960; Roberts, 1964). The ability of adult mice to resist fatal infection with Coxsackie B-3 virus is also related to maturing of the humoral immune response (Rager-Zisman and Allison, 1973). The co-

transfer of peritoneal exudate cells with antiviral antibodies, but not peritoneal cells alone, protected animals against the lethal disease. However, antibody alone is probably not sufficient to produce resistance. Resistant mice which had been treated with the immunosuppressive agent cortisone before infection with Coxsackie B-3 virus, developed high levels of viremia and many died despite the presence of normal titres of neutralizing antibody (Woodruff, 1979). The immigration of mononuclear inflammatory cells into target organs was greatly reduced. These observations suggest that both mononuclear cells and antibody are required for recovery from Coxsackie B-3 virus infection.

It has been noted that the genetically acquired resistance of chickens to Marek's disease is controlled by two genes. In addition to the Ly-4 locus, which determines the presence of a receptor on T lymphocytes for virus adsorption, the B locus, located within the major histocompatibility complex of the chicken, plays a role in resistance. The dominant B^{21} allele confers resistance to the disease upon strains of chickens which have this allele (Longenecker and Gallatin, 1977). It has been suggested that the B^{21} allele may give the host the ability to immunologically restrain the proliferation of MDV-transformed cells, (Longenecker et al., 1975 , quoted in Longenecker and Gallatin, 1977).

The infection of cats with feline leukemia or sarcoma viruses frequently, but not always, results in tumour formation. There is some evidence that an immune response to a cell surface antigen present on leukemia cells may play a role in determining the outcome of infection. It has been observed that cats which never developed tumours, or had tumours that regressed, had high titres of anti-FOCMA antibodies

(Essex, 1977). FOCMA, an acronym for feline oncornavirus associated cell membrane antigen, is an unique antigen which is present on the surface of feline lymphoma and virus-infected cells. It is thought not to be a structural virus protein (Essex, 1977) and may require a host function for its expression. Cats that died of tumours had either no anti-FOCMA antibody or very low levels. No correlation between survival and the presence of antibodies directed against structural proteins of the virus was demonstrable. Thus, tumour formation appears to be related to the cat's ability to generate a humoral immune response against FOCMA. This correlation could have two explanations. One possibility is that cats which develop leukemia are unable to make FOCMA. The other is that these cats may be genetically unable to immunologically respond to the antigen.

The examples described so far represent situations in which disease results from a direct effect of the virus, and resistance lies either in the ability of target cells to resist virus infection or in an immune response to the virus. However, the immune response occasionally acts to the detriment of the host: the immune destruction of an infected cell, when the viral cytopathic effect is relatively benign, can result in disease. Lymphocytic choriomeningitis (LCM) is a viral disease of mice. The pathogenesis of the acute disease has been attributed not to a direct effect of the virus but rather to the host's cell-mediated immune response against virus-infected cells (Doherty and Zinkernagel, 1974). The severity of chronic disease associated with virus infection is genetically controlled, and has been correlated to the histocompatibility type of the host (Oldstone et al., 1973).

Since the genes associated with the major histocompatibility complex are known to determine the nature and extent of the host's immune response to a wide variety of antigens (Benacerraf and Germain, 1978), it has been suggested that LCM disease is a function of the ability of the host to recognize viral antigen and mount a cell-mediated immune response, (McDevitt et al., 1974; Oldstone, 1975).

As has already been pointed out, the host possesses a number of non-specific defense mechanisms which help to limit virus replication or spread. These non-specific defenses may also be genetically regulated. For example, the genetically determined resistance of RV mice to infection with West Nile virus has been partially related to the ability of mouse embryo fibroblasts from resistant mice to produce defective interfering virus particles which interfere with normal virus replication (Darnell and Koprowski, 1974).

Bang (1976) has pointed out that the inheritance of susceptibility to Newcastle disease virus correlated with the genetically determined levels of interferon in certain mouse strains. It was suggested that the extent of interferon production may have been responsible for limiting virus replication and possibly altered the course of the disease.

The age-acquired resistance to herpes simplex virus (HSV) infections in mice is also genetically controlled. Resistance is a dominant phenotype and is under the regulation of at least two genes (Lopez, 1975). Resistance was originally thought to be associated with the ability of the maturing macrophage to restrict virus replication in vitro (Johnson, 1964). However, F_1 (resistant X susceptible) progeny,

which themselves are resistant to the lethal virus infection, have been shown to possess macrophages which support virus replication as well as cells derived from the susceptible parent (Lopez, 1977). T lymphocyte function is very important in survival (Lopez, 1977; Rager-Zisman and Allison, 1976), but it was not possible to demonstrate an absence of T lymphocyte responsiveness in susceptible mice.

A strong correlation between the ability to survive HSV infection and the capacity to reject bone-marrow grafts was noted by Lopez (1977) and confirmed by Kirchner et al. (1977). The ability of F_1 progeny to reject parental bone-marrow grafts is controlled by the Hh-1 locus (Cudkowicz, 1975; Clark et al., 1977b). The effector cell which mediates allogeneic resistance is thought to be identical to the effector cell that is responsible for natural killer (NK) activity (Harmon et al., 1977). Furthermore, animals which demonstrated high levels of allogeneic resistance were resistant to HSV infection while animals with low levels of allogeneic resistance were susceptible (Lopez, 1977). Thus, these observations suggested that NK cells played a role in resistance against HSV infection.

Support for this hypothesis is derived from work by Rager-Zisman and Allison (1979). These people showed that treatment of mice with cyclophosphamide, which increased their susceptibility to fatal HSV infection, decreased levels of spleen NK activity. In addition, the treatment of mice with anti-interferon antibodies, a procedure known to inhibit NK activity (Djeu et al., 1979a), markedly accelerated the appearance of disease and death in HSV-infected mice (Gresser et al., 1977).

NK cells may also play a role in the resistance of mice to murine leukemia virus (MULV). As has already been pointed out, the resistance of mice to murine leukemia virus is under complex controls. The Fv-2 gene regulates absolute resistance to the defective spleen focus-forming virus (SFFV) component of Friend virus disease (Lilly and Pincus, 1973), and resistance is recessive. It has been shown that treatment with the bone marrow-seeking isotope ^{89}Sr ablates resistance to the virus (Kumar et al., 1974). This suggested that a marrow-dependant cell or "M cell", played an important role in Fv-2 regulated resistance against Friend virus disease. Since ^{89}Sr treatment of mice also markedly reduces NK activity (Haller and Wigzell, 1977), a relationship between M and NK cells may exist. Thus, Fv-2 mediated resistance to Friend disease may involve a requirement for functioning NK cells.

1.3 Natural Killer (NK) Cell-mediated Cytotoxicity

Natural killer (NK) lymphocytes were originally detected by their ability to lyse tumour cells in vitro. NK lymphocytes have now been demonstrated in many species, including man, mice, guinea pigs and rats (Altman and Rapp, 1978; reviewed by Pross and Baines, 1977; Herbermann and Holder, 1978, and Herberman et al., 1979).

Several lines of evidence indicate that NK lymphocytes have some importance in vivo. At one time, it was thought that T lymphocytes were responsible for recognition and elimination of altered cells such as tumour cells or virus-infected cells. It was found, however, that congenitally athymic "nude" mice were no more susceptible to spontaneous tumour formation or to challenge with chemical carcinogens than normal

mice. "Nude" mice have been shown to have substantial NK activity (Herberman and Holden, 1978). Furthermore, Herberman et al. (1977) have demonstrated that the injection of normal or nude mice with tumour cells augmented endogenous NK activity. Those tumours which did arise in nude mice were not susceptible to NK activity (Herberman and Holden, 1978). These findings suggest that NK cells may have a role in immunosurveillance against tumours (Baldwin, 1977). Further evidence for this idea was provided by studies on strains of mice which have genetically determined differences in NK activity (Kiessling et al., 1975). Chimeras were created by repopulating irradiated F_1 recipients with bone marrow, a source of NK cells, from parents which expressed "high NK" or "low NK" activity. The recipients were then challenged with histocompatible tumour cells. It was observed that the presence of bone marrow cells from the "high NK" parent correlated with a reduced (50%) tumour take in the F_1 recipients, in contrast to the 100% tumour formation that was seen when "low NK" cells were used to repopulate the F_1 animals (Haller et al., 1977). Furthermore, preparations of spleen NK cells which had been partially purified by removal of B and T lymphocytes and macrophages (Kiessling et al., 1976), or by positive selection for an NK cell-specific antigen (Kasai et al., 1979), were able to suppress tumour growth in vivo when inoculated with tumour cells into recipient mice.

In addition to a role in immunosurveillance against tumours, NK cells may also be important in certain viral or bacterial infections. Many different viruses have been shown to induce NK activity in vivo (Herberman et al., 1977; rev. Herberman and Holden, 1978; rev. Welsh,

1978). This rise in activity appeared within one day of infection, and peaked at three days (Welsh and Zinkernagel, 1977; Welsh, 1978). It was found in the spleen, peripheral lymph nodes, peritoneal cavity, peripheral blood and the bone marrow. This wide-spread activity thus preceded the development of detectable levels of specific effector cells by a few days. It has already been mentioned that the genetic resistance of mice to fatal HSV infection correlated strongly with the genetically determined levels of NK activity in these strains (Lopez, 1977); manipulations of NK activity induced corresponding alterations in susceptibility to the virus (Rager-Zisman and Allison, 1979). Thus, NK cells may represent an early non-specific defense mechanism against certain infectious diseases.

In view of the potential importance of naturally occurring cytotoxic cells in host defense, considerable effort has been expended in their characterization. Although NK effector cells derived from different species demonstrate considerable variability in their properties, they do possess some common properties (reviewed by Herberman and Holden, 1978). NK cells are small, non-adherent, non-phagocytic lymphocytes which lack detectable surface immunoglobulin (Kiessling et al., 1975; Herberman et al., 1975; Shellman, 1973; Altman and Rapp, 1978; Welsh, 1978). It has already been pointed out that NK cells can be demonstrated in athymic "nude" mice; however, they probably express low levels of a thymus-associated antigen (Herberman et al., 1978), and also a surface receptor for the Fc end of immunoglobulin (Herberman et al., 1977; Arnaud-Battandier et al., 1978; Saksela et al., 1979). In addition to expression of these two markers, the mouse NK cell also

bears two NK cell-specific antigens on its surface, NK-1 (Glimcher et al., 1977) and Ly-5 (Cantor et al., 1979).

Treatment of mice with the bone-seeking isotope ^{89}Sr abrogated NK activity in the spleens of mouse strains which normally have high levels of activity (Haller and Wigzell, 1977). Moreover, the adoptive transfer of bone marrow from high or low reactive donors into irradiated recipients resulted in the phenotypic expression of high or low levels of NK activity, respectively (Haller et al., 1977). These observations suggest that the precursor NK cell is a bone marrow-derived cell. NK activity is relatively resistant to irradiation (Hochman et al., 1978), but it is sensitive to cyclophosphamide treatment (Herberman and Holden, 1978).

Many studies have been carried out in an attempt to establish the specificity of killing by NK cells (reviewed by Welsh, 1978; Herberman and Holden, 1978). The original observation that NK cells lysed tumour cells in vitro led to a search for virus-specific or tumour-specific antigens which were specifically recognized by NK cells (Ono et al., 1977; Herberman et al., 1974). Becker et al. (1976) demonstrated that the susceptibility of a cell to endogenous NK-mediated lysis did not correlate with the expression of murine C-type viral antigens. Further, unlike T lymphocyte cytolysis, NK cell-mediated lysis did not appear to be restricted to histocompatible target cells (Becker et al., 1976). NK cells killed syngeneic, allogeneic and even certain xenogeneic targets (Welsh and Zinkernagel, 1977; Wolfe et al., 1976; rev. Welsh, 1978).

After induction in vivo by viruses, chemicals or tumour cells,

NK cells were able to lyse most continuous cell lines and early passage fibroblasts, regardless of their susceptibility to endogenous NK cells (Nunn et al., 1977). However, target cells differed dramatically in their susceptibility to NK-mediated lysis, and the basis for this is not understood. Welsh (1978) has pointed out that primary cultures of peritoneal cells are lysed by induced NK cells from some, but not all, strains of mice. He has suggested that NK cells have two levels of recognition. The first level involves a non-specific recognition of transformed cell membranes, a feature which would be common to most continuous cell lines. The second involves specific recognition of an antigen on primary peritoneal cells, rendering only certain peritoneal exudate targets susceptible to lysis by effectors from different mouse strains (Welsh, 1978; Welsh et al., 1979).

The mechanism by which NK cells lyse target cells has been studied (reviewed by Kiessling and Wigzell, 1979). The interaction between effector and target cells was inhibited by the presence of EDTA, indicating a requirement for divalent cations. The binding of the target cell by the effector cell proceeded at 4°C but lysis only occurred at temperatures between 20° -37°C. Moreover, inhibitors of cell metabolism, such as sodium azide, suppressed NK-mediated lysis (Roder et al., 1978; Kiessling and Wigzell, 1979). These observations suggest that energy was required for lysis, but not for binding, of the target cell. In the mouse, lysis proceeded rapidly and was essentially complete within 2-4 hrs of incubation (Welsh, 1978). In humans, however, NK lysis proceeded linearly throughout the 18 hrs of incubation with the target cells (Santoli and Koprowski, 1979).

Because NK cells bear an Fc receptor for immunoglobulin, it has been important to determine whether lysis of targets was mediated through an intervening antibody. Such antibody-dependant cellular cytotoxicity (ADCC) can be mediated by a variety of Fc receptor-bearing cells, including polymorphonuclear leukocytes and macrophages. However, ADCC can be blocked by the presence of aggregated gammaglobulin or anti-immunoglobulin, while NK-mediated lysis is not. Thus, while the NK effector cell may be able to mediate ADCC (de Landazuri et al., 1979; Ojo and Wigzell, 1978; Santoli and Koprowski, 1979), the mechanisms of NK-mediated lysis and ADCC are probably distinct (Koren et al., 1978; Koren and Williams, 1978; Kiessling et al., 1976; Santoli and Koprowski, 1979).

Reference has already been made to the fact that NK activity can be augmented in vivo (reviewed by Herberman and Holden, 1978; Welsh, 1978). In mice, injection of a number of viruses, including C-type viruses, Kunjin virus, Semliki Forest virus, lactic dehydrogenase virus, minute virus of mice, lymphocytic choriomeningitis virus, mouse hepatitis virus, polyoma virus, Sendai virus, mouse adenovirus, Coxsackie virus and Pichinde virus, induced an augmented NK response which peaked three days after infection (McFarland et al., 1977; Herberman et al., 1978; Welsh, 1978). The bacteria Bacillus Calmette-Guerin (BCG) and Corynebacterium parvum had a similar effect (Herberman et al., 1977; Ojo et al., 1978; Wolfe et al., 1976, 1977), as did interferon and interferon-inducing agents such as polyinosinic:cytidilic acid (poly I:C), statolon and tilorone (Djeu et al., 1979; Gidlund et al., 1978; Oehler et al., 1978). The injection of mouse tumour or thymus

cells also induced NK activity which peaked after three days (Herberman et al., 1977).

The ability of such a heterogenous group of agents to induce or augment NK activity was also observed in rats. Oehler et al. (1978c) showed that C. parvum, lymphocytic choriomeningitis virus, Kilham rat virus and poly I:C increased the ability of rat spleen cells to lyse lymphoma targets in vitro.

Similar information on induction of human NK activity is available. An increase in cytotoxic activity following vaccination with swine influenza virus was noted (Herberman and Holden, 1978). NK cell activity has been generated in mixed leukocyte cultures in vitro (Jondal and Targan, 1978), after incubation with virus-infected fibroblasts (Santoli et al., 1978b) or tumour-derived cell lines (Trinchieri et al., 1978a, b) and after the addition of interferon or interferon inducers to cultures of human lymphocytes (Trinchieri et al., 1978b).

The extreme heterogeneity of agents which were found to induce NK activity in vivo and in vitro provided a clue to the mechanism behind the augmented activity: all agents were able to induce interferon (reviewed by Welsh, 1978). Studies to examine the role of interferon in induction of NK activity were therefore initiated. Mice which had been injected with LCMV secreted detectable levels of interferon within one day of infection (Welsh, 1978a). High levels were seen for the first three days after which titres dropped to low levels. The kinetics of interferon release thus closely parallelled the kinetics of NK activity. The ability of tilorone, statolon and Newcastle disease virus to augment mouse spleen cell cytotoxicity against ^{51}Cr-labelled YAC-1 tumour targets

was abrogated by the presence of anti-interferon antibody (Gidlund, et al., 1978), suggesting that the augmentation was mediated by interferon. It has already been mentioned that interferon alone was able to induce NK activity both in vivo (Welsh, 1978; Gidlund et al., 1978) and in vitro (Djeu et al., 1979a). Thus, interferon appeared to be an important mediator of induction of mouse NK activity.

The role of interferon in the activation of human NK lymphocytes has also been studied. In vitro cultures of human lymphocytes incubated with anti-lymphocyte serum, antigen, mitogens (Neumann and Sorg, 1977), tumour-derived or virus-transformed cell lines, or virus-infected cells all secreted interferon into the supernate (Trinchieri et al., 1978; Trinchieri and Santoli, 1978). The production of interferon in these cultures correlated strongly with the appearance of NK activity. It has been estimated that 70-90% of the NK activity seen in the usual 18 hour assay is attributable to the generation of interferon released into the supernate during the incubation, (Trinchieri and Santoli, 1978; Trinchieri et al., 1978; Santoli et al., 1978). The NK activity of human lymphocytes can also be augmented by directly adding interferon to lymphocyte preparations (Einhorn et al., 1978; Zarling et al., 1979). Thus, like the mouse system, these observations suggested that interferon plays an important role in induction of human NK activity.

A most interesting and potentially important observation was that pretreatment of target cells with interferon for several hours before assay completely protected them against NK-mediated lysis (Trinchieri and Santoli, 1978). These authors have proposed that interferon induces very efficient natural killer cells, but simultan-

eously protects normal cells from lysis. The implication then is that NK cells represent an inducible selective defense mechanism against tumours and virus-infected cells.

The augmentation of NK activity by various agents is superimposed on a background of endogenous activity that varies from one strain of animals to the next. Mice can be classified into "high reactive" and "low reactive" strains on the basis of the magnitude of cytotoxicity in vitro against ^{51}Cr-labelled YAC-1 Moloney virus-induced lymphomas (Kiessling et al., 1975c). The genetic basis for the levels of activity was examined in breeding experiments. High reactivity appeared to be dominant in F_1 (high X low) progeny, and the in vitro NK activity correlated well with in vivo resistance to YAC-1 tumour cells (Kiessling et al., 1976). Analysis of test-cross progeny for NK activity in vitro has suggested that multiple genes were involved, and that an H-2-linked gene was present (Kiessling et al., 1975; Petranyi et al., 1976). It has been proposed that the H-2 influence may be a manifestation of an Ir gene function.

A genetic component has been found in the strain distribution of NK activity in rats as well. In contrast to mice, high reactivity was recessive. There was no evidence for linkage to the rat (Ag-B) major histocompatibility locus (Oehler et al., 1978b). Genetic control of NK activity may also occur in guinea pigs; two strains of guinea pigs, 2 and 13, differed in their levels of endogenous NK activity (Altman and Rapp, 1978). Levels of human NK activity may also be genetically controlled; correlation to HLA types has been reported (Herberman and Holden, 1978).

The mechanisms underlying genetic differences among strains of mice in NK activity have been examined. Mice were thymectomized or splenectomized prior to assay for NK activity in vitro (Haller et al., 1978). Levels of NK activity in thymectomized, irradiated, bone-marrow reconstituted recipients were dependant only on the genotype of the bone marrow donor; that is, "high NK" or "low NK" donors conferred the "high NK" and "low NK" phenotypes, respectively, on the recipient. The presence of thymocytes derived from "high NK" or "low NK" animals had no effect on the marrow-dependant levels of NK activity. Splenectomy of mice 4 weeks prior to assay did not alter levels of NK activity in peripheral locations. Nor did splenectomy affect the reconstitution of irradiated young mice with syngeneic bone marrow cells. These observations suggest that the spleen did not affect peripheral NK activity or the reconstitution of NK activity in irradiated animals. Secondly, neither help nor suppression by T lymphocytes was demonstrable for endogenous NK activity.

In contrast to this situation, the host appears to possess several mechanisms for controlling NK activity which has been augmented by various agents. C57Bl/6 mice, which were injected with a syngeneic chemically induced tumor, were assayed for cytolytic activity by in vitro lysis of target cells. Activity was present at one week and at 4-5 weeks after grafting, but was absent during the second week. Lymph node cells obtained during this mute period could suppress the in vitro cytotoxicity of lymphocytes collected 28 days after grafting. Fractionation of these cells according to size revealed that cytotoxic cells were indeed present - but so was a larger, suppressive cell. This

suppressor was adherent but non-phagocytic, and was sensitive to treatment with antibodies to theta antigen and complement. Thus, a T suppressor cell was present which inhibited the activity of the effector cells (Schaaf-Lafontaine, 1978).

Macrophages have also been examined for a possible regulatory effect on NK activity (reviewed by Cudkowicz and Hochman, 1979). The ability of irradiated F_1 mice to reject parental bone marrow grafts, which is thought to be mediated by NK cells, is abolished by pretreating the F_1 recipients with carrageenan or silica (Cudkowicz and Yung, 1971). Since these agents are toxic to macrophages, the implication is that macrophages may be involved in activation of NK cells. Macrophages are required for the poly I:C induction of mouse NK activity *in vitro*; this has been attributed to a requirement for these cells in the production of interferon (Djeu *et al.*, 1979b). Oehler and Herberman (1978) have observed that silica reduced rat NK activity when injected one to three days prior to assay. In spite of this treatment, poly I:C was still able to enhance NK activity, even in adult thymectomized rats. This suggests that in rats, T lymphocytes are not required in the activation of NK cells, and further, that the poly I:C-induced enhancement can procede without healthy macrophages.

In addition to a role in activation of NK cells, macrophages may also be able to mediate suppression. Spina and Hofman (1979) have reported that an adherent, esterase-positive cell inhibited human spontaneous cytotoxicity *in vitro*; it was thought to contribute significantly to the loss of cytotoxicity that is observed in cultures over a period of time. The inhibitory effect of macrophages may be due to production of

prostaglandins, agents which have been shown to have a suppressive effect on NK cell activity (Droller et al., 1978). These observations suggest that macrophage-like cells may regulate the activity of the NK cell system, possibly by the production of soluble mediators.

1.4 Synopsis of the Study

Several members of the arenaviridiae cause fatal diseases in humans, including the causative agents of Lassa fever and Argentine and Bolivean hemorrhagic fever. Our present knowledge of the pathogenesis of these diseases and the nature of the human immune response against them has not provided methods for prevention or treatment. However, the fact that these viruses are biohazards has imposed restrictions on extensive study.

In many respects, the pathogenesis of Pichinde virus infection in Syrian hamsters resembles that of fatal human arenavirus infections (Rawls and Leung, 1979). A comparison of the factors leading to fatal Pichinde virus disease in the susceptible and resistant strains of hamsters may contribute to the understanding of the pathogenesis of arenavirus-induced diseases. The information derived from the study of hamsters may lead to practical approaches for preventing or controlling human arenavirus infections.

The present studies were begun in order to establish whether susceptibility to the intraperitoneal infection of Pichinde virus was under genetic control of the host, and to determine the basis for the susceptibility of MHA hamsters to a lethal infection with this virus. Experiments to determine the genetics of survival and the ability to limit viremia following infection with Pichinde virus were carried out.

The results are consistant with the hypothesis that a single autosomal dominant gene controls these phenotypes.

Previous work by Buchmeier and Rawls (1977) had implicated a role for the immune response in recovery from Pichinde virus infection. However, both strains of animals appeared to have adequate humoral immunity, as assessed by production of antibodies. Therefore, experiments were undertaken to test the hypothesis that MHA hamsters possessed a genetic defect in the ability to generate a cell-mediated immune response against Pichinde virus antigens. Such a defect would be expected to result in uncontrolled virus replication and death of the animals. It was observed that, while MHA hamsters were unable to respond with footpad swelling to a footpad challenge of virus, this normally susceptible strain survived the infection when the virus was given by this route. Furthermore, the animals surviving footpad challenge were protected against a subsequent intraperitoneal challenge of virus. This observation suggested that susceptible MHA hamsters were able to generate a protective immune response against Pichinde virus.

During studies on cytotoxic lymphocytes, it was found that susceptible MHA hamster spleens possessed higher levels of an endogenous cytotoxic activity against tumour cells in vitro than did the resistant LSH hamsters. This lytic activity was augmented by Pichinde virus infection to a greater extent in the MHA strain than in LSH hamsters. The cell which mediated the cytotoxicity had properties which are characteristic of natural killer (NK) cells.

An analysis of the target cells in the spleen early in infection was undertaken. It was found that cells with sedimentation characteris-

tics in an albumin gradient and adherent properties similar to macrophages were infected to a similar extent in both strains of hamsters. However, in the susceptible MHA strain a second population of cells was found to be infected by the virus. This cell was non-adherent and had sedimentation characteristics of lymphocytes. On the basis of these observations it is concluded that a major factor in the fatal outcome of intraperitoneal injection with Pichinde virus in the MHA hamsters is the presence of a primary target cell which is present in reduced amounts or absent in the spleens of LSH hamsters.

Of interest was the observation that the putative target cell co-purified with NK cell activity. Therefore, the hypothesis generated from the observations is that MHA hamsters have an additional target cell, defined by its NK activity, for Pichinde virus replication which the LSH strain lacks.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

The inbred MHA and LSH strains of hamsters, the outbred LVG strain, and F_1(LVG X MHA) progeny were obtained from Charles River (Lakeview, NJ). F_1(LSH X MHA) and back-cross progeny were bred in the animal quarters at McMaster University. The hamsters had access to Purina chow and water ad lib. They were housed in wire-topped polycarbonate cages with no more than 3 animals per cage. Animal rooms received 14 hrs of light daily between 2:00 A.M. and 4:00 p.m., a time period chosen to facilitate breeding.

2.2 Cell Lines and Cell Culture

Baby hamster kidney (BHK) cells were obtained from the Imperial Cancer Research Foundation in London, England, by Dr. C.R. Howard (London School of Hygiene and Tropical Medicine). Vero cells, derived from an African green monkey kidney, were purchased from Flow Laboratories (Rockville, MD). These two cell lines were grown in Eagle's minimal essential medium (MEM F-15; Flow Laboratories, Mississauga, Ontario) containing 10% v/v heat-inactivated fetal bovine serum (FBS: Grand Island Biological Company, Grand Island, NY), 0.75 g/l sodium bicarbonate, 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO). Syrian hamster embryo (SHE) cells were a gift of Dr. J. Docherty (University Park, PA.). These cells were derived from cultures of 13 day old embryos; the cells represent a continuous cell line which had not been established by transformation with viruses. SHE cells were grown in medium 199 (Flow) supplemented with 10% v/v tryptose phosphate broth and antibiotics.

Two cell lines were derived from tumours induced in MHA and LSH

hamsters by adenovirus type 12. The method of establishing cell lines from tumours has been described in detail by Tompkins et al. (1974). Neonatal MHA and LSH hamsters were injected subcutaneously with 10^9 virus particles. Selected animals bearing tumours were sacrificed and the tumours were aseptically excised. The tumours were minced into small fragments with scissors, and the fragments were then monodispersed by incubation in 0.15% trypsin (GIBCO) for 1 hr at 37°C and then overnight at 4°C. The supernate, containing single cells or small clumps of cells, was decanted and poured into 75 cm^2 plastic tissue culture flasks (Corning Glass Works, Corning, NY) containing alpha MEM (Flow) supplemented with 10% v/v FBS, 0.75 g/ml sodium bicarbonate, 0.02 M hydroxyethylpiperazine N'-2 ethansulfonic acid pH 7.24 (HEPES) buffer (GIBCO), 3% v/v distilled water, and antibiotics. This medium is subsequently referred to as αMEM. The monodispersed cells from an MHA hamster were grown into monolayers, and established into a cell line designated MAD. A cell line derived from an LSH hamster was called LAD.

The herpes simplex virus type 2-transformed hamster cell line, 333-8-9T was obtained from Dr. Fred Rapp (Hershey, PA) and was also propagated in αMEM.

2.3 Viruses and Virus Assays

2.3.1 Pichinde Virus

The original isolate of Pichinde virus, strain AN 3739, was obtained by Trapido and Sanmartin (1971) from the blood of a rodent called Oryzomys albigularis. A stock of virus was made by infecting monolayers of BHK cells with 1-3 plaque-forming units (pfu) of virus per cell. The virus was allowed to adsorb for 90 min at 37°C. The

cells were then refed with F-15 MEM. The virus was harvested after 48 hr of incubation of the infected cells at 37°C. Aliquots of virus were dispersed into vials and stored at -90°C. This stock, which had a titre of 10^6 pfu/ml, was used in all animal studies. Animals were injected intraperitoneally (IP) with 2 X 10^3 pfu Pichinde virus diluted in 0.2 ml F-15 MEM unless otherwise noted.

Pichinde virus was assayed by plating 10-fold dilutions on confluent monolayers of Vero cells grown in 60 mm^2 plastic tissue culture dishes (Lux Scientific Corporation, Newbury Park, CA), according to the method described by Mifune et al. (1971). Briefly, the virus was adsorbed to Vero cells for 90 min at 37°C. The infected cells were then overlaid with MEM F-15 supplemented with the additives already described, plus 50 U/ml mycostatin (E.R. Squibb & Sons, Inc., Princeton, NJ), 0.75 g/l sodium bicarbonate and 1% w/v Bactoagar (DIFCO Laboratories, Detroit, MI). Following an incubation for 3 days at 37°C, a second agar overlay containing 0.1 g/l neutral red (GIBCO) was added. Plaques were enumerated after an additional incubation of 24 hr at 37°C.

2.3.2 Herpes Simplex Virus

Herpes simplex virus (HSV) type 1, strain KOS, was isolated from a lip lesion of a patient. The propagation of HSV has been described by Rawls et al. (1968). Stocks of the virus were prepared by infecting monolayers of Vero cells with an MOI of 1-3 pfu/cell. After adsorption for 60 min at 37°C, the infected cells were refed with MEM F-15. The cells were then incubated at 37°C until a complete cytopathic effect was visible. The supernate, containing virus, was centrifuged at 29,000 rpm for 1 hr in a Damon/IEC B60 ultracentrifuge. The pellet of virus

was then combined with a cell lysate, obtained by freeze-thawing infected cells and centrifuging at a low speed to remove debris. This stock had a titre of 1 X 10^9 pfu/ml, and was stored at -90°C.

2.3.3 Vaccinia Virus

A stock of vaccinia virus, strain WR, was made, prepared and titred as described by Niederkorn-Buchmeier (1977). Briefly, primary rabbit kidney cells were infected with 0.1 pfu/cell. The virus was allowed to adsorb for 1 hr at room temperature, and the infected cells were refed with MEM F-15. After an incubation of 48 hr at 37°C, the infected cells were freeze-thawed in the virus-containing medium, and debris was removed by low speed centrifugation. Aliquots of the supernate were frozen at -90°C; the stock had a titre of 2.5 X 10^7 pfu/ml.

2.3.4 Vesicular Stomatitis Virus

Vesicular stomatitis virus (VSV), strain HR-LT of the Indiana serotype, was obtained from Dr. L. Prevec, McMaster University. The preparation and method of titration of this virus have been described by SenGupta and Rawls (1979a). To briefly outline the method, BHK cells were infected with 5 pfu/cell. After an adsorption of 30 min at 37°C, the plates were refed with MEM F-15 and returned to the 37°C incubator for 18 hr. The virus-containing supernate was harvested and frozen in aliquots at -90°C. This stock had a titre of 1 X 10^7 pfu/ml.

2.3.5 Adenovirus

Adenovirus type 12, strain 1131, was a gift of Dr. S. Mak (McMaster University). Its preparation and method of assay have been described by Smiley and Mak (1978). For studies on oncogenicity of this virus,

ten-fold dilutions of the virus stock were made in phosphate buffered saline (PBS) and 0.1 ml of the appropriate dilutions were injected subcutaneously into neonatal hamsters.

2.4 Infectious Centre Assays

Infectious centre assays were performed on lymphoid cells from infected hamsters by making 10-fold dilutions of cells in medium and adding 0.2 ml of appropriate dilutions to drained monolayers of Vero cells which had been grown in 60 mm tissue culture dishes. After 1 hr at 37°C the monolayers were carefully overlayed with MEM F-15 medium containing 1% w/v agar. A second agar overlay containing neutral red (0.1 g/l) was added 3 days later and plaques were counted after an additional day of incubation at 37°C. Previously, it was found that infectious centres assayed by this method correlated well with the number of antigen-containing BHK cells (SenGupta and Rawls, 1979a).

2.5 Growth Curves of Pichinde Virus In Vitro

Peritoneal exudate cells were obtained by aseptically washing the peritoneal cavity with 100 ml Hanks' balanced salts solution containing 2% v/v FBS, antibiotics and 0.75% w/v sodium bicarbonate. The cell suspension was then centrifuged at 200 g for 10' at 4°C, and resuspended in αMEM. Suspensions of spleen cells were prepared as described for the ^{51}Cr release assay.

Cells were infected in suspension by mixing 2 X 10^7 viable nucleated spleen cells with 2 X 10^7 pfu Pichinde virus in a total volume of 2.0 ml αMEM. After an adsorption of 90' at 37°C, the infected cells were washed 3 times with 10.0 ml αMEM to remove any unadsorbed virus. The cells were then resuspended at a concentration of 2 X 10^6

cells/ml, and 1.0 ml was aliquoted into a polypropylene tube. The tubes were then incubated at 37°C. Duplicate samples were removed at 0, 2, 24 and 48 hr and frozen at -45°C. All samples were subjected to 3 cycles of freeze-thawing, and duplicate samples were pooled prior to assay for infectious Pichinde virus on monolayers of Vero cells.

2.6 ^{51}Cr Release Assay

The procedure described by Clark et al. (1977) was followed, with some modifications. Spleens which had been aseptically removed from sacrificed animals were minced with scissors and the fragments were gently pressed through a 60 gauge wire mesh screen. These cells were suspended in αMEM (supplemented as above), and large debris was removed by layering the cell suspension over FBS. The cells were then counted by trypan blue exclusion in a hemacytometer and adjusted to 1×10^7 viable nucleated cells/ml. Doubling dilutions of these spleen cells were made in 96 well flat-bottom tissue culture plates (Linbro), in a total volume of 0.1 ml αMEM.

Stocks of monodispersed target cells which had been suspended in αMEM containing 10% v/v dimethyl sulfoxide (DMSO) and frozen in liquid nitrogen were thawed out and grown in culture for 1 to 2 days. This procedure minimized experimental variation. The target cells were monodispersed by trypsinization and labelled with 250 μCi sodium ^{51}chromate (New England Nuclear, Boston, MA.) for 90 min at 37°C, in Hanks' balanced salt solution (HBSS) without Ca++ or Mg++ (GIBCO) supplemented with 10% v/v heat-inactivated FBS, 0.02 M HEPES, and 3% v/v water. The labelled cells were then washed 3 times in HBSS and

suspended in αMEM at a final concentration of 1 X 10^5 viable cells/ml. The target cells, in a volume of 0.1 ml, were then added to wells containing effector cells and incubated for 16 hr, unless otherwise noted, at 37°C in CO_2 incubator. The plates were centrifuged at 200 g for 10 min, and 0.1 ml supernate was removed and counted in a gamma counter (Beckman Gamma 300). Values for maximal release were obtained by mixing 0.1 ml of the labelled target cells with 0.1 ml water containing 1% v/v NP-40. Spontaneous release was determined by incubating the target cells in 0.1 ml αMEM for the duration of the assay and usually ranged between 35 and 50% of the maximal release. Results are expressed as the mean per cent specific ^{51}Cr release of 4 replicates, where

$$\% \text{ specific } {}^{51}Cr \text{ release} = \frac{\text{test cpm} - \text{spontaneous}}{\text{maximum} - \text{spontaneous}} \times 100\% .$$

The standard error of the mean for the data that will be presented was always less than 5% unless otherwise indicated.

2.7 Characterization of NK Cells

2.7.1 Adherence

A maximum of 4 X 10^7 spleen cells, at a concentration of 2 X 10^6 cells/ml, was incubated in 100 mm sterile plastic tissue culture dishes (Corning) for 60 min at 37°C. The supernate, which contained non-adherent cells, was decanted. The monolayer was then washed 3 times with 5.0 ml PBS lacking Ca++ or Mg++ to remove any remaining non-adherent cells, and the washes were added to the first supernate. Adherent cells were gently removed from the plastic with the aid of a rubber policeman and suspended in 5.0 ml PBS. Both adherent and non-adherent cell fractions were centrifuged at 200 g

for 10 min, resuspended in αMEM and adjusted to a final concentration of 1 X 10^7 viable nucleated cells/ml. The total recovery was usually between 75-95%.

2.7.2 Phagocytosis and/or Adherence of Carbonyl Iron Particles

Spleen cells, adjusted to a concentration of 1 X 10^8 cells in 7.0 ml αMEM, were incubated with 1.0 g carbonyl iron in 1.0 ml PBS for 45 min at 37°C with occasional shaking, as described by Lee et al. (1976). The carbonyl iron-containing cells were then held to the side of the tube with the aid of a magnet, and the carbonyl iron-resistant cells were aspirated, washed once and readjusted to 1 X 10^7 viable nucleated cells/ml. The yield of cells after this treatment represented 25-50% of the input depending on the source of spleen cells.

2.7.3 Lability of Cytotoxic Activity

Effector cells were diluted in 0.1 ml αMEM in microtitre plates, as described for the ^{51}Cr release assay. These cells were then incubated for 24 hr at 37°C in a CO_2 incubator prior to testing for cytotoxic activity against fresh ^{51}Cr-labelled MAD targets.

2.7.4 NH_4Cl Treatment

Spleen cells were treated with NH_4Cl as described by Shortman et al. (1972). The cell preparation, which had been centrifuged at 200 g for 10 min to pellet the cells, was suspended in 3.0 ml of 0.174 M NH_4Cl, a solution which is iso-osmotic to hamster serum. The cells were incubated in NH_4Cl for 10 min at 4°C, and then 1.0 ml of FBS was layered beneath the cell suspension. The cells were again centrifuged to pellet the remaining cells. This treatment destroyed 99.9% of the red blood cells as assessed by enumeration in trypan blue, while

approximately 90% of the white cells was recovered.

2.7.5 Requirement for Divalent Cations

To assess the requirement for divalent cations, 0.025 M ethylenediaminotetracetic acid (EDTA) diluted in αMEM was added to individual wells during the standard ^{51}Cr release assay either before the targets were added, or at various times during the 16 hr incubation, to give a final concentration of 0.005 M EDTA.

2.7.6 Expression of Surface Immunoglobulin

Immunoglobulin-bearing spleen cells were removed by suspending 2.2 X 10^7 cells in 2.0 ml of rabbit anti-hamster immunoglobulin antiserum diluted 1:10 in αMEM containing 0.2% w/v bovine serum albumin (αBSA) in place of FBS. After incubation for 30 min at 37°C, the cells were washed and resuspended in Low Tox rabbit complement (Cedarlane Laboratories, Hornby, Ont.) diluted 1:7.5 in αBSA. After a second incubation for 30 min at 37°C, the cells were washed in αMEM and resuspended at a concentration of 1 X 10^7 viable cells/ml prior to testing for NK activity.

2.8 Velocity Sedimentation of Spleen Cells

Spleen cells were separated by velocity sedimentation under unit gravity at 4°C in a STA-PUT apparatus (O.H. Johns Scientific Co., Ltd., Toronto, Ontario), as described by Miller and Phillips (1969) and modified by Miller (1973). Briefly, spleen cells suspended in 0.2% w/v bovine serum albumin (BSA) in PBS were layered beneath PBS in the sedimentation chamber (diameter, 24 cm). A stacking region of 0.35% BSA, and then a continuous gradient of 1.0 - 2.0% BSA in PBS, was formed beneath the cell layer. The cells were allowed to settle

through this gradient for 3.5 to 4.0 hrs at 4°C. Fractions of 50 ml were collected and the sedimentation velocity for each fraction was calculated as previously described (Miller and Phillips, 1969; Miller, 1973) using a computer program developed by Dr. David Clark (McMaster University). In one experiment, sedimentation chambers of a diameter of 12 cm were used, in which event 25.0 ml fractions were collected. The cells in the fractions were concentrated by centrifugation at 200 g for 10 min and resuspending the pellet in 1.0 ml of αMEM. The viable cell content in each fraction was then assessed by trypan blue exclusion in a hemacytometer. The concentration of each fraction was adjusted to 1 X 10^7 viable nucleated cells/ml; fractions were pooled when necessary to make up the required concentration. Each fraction was then assayed for NK activity by ^{51}Cr release from labelled MAD cell targets, and for infectious centres.

2.9 Footpad Swelling

The hind footpads of animals to be tested were pre-measured using a micrometer (Moore and Wright, Ltd., Sheffield, England). One footpad then received an injection of virus in a volume of 10λ using a Hamilton constant delivery syringe and a 27 gauge, 1/2" needle. In some cases, a similar volume of control antigen, which was a sonicated preparation of BHK cells, was injected into the other rear footpad. Daily measurements of both hind feet were then made.

2.10 Histological Studies on Footpads

Animals were sacrificed using ether, and the hind feet were amputated and fixed in 10% buffered formalin. The samples were decalcified and embedded in paraffin wax prior to sectioning and

staining with hematoxylin-eosin. The coded samples were scored by Dr. Jarrett Gardner at the Centre for Disease Control, Atlanta, GA.

2.11 Complement-fixation Test for Detection of Antibody

Antibodies against Pichinde virus antigens were quantitated by complement fixation. The procedure described by Kagan and Norman (1970) was adapted to microtitre plates.

Doubling dilutions of the sera to be tested were made in round bottom 96-well Microtiter plates (Cooke Engineering Company, Alexandria, Virginia) in a total volume of 25λ of veronal buffered diluent (VBD), using constant delivery (25λ) Microtiter dilution loops (Cooke Engineering Company, Alexandria, Virginia). To each of triplicate wells was added 5 units of antigen in a total volume of 25λ. One unit of antigen was defined as that amount of antigen which, in the presence of excess anti-Pichinde virus antibody, consumed 50% of the available complement. The positive antigen was prepared by sonicating and freeze-thawing Pichinde virus-infected BHK cells. This antigen preparation did not react with normal hamster sera, but had a high titre when reacted with immune sera from Pichinde virus-infected animals. In this case, a 1:320 dilution of the antigen preparation contained 1 unit of antigen, and therefore a dilution of 1:64 contained 5 units of antigen. As a negative control, all test sera were also assayed against a sonicated preparation of BHK cells, diluted 1:10 in VBD.

To each well containing antigen and antibody was added 5 C'H50 units of guinea pig complement (GIBCO) in a total volume of 50λ. The guinea pig complement had previously been titred against

sensitized sheep red blood cells; 1 C'H50 unit was that dilution of complement which lysed 50% of a 1.4% v/v suspension of sensitized sheep red blood cells. Each dilution of sera and both antigen preparations were screened for anticomplementary activity by adding 5 C'H50, 2.5 C'H50, 1.25 C'H50 and no complement, in the absence of antigen or antibody, respectively. In all tests, both known positive and known negative antisera were also included as controls.

The antigen and antibody were allowed to react with complement overnight at 4°C, and then warmed for 15 min at room temperature. Sheep red blood cells were washed 3 times in VBD, and a 2.8% v/v cell suspension was made. The sheep red blood cells were sensitized by incubating 1 volume of the washed 2.8% v/v cell suspension with 1 volume of haemolysin (GIBCO), diluted 1:2000, for 15 min at room temperature. This dilution of haemolysin had previously been determined as optimal. Individual wells then received 25λ of the 1.4% v/v sensitized sheep red blood cell suspension; the plates were incubated for 1 hr at 37°C, at which time the complement controls exhibited the appropriate amount of lysis. The wells were scored for the amount of lysis on a 0-4+ scale, where 0 represented 100% lysis and 4+ indicated a clear supernate with an intact red cell button. A reaction of 3-4+ was considered positive.

CHAPTER 3

RESULTS

3.1 Genetics of Susceptibility to Pichinde Virus Infection in Syrian Hamsters

LVG hamsters, a random-bred strain maintained in a closed colony, were used in the initial studies of susceptibility to Pichinde virus. LVG hamsters survived an intraperitoneal challenge of at least 10^6 pfu Pichinde virus (unpublished observations) whereas MHA hamsters were susceptible to as little as 35 pfu (Buchmeier and Rawls, 1977). These observations raised the possibility that susceptibility to the lethal virus infection was genetically determined. To test this idea, F_1(LVG X MHA) hamsters and back-cross progeny along with hamsters of parental strains were injected with Pichinde virus and observed daily for survival. The results are shown in Figure 1 and Table I. The survival of the F_1 hamsters and (F_1 X LVG) progeny was significantly different from that of MHA hamsters ($p<0.001$ for both) but not different from survival of LVG hamsters ($0.1<p<0.2$ and $0.4<p<0.5$, respectively). The survival of (F_1 X MHA) progeny did not differ significantly from the values expected for a dominant characteristic controlled by a single gene or linked genes ($p=0.9$).

Levels of viremia in these animals were determined 8 days after infection with Pichinde virus (Table II). As shown in Table II, Experiment 1, F_1(LVG X MHA) progeny limited virus replication in the blood to 2.45 ± 1.58 $\log_{10}$ pfu/ml, while LVG animals had a mean titre of 2.80 ± 1.13 $\log_{10}$ pfu/ml. MHA hamsters had a mean titre of 6.52 ± 0.64 $\log_{10}$ pfu/ml blood, which was significantly greater than either LVG or F_1(LVG X MHA) animals ($p<0.001$). Thus, the ability to limit viremia behaves as a dominant trait. Among a total of thirty-five (F_1 X LVG) back-cross progeny examined in Experiments 1 and 2,

Figure 1

Survival in F_1 progeny after challenge with 10^5 pfu Pichinde virus. LVG, MHA and F_1(LVG X MHA) animals were inoculated with 10^5 pfu Pichinde virus IP. Animals were caged individually and observed daily for survival.

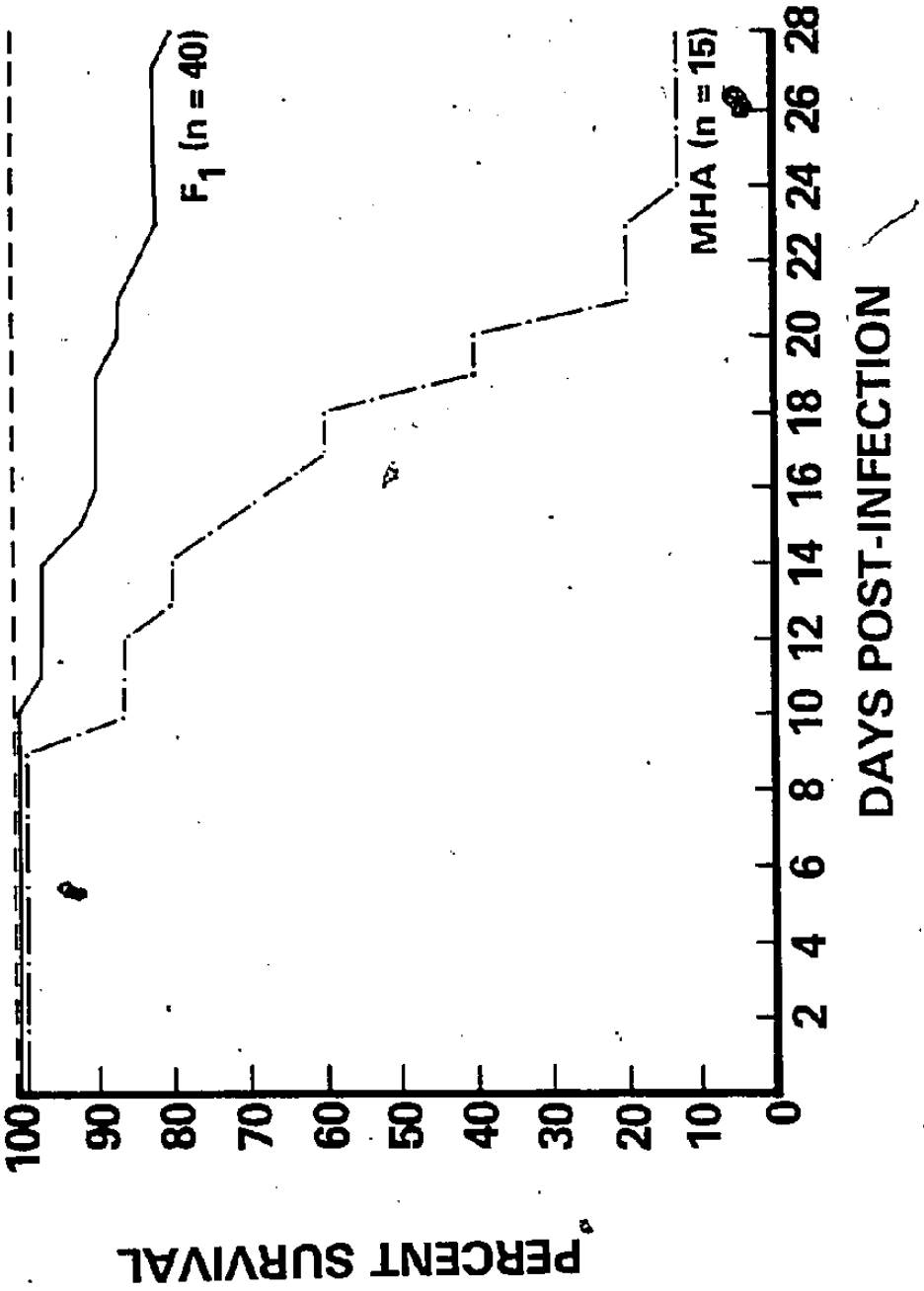

LVG (n = 15)
F1 (n = 40)
MHA (n = 15)
PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
0
2 4 6 8 10 12 14 16 18 20 22 24 26 28
DAYS POST-INFECTION

Table I

Inheritance of Susceptibility to Fatal Pichinde Virus Infections in Syrian Hamsters

Genetic Background	Number surviving per number tested[a]				Per cent survival
	Expt. 1	Expt. 2	Expt. 3	Total	
LVG	15/15	5/6	6/6	26/27	96.3
MHA	2/15	2/6	4/10	8/31	25.8
F_1 (LVG X MHA)	32/40	NT	8/8	40/48	83.3
F_1 X LVG	NT[b]	12/16	23/24	35/40	87.5
F_1 X MHA	NT	15/35	11/16	36/51	51.0[d]

[a] Animals which had been injected with 2000 pfu Pichinde virus IP were housed individually, and observed daily for survival.

[b] Not tested.

[c] The survival rates of F_1 and (F_1 X LVG) progeny were compared to those seen in the MHA and LVG strains using a χ^2 test for homogeneity; Yates correction for continuity was applied when expected values were ≤ 5. * $p>0.10$; ** $p<0.001$

[d] The survival rate in (F_1 X MHA) progeny was compared to that expected for the effect of 1 gene by applying a χ^2 test for goodness of fit. ($\chi^2=0.02$; $p=0.9$)

Table II

Inheritance of Ability to Limit Viremia after Pichinde Virus Infection

Genetic Background	n	Phenotype[c.]	Mean virus titre ± SD[d.] (log_{10} pfu/ml blood)
Experiment 1[a.]			
LVG	9	-	2.80±1.13
MHA	9	-	6.52±0.64
F_1 (LVG X MHA)	19	19 LVG	2.45±1.58
F_1 X LVG	21	17 LVG	3.65±0.73[e.]
		4 MHA	5.94±0.26[e.]
F_1 X MHA	16	9 LVG	3.83±0.82[e.]
		7 MHA	6.58±0.57[e.]
Experiment 2[b.]			
LVG	5	-	4.95±0.55
MHA	3	-	7.74±0.42
F_1 X LVG	14	14 LVG	3.71±1.24[e.]
F_1 X MHA	24	13 LVG	3.78±1.46[e.]
		11 MHA	6.95±0.54[e.]

[a] Animals were injected with $2X10^3$ pfu Pichinde virus IP.

[b] Animals were injected with $2X10^5$ pfu Pichinde virus IP.

[c] F_1 and back-cross progeny were classified as having an LVG phenotype if their virus levels fell within 1.96 standard deviations (SD) of the mean LVG virus titre. All titres above this limit were defined as manifesting the MHA phenotype.

[d] Individual animals were bled by cardiac puncture 8 days after infection with virus. Aliquots of blood were diluted 1:10 in medium and then stored at -90°C until assay for plaque-forming units on monolayers of Vero cells.

Table II (Footnotes cont'd)

[e] These titres represent the mean $\log_{10}$ pfu of each phenotypic group in the back-cross progeny.

thirty-one (89%) had titres which resembled the LVG parent. Among forty (F_1 X MHA) test-cross animals, twenty-two (55%) had titres of virus similar to their LVG parent and eighteen (45%) had titres which resembled the MHA strain. These results are consistant with the idea that a single dominant gene controls virus replication ($0.6<p<0.7$). No difference in survival or virus titre with respect to sex of the animal was observed. An analysis of coat-colour in F_1 and back-cross progeny failed to reveal a linkage of genes controlling this trait to those genes responsible for survival and limiting viremia after Pichinde virus infection (data not shown).

In much of the later work, an inbred strain, LSH, was utilized for comparative studies with the inbred MHA strain. While specific genetic crosses were not performed between LSH and MHA hamsters, the LSH hamsters were found to be similar to the LVG hamsters in their response to Pichinde virus. The animals survived challenge with Pichinde virus and the mean virus titer 8 days after infection was 3.4×10^3 pfu/ml blood. The LSH hamsters produced titres of anti-viral antibody comparable to LVG hamsters, and peritoneal exudate cells from these animals supported Pichinde virus replication in vitro as well as MHA peritoneal exudate cells (data not shown).

3.2 Studies on the Cell-Mediated Immune Response to Pichinde Virus in Syrian Hamsters

Studies were undertaken to analyze the mechanisms responsible for the observed genetic differences in susceptibility to fatal Pichinde virus infection in LVG, LSH and MHA hamsters. In the early studies, the histopathology of the virus-induced lesions in the susceptible and resistant

animals was compared (Murphy et al., 1977). Focal necrosis was observed in a number of organs, particularly the liver and the reticuloendothelial system. The severity of necrosis correlated with the extent of virus infection as quantified by serum titres and was thought to be responsible for the total outcome of the infection. Since the sites of necrosis lacked evidence of a mononuclear infiltrate (Murphy et al., 1977), the lesions did not appear to be of an immunopathological nature but were most likely caused by a direct viral cytopathic effect. These observations suggested that the main difference between the susceptible and resistant strains lay in the ability to limit virus proliferation. Because peritoneal exudate cells and primary kidney cells from either hamster strain supported virus replication equally well in vitro (Buchmeier and Rawls, 1977), an innate difference in the ability of target cells to support virus replication was an unlikely explanation for the observed differences in susceptibility in vivo.

Other information suggested that the limiting mechanism could be the development of an immune response in the resistant strains. A protective role for immunity was implied by the finding that cyclophosphamide treatment of LVG hamsters rendered them susceptible to a lethal Pichinde virus infection. Furthermore, it was observed that resistance in this strain developed post-natally (Buchmeier and Rawls, 1977), in association with the maturation of the host's immune response. However, it was also found that both strains of hamsters produced similar titres of antibodies against internal antigens of the virion (Buchmeier and Rawls, 1971), suggesting that immune recognition of viral antigens, as assessed by the humoral immune system, was not deficient in the suscep-

ible strain.

Therefore the hypothesis was proposed that MHA hamsters were genetically unable to generate an appropriate cell-mediated immune response against Pichinde virus, leading to uncontrolled virus replication and death of the host. In order to test this hypothesis, some studies of cell-mediated immunity were done.

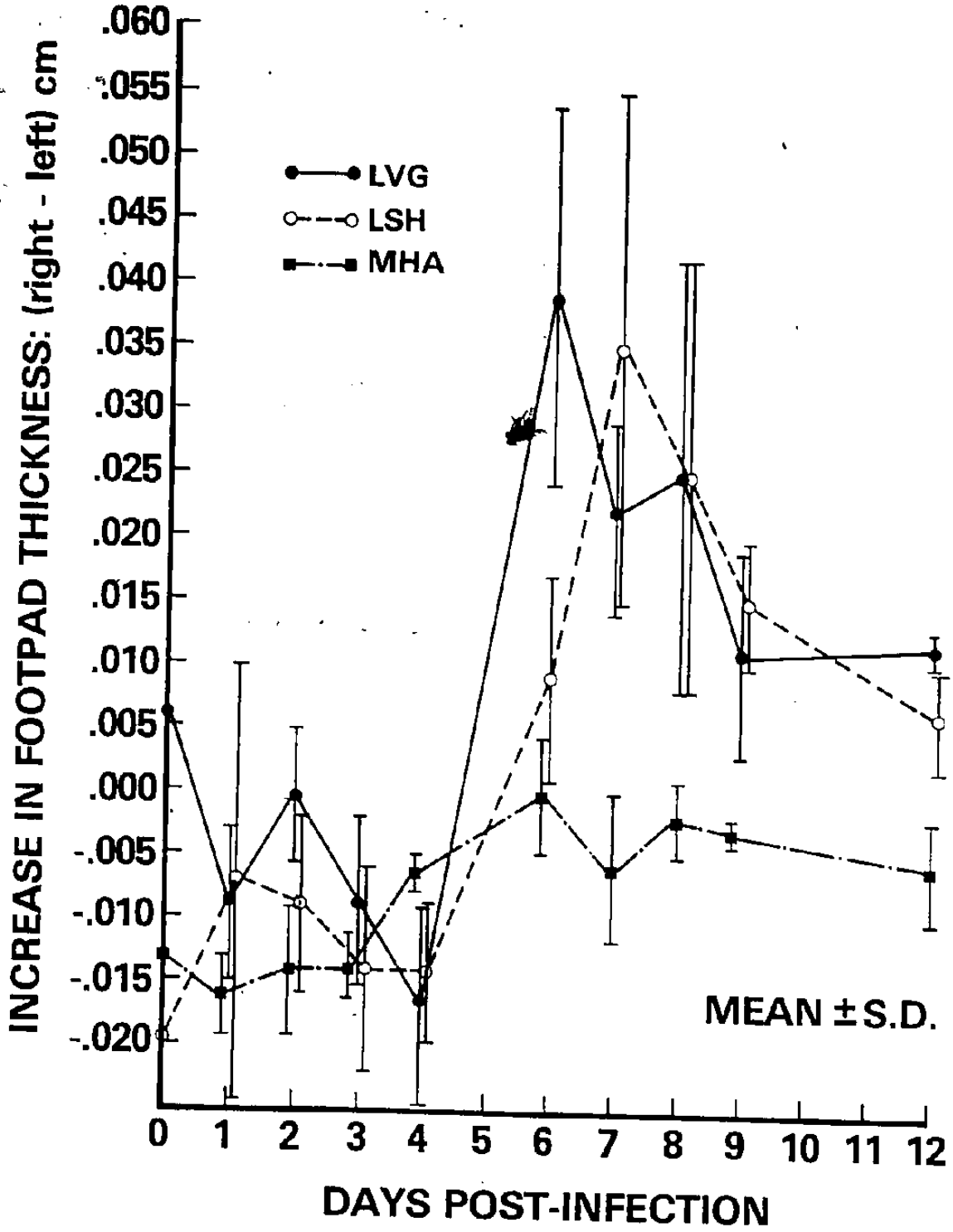
3.2.1 The Footpad Swelling Response to Pichinde Virus

Zinkernagel et al. (1977) have reported that swelling of the footpad is observed eight days after injection of lymphocytic choriomeningitis virus or vaccinia virus into the footpads of rats. The kinetics of the footpad swelling response coincided with the appearance of cell-mediated immunity, as measured by an increased weight of the draining lymph nodes, increased numbers of lymphocytes per node, and the presence of cytotoxic T-lymphocytes which specifically lysed histocompatible virus-infected cells. It was concluded that footpad swelling after a primary exposure to an antigen was a manifestation of cell-mediated immunity.

This test for cell-mediated immunity was performed using hamsters infected with Pichinde virus. The results are shown in Figure 2. A swelling response was observed in both LVG and LSH hamsters after a footpad injection of virus, and it peaked between 6 and 8 days after injection. No significant response was seen in MHA hamsters. This pattern of response was not elicited by control antigen alone, and was independant of the dose of virus given, over a range of 10^3 - 10^6 pfu Pichinde virus (data not presented).

Figure 2

Kinetics of the footpad swelling response after Pichinde virus injection in the footpads of Syrian hamsters. Groups of three LVG, LSH and MHA hamsters received an injection of 10^6 pfu/10λ Pichinde virus in the right hind footpads. The left pad received an injection of a sonicated preparation of BHK cells. Footpads were measured daily with spring-loaded calipers. The data are presented as the mean difference in thickness between the test (right) and control (left) footpads.


INCREASE IN FOOTPAD THICKNESS: (right - left) cm
.060
.055
.050
.045
.040
.035
.030
.025
.020
.015
.010
.005
.000
-.005
-.010
-.015
-.020
LVG
LSH
MHA
MEAN ± S.D.
0 1 2 3 4 5 6 7 8 9 10 11 12
DAYS POST-INFECTION

In order to define more closely the nature of the swelling in LVG and LSH hamsters, histological analyses were carried out on footpads from animals which had been injected with control antigen or Pichinde virus. Feet from these animals were amputated and then stored in buffered formalin prior to embedding in paraffin. Sections of these samples were decalcified and stained with hematoxylin-eosin. The slides were read by Dr. Jarrett Gardner at the Center for Disease Control, Atlanta, Georgia. The results are summarized in Table III. In both LVG and LSH hamsters, the control, virus-free preparation of BHK cells elicited no histological response, while an injection of Pichinde virus induced some edema and infiltration by both lymphocytes and macrophages 7-8 days later. This is typical of a cell-mediated immune response localized in the footpad. MHA hamsters showed no evidence of a cellular infiltration, as was expected from the lack of swelling.

To determine whether the footpad route of inoculation introduced sufficient virus to elicit a host reaction, footpad-injected hamsters were bled by cardiac puncture on day 8, at the time of maximum swelling, and again on day 13. Aliquots of blood were assayed for infectious Pichinde virus, and sera were assayed for antibodies to the virus by complement fixation. These results are shown in Table IV. The 6 MHA hamsters had a mean virus titre of 3.90 ± 0.83 $\log_{10}$ pfu/ml blood 8 days after infection; there was no detectable viremia by day 13. No virus was demonstrable in the LSH strain. The antibody response, as measured by complement fixation, was comparable to that elicited by IP immunization, and was greater in MHA hamsters than in the LSH strain. Thus, the failure of the MHA to respond to a footpad inoculation of Pichinde virus

Table III

Histology of the Footpad Swelling Response to Pichinde Virus

Strain	Antigen[a]	Histology of the Foot[b]			
		edema	PMN	lymphocytes	macrophages
LVG	Pichinde	1+	0	1-2+	1-2+
	BHK	0	0	Trace	Trace
LSH	Pichinde	1+	0	1-2+	1-2+
	BHK	0	0	Trace	Trace
MHA	Pichinde	0	0	Trace	Trace
	BHK	0	0	Trace	Trace

[a] Animals were injected with 10^6 pfu Pichinde virus in the right hind footpad, and with a sonicated preparation of BHK cells in the left hind footpad. Animals were sacrificed 7-8 days later and the hind feet were amputated, embedded in paraffin, sectioned and stained with hematoxylin-eosin. Duplicate sections were read for each animal by Dr. Jarett Gardner of the Center for Disease Control, Atlanta, Georgia.

[b] The amount of edema, and infiltration by polymorphonuclear leukocytes (PMN), lymphocytes and macrophages was scored on a 0-4+ scale, where 0 represents no response, and 4+, a maximum response.

Table IV

Viremia and Serum Antibody Following Footpad Inoculation of Pichinde Virus in LSH and MHA Hamsters

Strain	Number	Footpad Swelling[a]	Viremia[b]		CF Antibody[c]	
		Mean increment (cm) ± SD	Mean $\log_{10}$ pfu/ml ± SD		Mean $\log_{10}$ titer ± SD	
			day 8	day 13	day 8	day 13
LSH	6	0.040±0.018	<2.70	<2.70	<1.176	1.68±.17
MHA	6	0.007±0.007	3.90±0.83	<2.70	1.53±.12	2.14±.14

[a] The right hind footpads of each animal were measured. Each animal then received an injection of 10^6 pfu Pichinde virus in this footpad. The swelling response is presented as the mean difference between footpad thickness on day 7 and the thickness on day 0. (SD standard deviation).

[b] Animals were bled by cardiac puncture on day 8 and day 13. Samples of blood were diluted in medium and frozen at -90°C prior to assay for plaque formation on Vero monolayers.

[c] Samples of serum were also obtained, and titred for antibody against Pichinde virus in the standard complement fixation (CF) test.

with swelling cannot be attributed to lack of exposure to the viral antigens. Furthermore, this experiment demonstrates that the MHA hamster is able to limit viremia when the virus is injected via the footpad. It was also observed that the MHA hamsters survived the footpad inoculation of Pichinde virus (data not presented).

The footpad inoculation of Pichinde virus protected MHA animals against a second challenge of virus given intraperitoneally, as shown in Table V. This second dose of virus, given by a normally lethal route, induced augmented titres of complement-fixing antibody, implying that a memory response had occurred in response to a second exposure of the antigen. In addition, no viremia was detectable. These observations suggest that, when challenged by an appropriate route, MHA hamsters are capable of resisting Pichinde virus infection. At least two mechanisms can be proposed to account for this. One possibility is that cell-mediated immunity, as measured by footpad swelling, is unimportant in determining the outcome of Pichinde virus infection. An alternative explanation is that inoculation of virus by the intraperitoneal route overwhelms the defenses of MHA hamsters, while the footpad route of inoculation delays virus spread sufficiently to allow the host's immune responses to clear the virus.

3.2.2 Cytotoxic T Lymphocytes Specific for Pichinde Virus

Cytotoxic T lymphocytes, which specifically lyse histocompatible target cells bearing the particular antigenic determinant which elicited their appearance, usually become detectable by 3 days and peak 4-8 days after primary exposure to the antigen. Spleen cells from hamsters which had been infected with Pichinde virus, or from control uninfected animals,

Table V

Effect of Footpad Inoculation on a Subsequent IP Challenge of Pichinde Virus in MHA Hamsters

First Inoculation[a] (pfu)	Second Inoculation[b] (pfu)	Number	Viremia: Mean $\log_{10} \pm$ SD pfu/ml	CF Antibody Mean $\log_{10}$ titre $\pm$ SD
IP-$2X10^3$	-	2	5.91±0.10	1.58±0.17
FP-10^6	-	6	3.90±0.80	1.53±0.12
FP-10^6	IP-$2X10^3$	2	<2.70[c]	>2.68[c]

[a] MHA hamsters were inoculated either IP or via the footpad (FP), with the amount of Pichinde virus indicated. Animals were bled at 8 days after injection, and samples were assayed for pfu on Vero monolayers, and for CF activity.

[b] Footpad-inoculated MHA hamsters were reinjected with Pichinde virus 4 weeks after the primary exposure to the virus. The animals were bled and assayed as above.

[c] Limits of detection of the assays.

were tested for the presence of cytotoxic T lymphocytes in a ^{51}Cr release assay, using syngeneic and allogeneic tumour cells infected with Pichinde virus as targets. The kinetics of cytotoxic activity induced following Pichinde virus infection are illustrated in Figure 3.

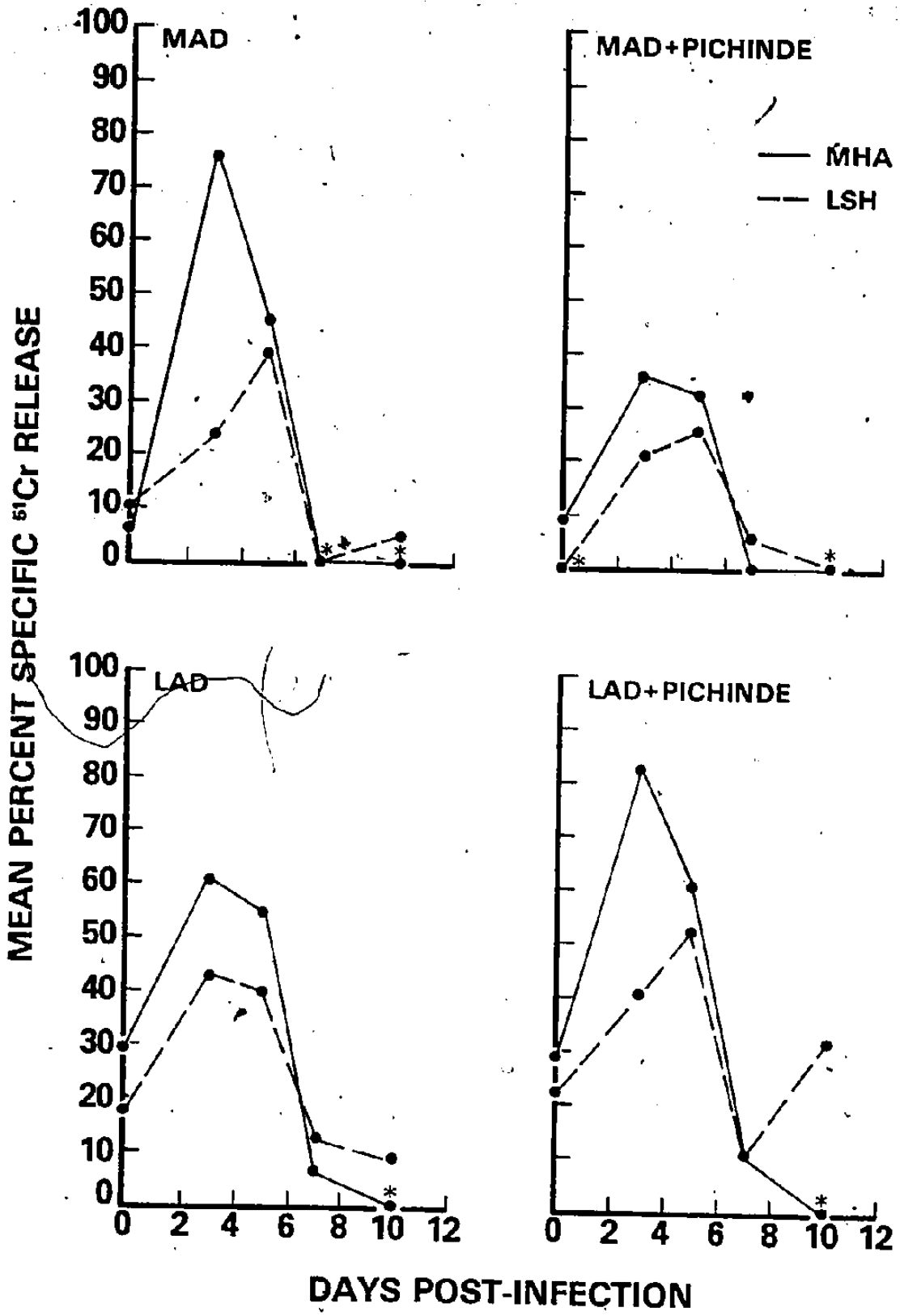
Spleen cells from uninfected control MHA hamsters exhibited detectable levels of cytotoxicity against both syngeneic and allogeneic tumour cell targets, and this activity increased after intraperitoneal infection with Pichinde virus. The peak of activity was reached on the third day after infection. Spleen cells from LSH hamsters, which usually showed lower endogeneous levels of cytotoxicity (see Appendix), showed less enhancement after viral infection. Syngeneic combinations of cytotoxic effectors and targets yielded no greater specific ^{51}Cr release than did the allogeneic combinations, and infection of the target cells with Pichinde virus did not increase their susceptibility to lysis. The kinetics of this cytotoxicity and the lack of specificity of lysis strongly suggested that the activity was not attributable to classic T lymphocyte-mediated lysis.

3.3 Natural Killer (NK) Lymphocytes in Syrian Hamsters

The demonstration that a naturally occurring cytotoxic activity against tumour cells existed in hamsters, and that this activity increased and peaked 3 days following Pichinde virus infection, raised the possibility that the cytotoxic effector cell was the NK lymphocyte. This type of cell-mediated lysis had not been previously described in hamsters. The data also suggested that the endogenous lytic activity was high in MHA hamster spleen cells, and that Pichinde virus infection augmented the activity to a greater extent in MHA than in LSH hamsters. A statistical analysis on pooled data which supports this suggestion is presented in the Appendix.

Figure 3

Kinetics of cytotoxic activity in the spleen following Pichinde virus infection. Groups of 2 hamsters were injected with 2000 pfu Pichinde virus IP and spleens were removed from uninfected animals (day 0) and animals that had been infected 3, 5, 7 or 10 days previously. The spleen cells were assayed for cytotoxicity against MAD and LAD targets which had or had not been infected with Pichinde virus 24 hrs previously. This data was obtained using an effector-to-target cell ratio of 100:1; similar patterns of lysis were obtained at ratios of 50:1 or 25:1. The asterisk indicates that the values were less than the spontaneous release.


MAD
MAD+PICHINDE
MHA
LSH
LAD
LAD+PICHINDE
MEAN PERCENT SPECIFIC ^{51}Cr RELEASE
DAYS POST-INFECTION
0
10
20
30
40
50
60
70
80
90
100
0
2
4
6
8
10
12

Thus, an investigation of the properties of the hamster effector cell and its role in Pichinde virus infection was initiated.

3.3.1 Parameters of the ^{51}Cr Release Assay for Hamster NK cells

Very little is known concerning the mechanism of NK-mediated lysis. As with cytotoxic T lymphocyte-mediated killing (rev. Cerottini and Brunner, 1974), contact with the target cell appears to be necessary (Kiessling & Wigzell, 1979). The interaction between cytotoxic T-lymphocytes and the target cell is facilitated by the presence of Ca++ and Mg++ ions (Martz, 1976) and killing is inhibited by the presence of EDTA during the incubation (Plaut et al., 1976).

3.3.1.1 Requirement for Divalent Cations

To determine whether the presence of EDTA had a similar effect on cytotoxicity mediated by the hamster effector cell, spleen cells from Pichinde virus-infected MHA hamsters were incubated with ^{51}Cr-labelled MAD target cells with or without addition of EDTA to the medium. As shown in Table VI, EDTA inhibited the cytotoxic activity of the effector cells almost completely. Thus, divalent cations are required for the productive interaction between hamster effector cells and the ^{51}Cr-labelled targets.

3.3.1.2 Kinetics of Cytolysis

The lysis of ^{51}Cr-labeled target cells by the mouse NK lymphocyte is essentially complete within 4 hrs of incubation (Welsh, 1978). In contrast, human NK-mediated cytotoxicity proceeds at a linear rate for at least 18-20 hrs (Santoli and Koprowski, 1979). A kinetic study of ^{51}Cr release from MAD targets in the presence of hamster effector cells was carried out to determine the optimal incubation time in the

Table VI

Requirement for Divalent Cations

Treatment[a]	Mean percent specific ^{51}Cr release ± SEM		
	100:1[b]	50:1	25:1
None	48.3±2.1	36.3±0.9	23.8±0.4
EDTA	4.4±0.7	7.5±0.4	8.4±0.8

[a] Effector cells from Pichinde virus-infected MHA spleens were incubated with ^{51}Cr-labeled MAD targets for 16 hrs at 37°C. For treatment with EDTA, 0.025M EDTA was added to each well at the time the labelled targets were added, to give a final concentration of 0.005M EDTA.

[b] Effector-to-target cell ratios.

hamster system. Spleen cells were incubated with targets for 4, 7.5 or 16 hrs, and per cent specific ^{51}Cr release was determined. The results are shown in Figure 4. The data obtained from a 24 hr incubation are not presented because the spontaneous release at this time was almost 90% of the maximum, and the specific release consequently was of the order of 10%. Unlike results obtained in the mouse system, lysis of the ^{51}Cr-labelled target cells proceeded slowly. Use of a 16 hr incubation gave high specific release with relatively low spontaneous release and this incubation period was used in all further assays. Furthermore, the addition of EDTA after 4 hrs or 16 hrs of incubation to prevent further interactions, followed by a second incubation of 1 to 3 hrs to permit any residual ^{51}Cr to escape from the damaged targets, did not give significantly higher specific ^{51}Cr-release values (Table VII). This indicates that further cation-dependant interactions between the effectors and targets were occurring between 4 and 16 hrs of incubation, and that the increase in specific release was not attributable to delayed release of the label after the target cell was damaged.

3.3.1.3 Dependance of Lysis on the Presence of Cells

A quantitative relationship exists between the concentration of effector cells and the degree of lysis of the target cells in T lymphocyte-mediated cytolysis (Cerottini and Brunner, 1974). If the lysis of tumour target cells by the hamster effector cell is a cellular function, then a quantitative relationship should be demonstrable in this system as well. Such a relationship is implicit from the observation that doubling dilutions of spleen cells mediate proportionate reductions in the amount of lysis. This conclusion was confirmed and extended by experiments

Figure 4

Kinetics of cytolysis. Spleen cells from control hamsters or hamsters which had been infected with Pichinde virus 3 days previously were incubated for various times with ^{51}Cr-labelled MAD target cells, using an effector-to-target cell ratio of 25:1. Comparable data were obtained using ratios of 50:1 and 100:1.

●——● LSH control

●——● Pichinde virus-infected LSH

■——■ MHA control

■——■ Pichinde virus infected MHA

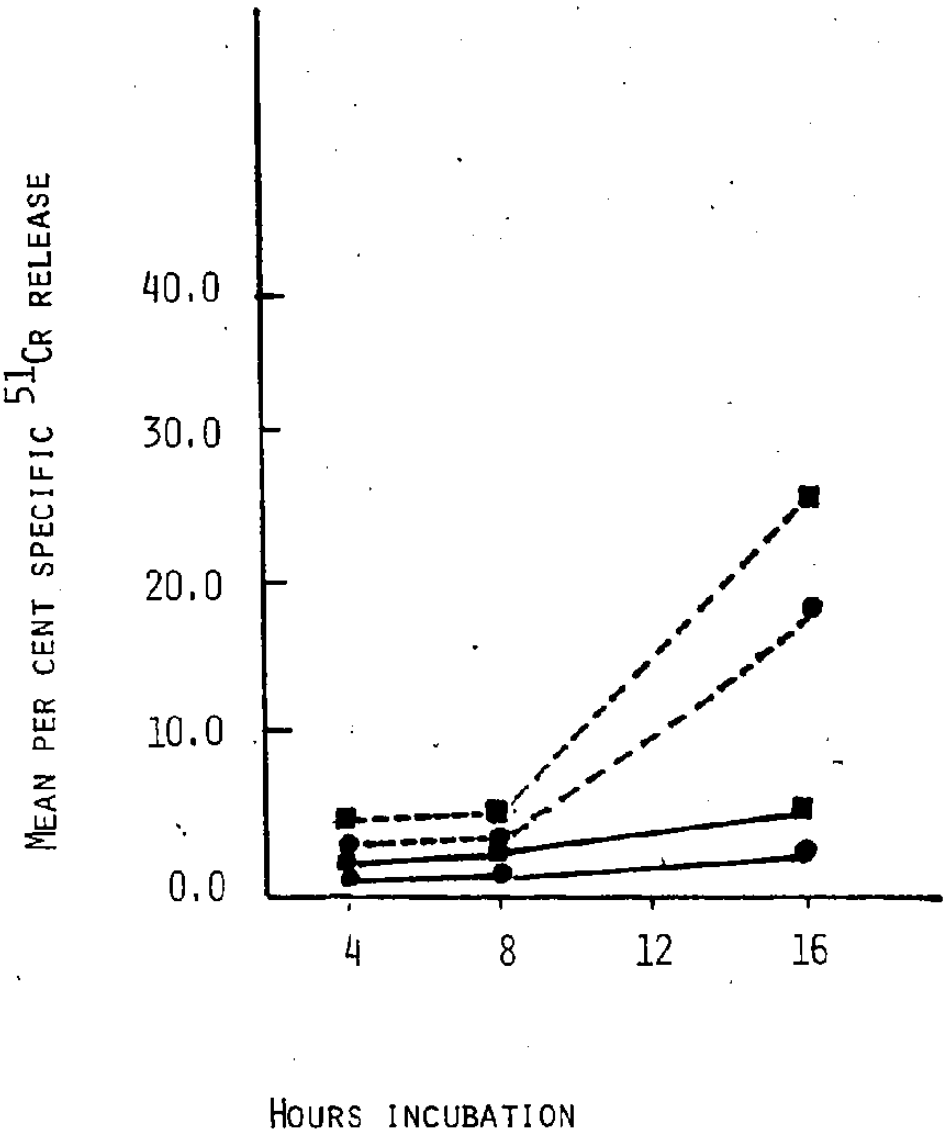

MEAN PER CENT SPECIFIC ^{51}Cr RELEASE
40.0
30.0
20.0
10.0
0.0
4
8
12
16
HOURS INCUBATION

Table VII

Effect of EDTA Added at the End of the Incubation Period

Length of Incubation[a]	Treatment[b] with EDTA	Time of Harvest	Mean per cent specific ^{51}Cr release±SEM[c]
4	none	4 hrs	12.2±0.6
	EDTA t=0	4 hrs	<SR
	EDTA t=4	4+1 hrs	10.7±1.0
	EDTA t=4	4+2 hrs	16.3±1.3
	EDTA t=4	4+3 hrs	10.6±1.0
16	none	16 hrs	48.3±2.1
	EDTA t=0	16 hrs	4.4±0.7
	EDTA t=16	16+1 hrs	52.6±1.9
	EDTA t=16	16+2 hrs	49.8±3.5

[a] Effector cells from Pichinde virus-infected MHA spleens were incubated with ^{51}Cr-labelled MAD targets for 4 hrs or 16 hrs.

[b] EDTA, at a final concentration of .005M, was either present during the assay (t=0) or added at the end of the 4 hr incubation period (t=4) or the 16 hr incubation period (t=16). In the event that EDTA was added at the end of the incubation, the cells were incubated for a further 1, 2 or 3 hrs at 37°C prior to assaying for ^{51}Cr in the supernate.

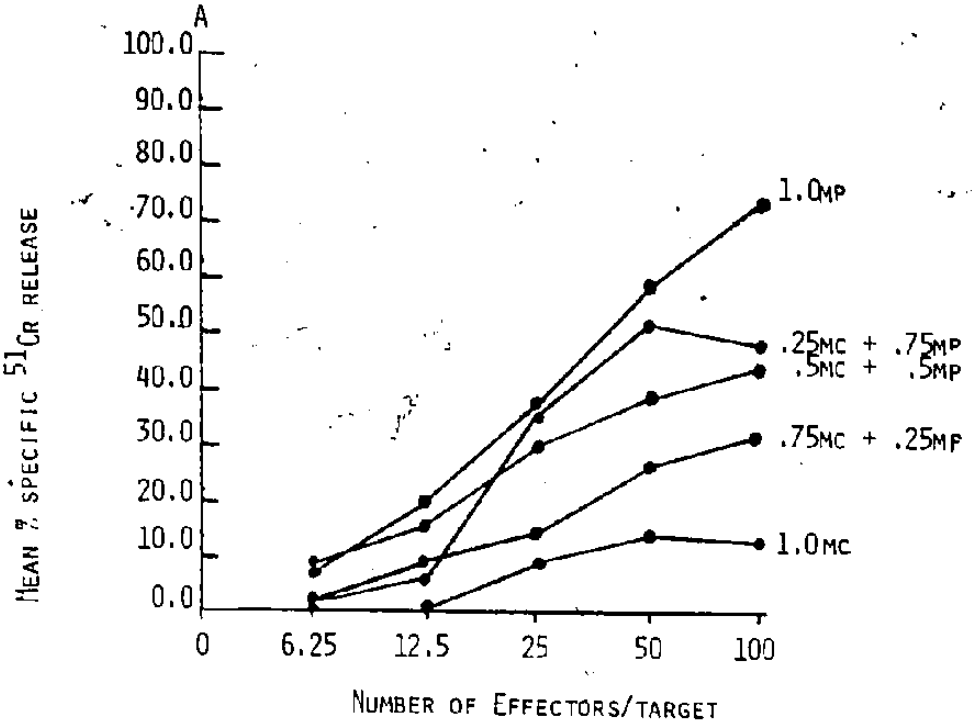
[c] Cells were tested for cytotoxic activity against ^{51}Cr-labelled MAD targets using an effector-to-target cell ratio of 100:1. Similar data were obtained using ratios of 50:1, 25:1, 12.5:1 and 6.25:1.

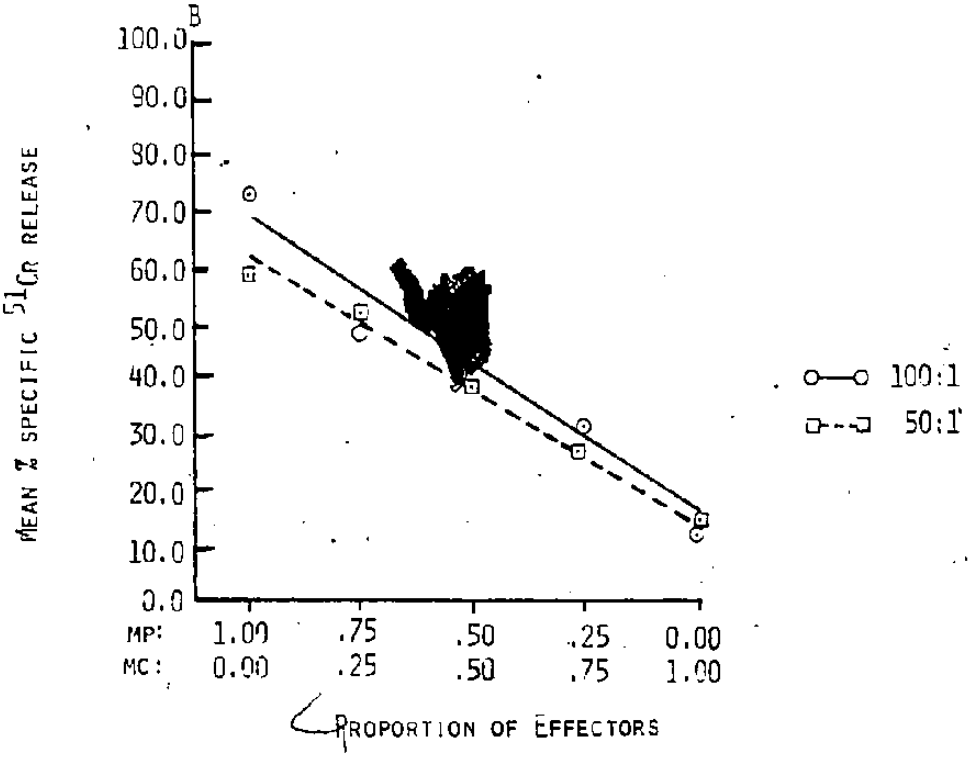
Figure 5

Relationship between number of effector cells and the amount of lysis.

A. Spleen cells were obtained from control MHA hamsters (MC) and hamsters which had been infected IP 3 days previously with Pichinde virus (MP). The concentrations of each were adjusted to 1 X 10^7 cells/ml, and then various volumes of MC cells were added to MP cells as indicated in the figure to give a final volume of 1.0 ml. Dilutions of these mixtures were then assayed for cytotoxic activity against ^{51}Cr-labelled MAD targets.

B. The per cent specific ^{51}Cr release in each mixture was plotted against the relative number of effector cells from control and Pichinde virus-infected hamsters.


A
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
MEAN % SPECIFIC ^{51}Cr RELEASE
1.0MP
.25MC + .75MP
.5MC + .5MP
.75MC + .25MP
1.0MC
0
6.25
12.5
25
50
100
NUMBER OF EFFECTORS/TARGET


B
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
MEAN % SPECIFIC ^{51}Cr RELEASE
MP: 1.00 .75 .50 .25 0.00
MC: 0.00 .25 .50 .75 1.00
PROPORTION OF EFFECTORS
100:1
50:1

in which mixtures of spleen cells from control and Pichinde virus-infected animals were assayed for cytotoxic activity. As illustrated in Figure 5A, the actual per cent release was observed to increase in proportion to the relative number of effector cells derived from Pichinde virus-infected hamsters, i.e., from hamster spleens exhibiting augmented cytotoxic activity. The relationship between per cent release and the relative number of cytotoxic cells derived from Pichinde virus-infected hamsters appeared to be a linear one (Figure 5B). These data suggested that lytic activity behaved as a cellular function.

3.3.1.4 Role of virus-specific antibodies in Pichinde virus-induced NK activity

To assess the role of virus-specific antibody in the augmented cytotoxic activity which is induced by Pichinde virus infection, control and Pichinde virus-infected hamsters received injections of saline, normal hamster serum, or a high titered immune hamster anti-Pichinde virus serum. On the day of assay, hamsters were bled by cardiac puncture and the serum samples were used to check the efficiency of the transfer. Spleen cells from these animals were then tested for cytotoxic activity against ^{51}Cr-labelled MAD targets, and for infectious centres. As the data in Table VIII show, the passive transfer of immune serum was successful; the sera from all animals which received an inoculation of hamster anti-Pichinde virus antibody had a CF titre greater than 1:32, a value that is comparable to that observed in hamsters eight days after an intraperitoneal injection of Pichinde virus. No other animals had detectable antibodies against the virus.

Table VIII

Effect of Passive Transfer of Antibody on NK Activity

and on Development of Infectious Centres

Treatment of Animals		Strain of hamster	Antibody Titre[c.]	Mean % Specific[d.] ^{51}Cr release±SEM	Infectious centres[e.] per 10^5 cells
Antisera[a.]	Infection with[b.] Pichinde virus				
none	no	LSH	<1	14.9±2.1	-
		MHA	<1	17.8±1.6	-
none	yes	LSH	<1	33.1±0.5	395
		MHA	<1	46.9±1.8	718
NHS	no	LSH	<I	13.9±1.5	-
		MHA	<1	26.6±0.9	-
NHS	yes	LSH	<1	40.8±2.0	130
		MHA	<1	66.6±1.9	1850
HαPich	no	LSH	>1:32	15.7±0.8	-
		MHA	>1:32	28.8±0.6	-
HαPich	yes	LSH	>1:32	32.8±1.2	603
		MHA	>1:32	40.0±2.3	835

Table VIII (Footnotes)

[a] Groups of 3 hamsters were injected with saline, with normal hamster serum (NHS) from F_1 (LVG X MHA) hamsters, or with immune sera obtained from F_1 hamsters which had received 2 IP injections of Pichinde virus (HαPich). The immune sera had a CF titre of 1:640. Each animal received 0.5 ml of sera in 2 sites on the rear flanks one day before injection of Pichinde virus, i.e., 4 days before assay.

[b] Animals were injected with 2000 pfu Pichinde virus IP 3 days before assay.

[c] A pool of sera, obtained from each of the 3 animals in each group, was assayed for complement fixation using excess amounts of a defined antigen and 5 units of complement, as described in Materials and Methods. All sera were negative against a control antigen preparation of uninfected cells.

[d] Spleen cells were assayed for cytotoxic activity against ^{51}Cr-labelled MAD targets using an effector-to-target cell ratio of 100:1. Similar data were obtained using ratios of 50:1, 25:1, 12.5:1 and 6.25:1.

[e] Log_{10} dilutions of spleen cells were plated on monolayers of Vero cells to assay for infectious centres.

A number of interesting points can be made from the data. The transfer of immune serum, as compared to normal serum, had little effect on the endogenous cytotoxic activity of normal spleens. Secondly, immune serum did not increase the augmented cytotoxicity seen in Pichinde virus-infected animals above levels seen in recipients of normal hamster serum. These data suggest that transfer of antibody has little effect on the Pichinde virus-induced augmentation of NI activity in either strain of hamsters.

The transfer of immune serum did not appear to increase the number of infectious centres in the spleens of Pichinde viurs-infected MHA or LSH hamsters above levels that were present in the spleens of hamsters which received only an injection of saline. However, a small increase in NK activity and the number of infectious centres was noted in Pichinde virus-infected MHA recipients of normal hamster serum above levels observed in MHA animals that received an injection of saline. In contrast, LSH animals that received an injection of normal hamster sera showed a decrease in infectious centres compared to the number observed in LSH hamsters that received saline alone. The significance of these changes is unclear.

These observations suggest that hamster cell cytotoxicity is not attributable to antibody-dependant cell-mediated cytotoxicity. Furthermore, the development of Pichinde virus infectious centers seems to be unaltered by the presence of anti-Pichinde virus antibodies in the infected animals.

3.3.2 Properties of the Hamster Effector Cell

The lack of antigenic specificity and the early development of cytotoxicity after virus infection suggested that the cytotoxic cell in hamsters was an NK cell. To further test this idea, characteristics of the hamster effector cell were studied. Properties of NK cells in different species have been summarized by Herberman and Holden (1978). In other systems, the NK effector is a small, non-adherent, non-phagocytic lymphocyte lacking surface immunoglobulin. Mouse NK cell activity is extremely labile in culture. These criteria for NK activity were applied to the putative hamster NK cell.

3.3.2.1 Adherence to Plastic

Spleen cells from infected LSH and MHA hamsters were incubated on plastic dishes, as described in Materials and Methods, and the adherent and non-adherent fractions were tested for cytotoxicity against ^{51}Cr-labelled MAD targets. A representative experiment is shown in Figure 6. Cytolysis was seen in the presence of both adherent and non-adherent cells from either hamster strain, but most of the lytic activity was associated with the non-adherent fraction.

3.3.2.2 Phagocytosis and/or Adherence of Carbonyl Iron

Cells from the spleens of control or infected MHA and LSH hamsters were incubated with carbonyl iron to remove phagocytic and/or adherent cells. Carbonyl iron-containing cells were removed with the aid of a magnet, and the residual cells were tested for cytotoxic activity against ^{51}Cr-labelled MAD target cells. A typical result is shown in Figure 7. Treatment of the effector spleen cells from either Pichinde virus-infected or control animals with carbonyl iron did not

Figure 6

Adherence of effector cells to plastic. Spleen cells from 6 infected LSH or MHA hamsters were incubated in plastic petri dishes for 1 hr at 37°C. Non-adherent cells, obtained by decanting the supernate and washing the monolayer with PBS minus Ca++ and Mg++, and adherent cells, obtained by scraping the remaining cells off the plastic dish with the aid of a rubber policeman, were tested for cytotoxicity against ^{51}Cr-labelled MAD targets.

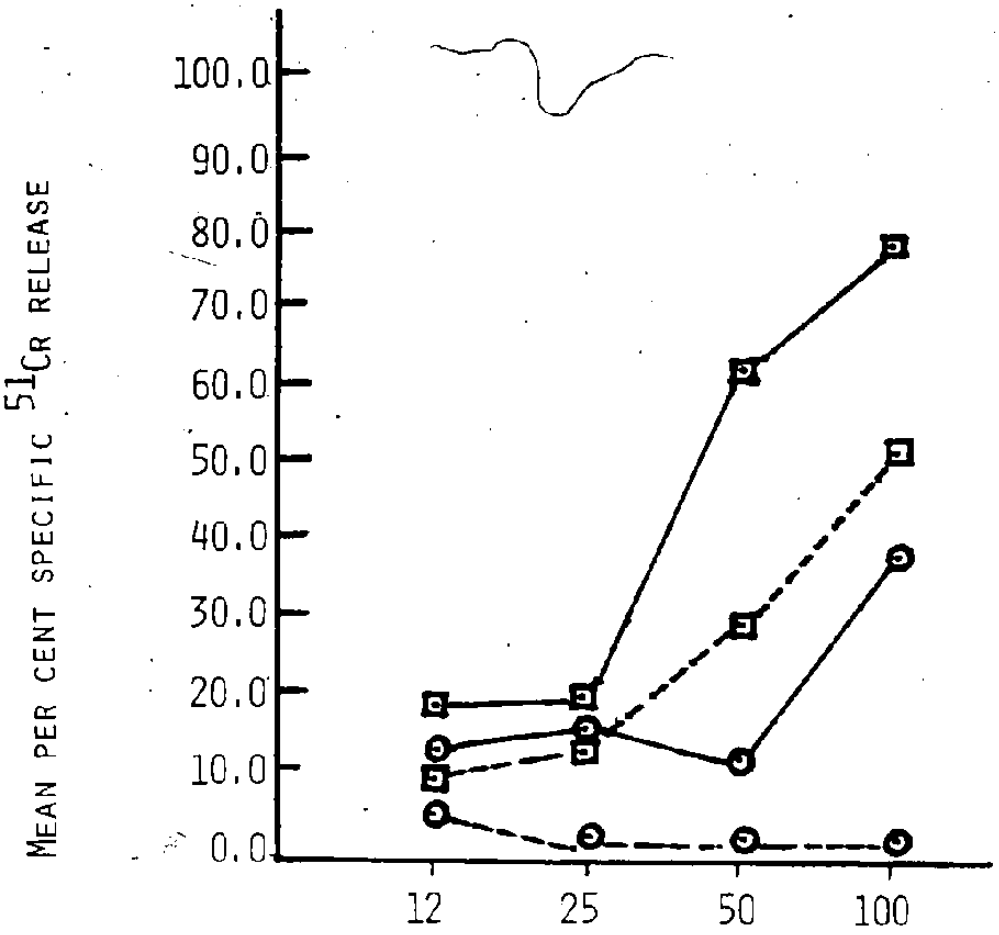
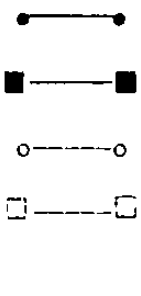

MEAN PER CENT SPECIFIC ^{51}Cr RELEASE
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
12
25
50
100
NUMBER OF EFFECTORS PER TARGET
MHA NON-ADHERENT
MHA ADHERENT
LSH NON-ADHERENT
LSH ADHERENT

Figure 7

Effect of carbonyl iron pretreatment on cytotoxic activity. Spleens from control animals or animals which had been infected with Pichinde virus 3 days previously were excised. 1 X 10^8 cells from each group were incubated with 1 g carbonyl iron for 45' at 37°C. Carbonyl iron-containing cells were removed using a magnet, and the remaining cells were assayed for cytotoxic activity against ^{51}Cr-labelled MAD targets.

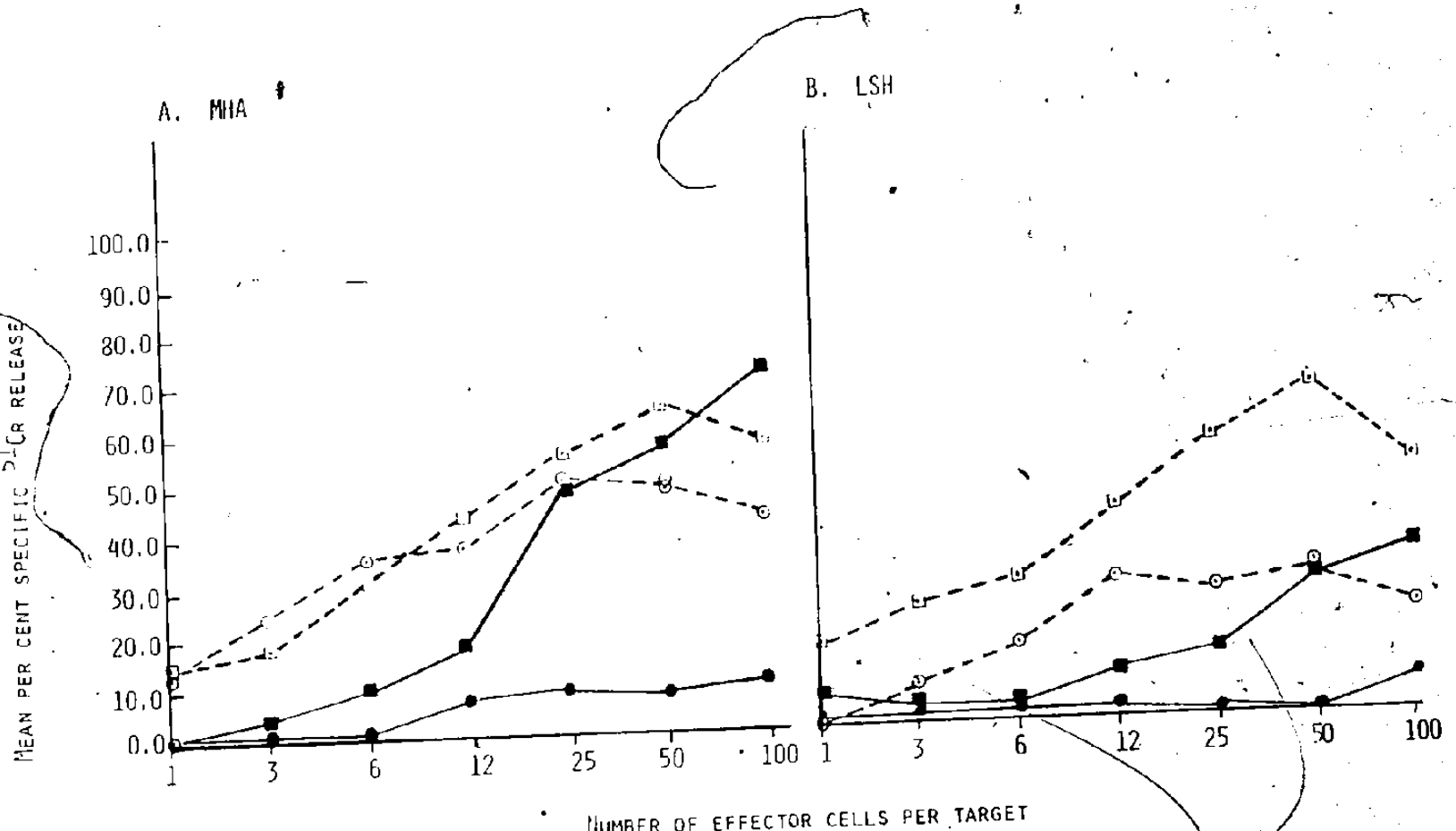


●——● cells from control animals

■——■ cells from Pichinde virus-infected animals

o——o control cells treated with carbonyl iron

□——□ cells from Pichinde virus-infected animals, treated with carbonyl iron


A. MHA
B. LSH
MEAN PER CENT SPECIFIC ^{51}Cr RELEASE
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
1
3
6
12
25
50
100
NUMBER OF EFFECTOR CELLS PER TARGET

abrogate the cytotoxicity of either strain of hamster. On the contrary, an enhancement of killing was observed. This experiment also demonstrates that LSH hamsters do not lack the effector cell; rather, the activity of the cells appears to be masked in LSH spleens.

3.3.2.3 Lability of Cytotoxic Activity

LSH and MHA spleen cells, obtained from control or Pichinde virus-infected animals, were pre-incubated for 24 hrs at 37°C prior to adding fresh ^{51}Cr-labelled MAD target cells. This treatment abrogated the cytotoxic activity, demonstrating its labile nature (Table IX). No decrease in cell viability as assessed by trypan blue exclusion was observed (data not presented).

3.3.2.4 Effect of NH_4Cl Pretreatment of Spleen Cells on Cytotoxic Activity

The incubation of spleen cells in an iso-osmotic NH_4Cl solution results in lysis of the red blood cells; it also inhibits human NK, but not mouse NK, activity (Herberman and Holden, 1978). The effect of NH_4Cl pretreatment on hamster effector cells was investigated. Spleen cells from Pichinde virus-infected MHA hamsters were briefly incubated in 0.174 M NH_4Cl. The cytotoxic activity of the cells against MAD targets was then compared to control cells which had not been treated with NH_4Cl. As shown in Table X, this treatment actually enhanced the lytic activity of the effector cells. Thus, the effector cell of MHA hamsters appears to be resistant to inhibition by NH_4Cl.

3.3.2.5 Expression of Surface Immunoglobulin on Effector Cells

NK lymphocytes in mice or humans do not express immunoglobulin on their surfaces (Herberman and Holden, 1978). To test for the

Table IX

Lability of Cytotoxic Activity

Effector cells		Mean per cent specific ^{51}Cr release ± SEM[b]	
Strain of hamster	Infection with Pichinde virus[a]	Untreated	Preincubated at 37°C[c] for 24 hrs
LSH	No	3.3±1.3	<SR[d]
LSH	Yes	27.9±3.5	<SR
MHA	No	7.8±0.6	<SR
MHA	Yes	96.5±3.1	<SR

[a] Spleen cells were obtained from control animals, or animals that had been infected 3 days previously with Pichinde virus IP.

[b] ^{51}Cr-labelled MAD cells were used as targets in the assay. These data were obtained using an effector-to-target cell ratio of 50:1. Similar results were obtained using ratios of 100:1 or 25:1.

[c] Spleen cells were diluted on microtitre plates as described for the ^{51}Cr release assay in Materials and Methods, and incubated at 37°C for 24 hrs in a CO_2 incubator. Freshly prepared, ^{51}Cr-labelled MAD targets were then added and lysis was measured after a further 16 hrs incubation.

[d] <SR, less than spontaneous release.

Table X

Effect of NH_4Cl Pretreatment on Lytic Activity

Treatment[a]	Mean per cent specific ^{51}Cr release ± SEM		
	100:1[b]	50:1	25:1
none	31.4±3.4	26.8±2.2	17.5±1.1
NH_4Cl	66.9±1.6	52.6±3.4	38.9±1.3

[a] Spleen cells from Pichinde virus-infected MHA hamsters were incubated with 0.174 M NH_4Cl for 10' at 4°C, as described in Materials and Methods. The cells were washed and recounted prior to assaying for NK activity against ^{51}Cr-labelled MAD targets.

[b] Effector-to-target cell ratio.

presence of immunoglobulin on the surface of hamster cytotoxic cells, spleen cells were pretreated with rabbit anti-hamster sera plus rabbit complement, and then tested for lytic activity against ^{51}Cr-labelled MAD cells. Controls included cells treated with complement alone and normal rabbit serum instead of anti-hamster immunoglobulin. This treatment did not reduce the cytotoxic activity of LSH or MHA spleen cells (Table XI), suggesting that the hamster effector cells did not possess surface immunoglobulin. An increase in the lytic activity of treated effector cells derived from MHA hamsters was noted; the significance of this increase is unclear.

3.3.2.6 Distribution of the Effector Cell

Mouse NK activity has been demonstrated in the spleen, bone marrow, lymph nodes, peripheral blood and in the peritoneal exudate. No activity has been associated with the thymus (Herberman and Holden, 1978). In the hamster, cytotoxic activity has been observed in spleen and peritoneal exudate cells after an IP injection of Pichinde virus, and in the popliteal lymph nodes following a footpad injection of virus (Table XII). Other potential sources of NK activity have not been tested.

3.3.2.7 Specificity of Induction

Many viruses, including lymphocytic choriomeningitis virus, Pichinde virus, measles virus and vesicular stomatitis virus (VSV) have been shown to elicit augmented NK activity in mice infected *in vivo* (Welsh, 1978; Herberman *et al.*, 1977). The observation that Pichinde virus infection augments NK activity in MHA hamsters to a greater degree than in LSH hamsters led to the screening of other viruses for a similar

Table XI

Expression of Surface Immunoglobulin on Hamster Effector Cells

Effector cells[a]	Treatment	Mean per cent specific ^{51}Cr release ± SEM 100:1[c]	50:1	25:1
LSH	none	3.4±2.2	4.1±2.5	0.8±1.8
	NRS+C'	6.7±0.7	5.4±0.7	3.7±0.7
	anti-Ig+C'[b]	8.4±1.6	2.9±2.5	3.6±1.2
MHA	none	23.9±0.7	17.4±1.3	8.3±1.5
	NRS+C'	29.3±1.8	20.0±2.7	7.7±0.7
	anti-Ig+C'[b]	47.1±0.9	27.6±1.5	12.6±2.5

[a] Spleen cells from hamsters which had been infected IP with Pichinde virus 3 days previously were used as effectors.

[b] Spleen cells were incubated in rabbit anti-hamster immunoglobulin antisera (anti-Ig) or normal rabbit serum (NRS) for 30' at 37°C as described in Materials and Methods. The cells were then washed and resuspended in Low Tox rabbit complement for a further 30' incubation, and then recounted prior to testing for cytotoxic activity. The anti-hamster immunoglobulin antiserum stained about 11% of spleen cells in an immunofluorescence assay, and the recovery of cells after treatment with the antiserum and complement was 84%.

[c] Effector-to-target cell ratios.

Table XII

Organ Distribution of Hamster NK Activity

Effector Cells: Strain of hamster	Infection with Pichinde virus	Source of NK cells	Mean per cent specific ^{51}Cr release ± SEM[c.] 100:1	50:1	25:1
MHA	no	spleen[a.]	NT[d.]	2.9±3.5	3.6±0.9
MHA	yes		55.9±2.3	52.9±3.8	34.9±3.0
MHA	no	peritoneal[a.] exudate	14.9±1.5	NT	NT
MHA	yes		28.0±3.6	NT	NT
MHA	no	popliteal[b.] lymph node	NT	-0.1±3.4	-0.5±2.8
MHA	yes		45.3±3.1	45.4±0.3	30.1±2.7

[a] MHA hamsters were infected with 2000 pfu Pichinde virus IP 3 days before assay.

[b] MHA hamsters received an injection 1000 pfu Pichinde virus in 25λ diluent, in each hind footpad 3 days prior to assay.

[c] Effector cells were assayed for cytotoxicity against ^{51}Cr-labelled MAD targets at various effector-to-target cell ratios.

[d] NT, not tested.

effect. The results of a typical experiment are presented in Figure 8. Infection of LSH hamsters with herpes simplex virus resulted in increased NK activity relative to control LSH animals but did not significantly raise the endogenous levels of NK activity in MHA hamsters. Vaccinia virus infection did not augment lytic activity in either hamster strain. As usual, Pichinde virus infection augmented activity in both strains, but to a greater extend in MHA than in LSH hamsters. Thus, the in vivo induction of hamster NK activity does not appear unique to Pichinde virus.

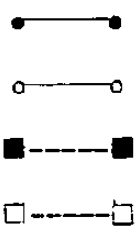
3.3.2.8 Specificity of Killing

Induced NK lymphocytes exhibit a broader spectrum of specificity of killing than endogenous NK cells, but neither are histocompatibility-restricted, or specific for the inducing agent (Becker, 1976; Welsh, 1978). To assess the specificity of killing by the hamster effector cells, spleen cells from uninfected animals or animals which had been infected 3 days previously with Pichinde virus were assayed for cytotoxic activity against syngeneic or allogeneic tumour cells, a continuous cell line, and embryo fibroblasts (Figure 9). The targets were susceptible to lysis, although with differing efficiencies. It can be seen that MHA spleen cells showed higher endogenous levels of cytotoxicity than did LSH spleen cells, and Pichinde virus infection augmented the activity to a greater extent in MHA than in LSH hamsters. Thus, the hamster cytotoxic effector cell is not specific for histocompatibility antigens and kills continuous cell lines and embryo fibroblasts in addition to tumour cells.

The effect of virus infection of the target cells on susceptibility to lysis by spleen cells was also investigated. Spleen cells

Figure 8

Specificity of induction of hamster NK cells. Groups of 3 hamsters were infected with 2000 pfu virus IP 3 days before assay for NK activity on MAD target cells.



control

Pichinde virus

Herpes simplex virus

Vaccinia virus

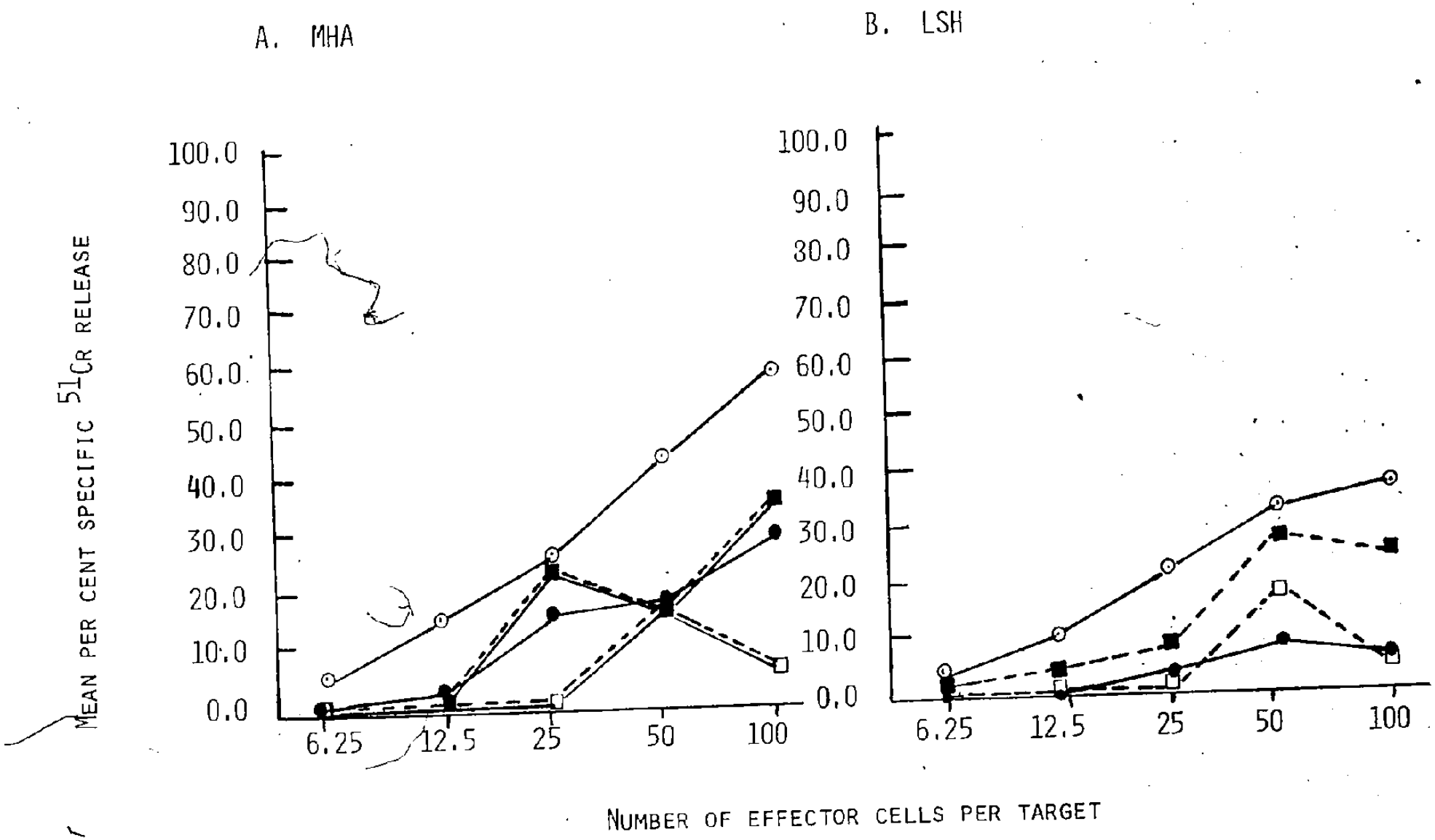
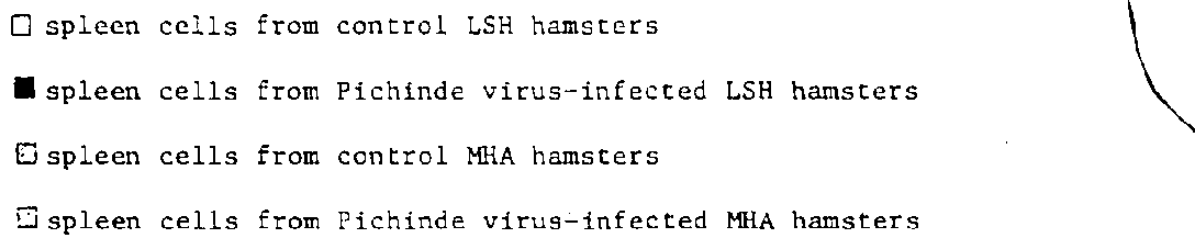
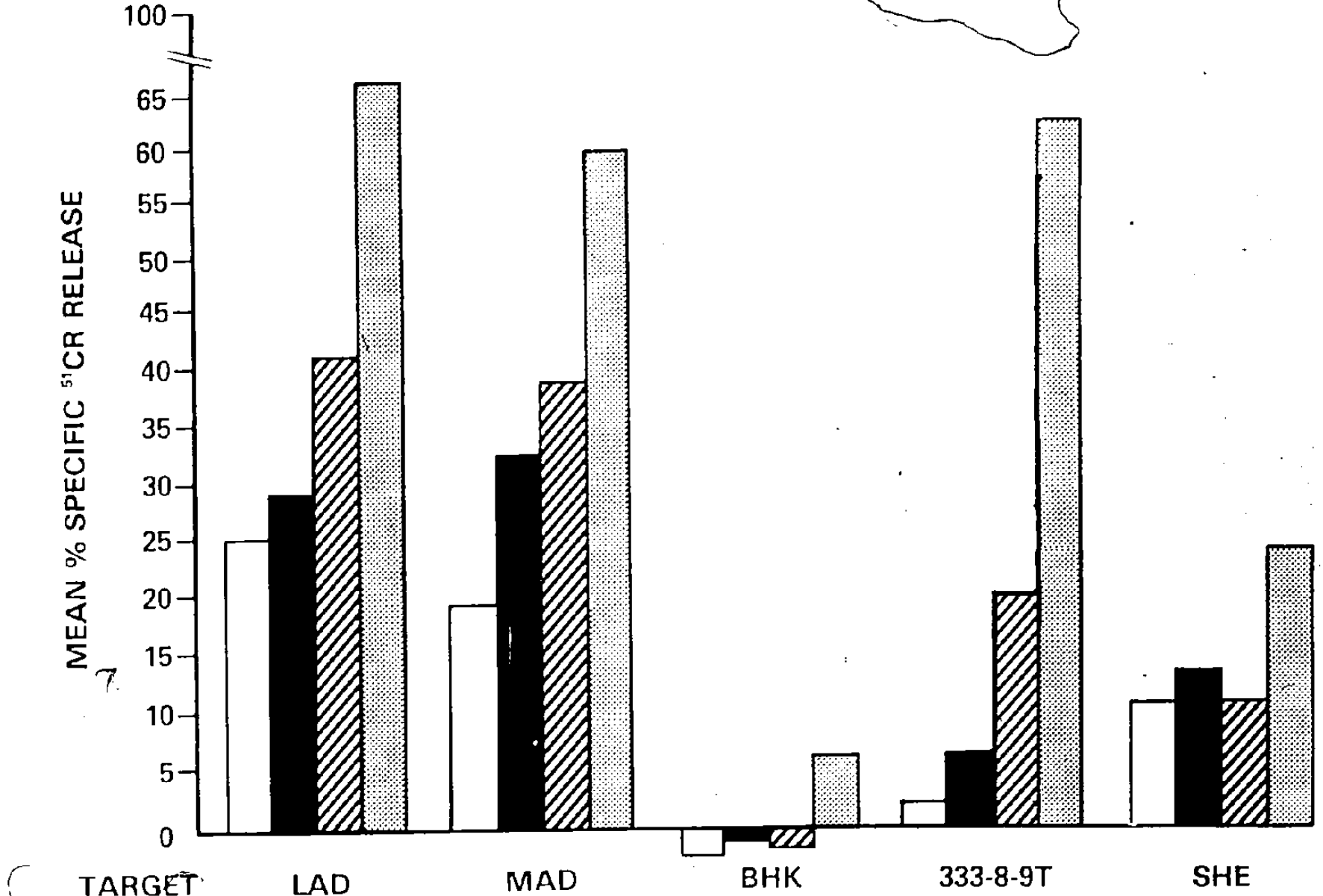

A. MHA
B. LSH
MEAN PER CENT SPECIFIC ^{51}Cr RELEASE
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
6.25
12.5
25
50
100
NUMBER OF EFFECTOR CELLS PER TARGET

Figure 9

Specificity of lysis of target cells by hamster NK cells. Spleen cells from uninfected animals or animals which had been injected with 2000 pfu Pichinde virus 3 days previously were assayed for cytotoxic activity against various ^{51}Cr-labelled targets. The LAD and MAD cell lines are tumour cells derived from adenovirus type 12-induced tumours, whereas the 333-8-9T cells were obtained from a tumour induced by transformation with herpes simplex virus type 2. BHK cells are a continuous cell line derived from a baby hamster kidney. SHE cells are fibroblasts obtained from Syrian hamster embryos. These data were obtained using an effector-to-target cell ratio of 100:1; similar data were obtained using ratios of 50:1 and 25:1.




MEAN % SPECIFIC ^{51}CR RELEASE
100
65
60
55
50
45
40
35
30
25
20
15
10
5
0
TARGET
LAD
MAD
BHK
333-8-9T
SHE

from control hamsters or hamsters which had been infected 3 days previously with Pichinde virus were assayed for lytic activity against ^{51}Cr-labelled MAD cells which had previously been infected with different viruses. The duration of infection for each virus was chosen to coincide with maximal viral antigen expression of the surface of infected cells, and was such that cytopathic effects to the targets during the assay were low. Once again, the usual pattern of response was observed (Figure 10): the endogenous lytic activity of MHA hamsters was greater than LSH hamsters, and Pichinde virus-induced augmentation was also greater in the MHA strain. Infection of the MAD targets with Pichinde, herpes simplex, adenovirus type 12 or vaccinia viruses did not enhance the lysis by any effector cell. The infection of MAD targets with vesicular stomatitis virus (VSV), however, did lead to increased specific ^{51}Cr release. This could be due to either an increased susceptibility of the infected targets to lysis, or to an *in vitro* induction of NK activity by the virus.

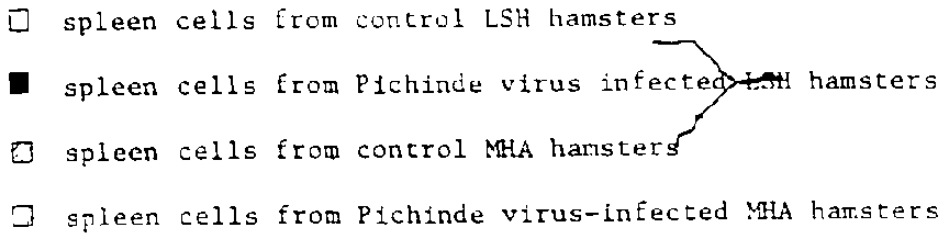
3.3.3 Inheritance of NK Activity in Syrian Hamsters

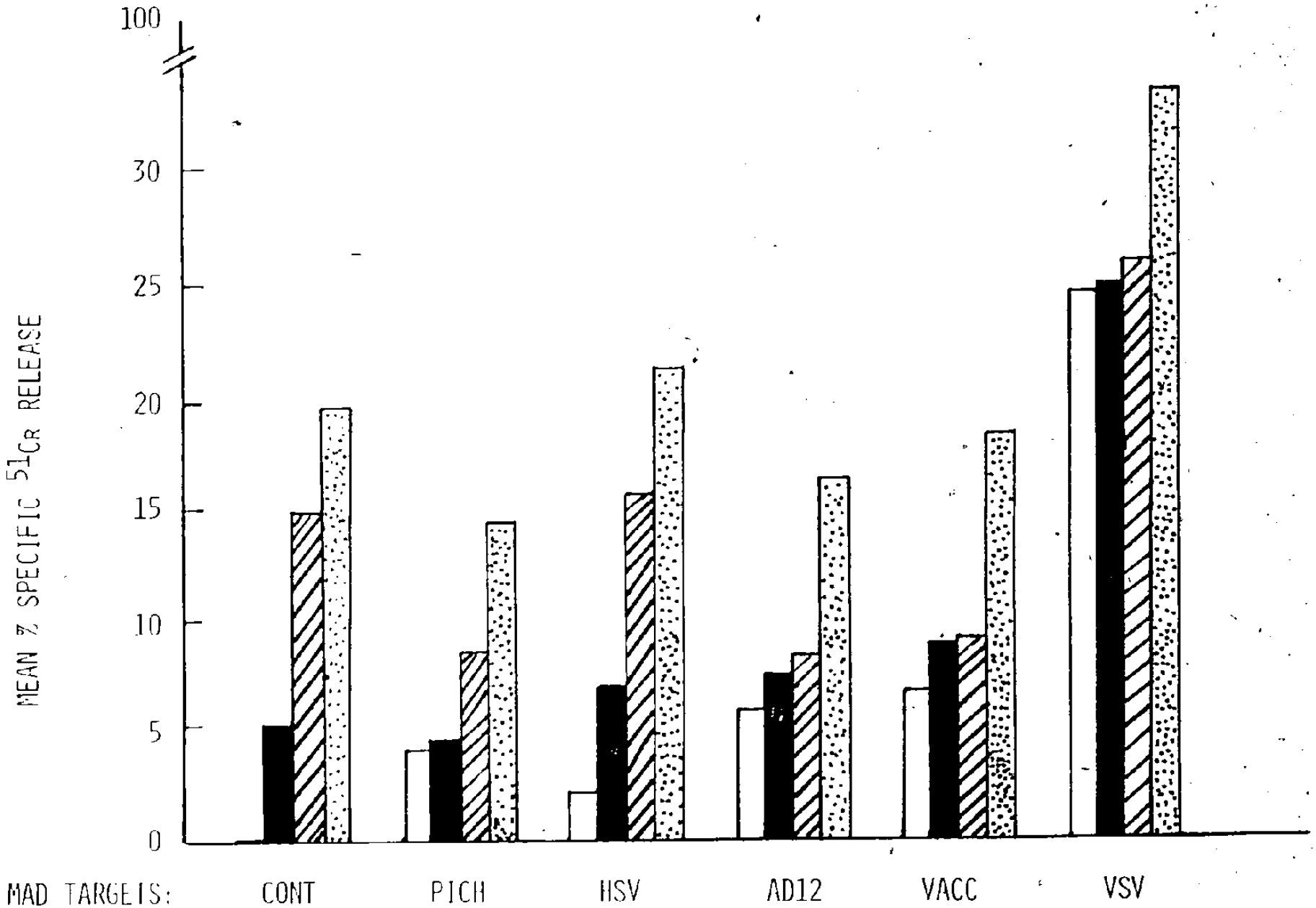
As has been previously mentioned, genetic studies on susceptibility to Pichinde virus infections were not performed on the LSH strain. However, the inbred LSH animals do not differ from the outbred LVG animals with respect to survival, ability to limit viremia, antibody production, and ability of peritoneal exudate cells to support Pichinde virus replication *in vitro*.

NK activity in the spleens of LVG and MHA hamsters was compared. The mean percent specific ^{51}Cr release value ($\pm$SEM) obtained for uninfected LVG animals was -14.5 ± 0.8 compared to 9.6 ± 1.8 per cent release in MHA hamsters. Values obtained 3 days after infection with Pichinde virus were

Figure 10

Effect of virus infection of MAD targets on cytolysis by hamster NK cells. Spleen cells were obtained from control hamsters or hamsters which had been infected 3 days previously with 2000 pfu Pichinde virus IP. These were tested for cytotoxic activity against MAD target cells which had been infected with different viruses at a MOI of 1-3 pfu/cell except adenovirus type 12, in which case an MOI of 1000 particles per cell was used. MAD cells were infected with Pichinde virus (PICH) for 32 hrs prior to the assay; with herpes simplex virus (HSV) for 8 hrs; with adenovirus type 12 (AD12) for 8 hrs; with vaccinia (VACC) virus for 8 hrs; or with vesicular stomatitis virus (VSV) for 4 hrs. The data in this Figure were obtained using an effector-to-target cell ratio of 50:1; similar data were obtained using ratios of 100:1 and 25:1.




100
30
25
20
15
10
5
0
MEAN % SPECIFIC ^{51}Cr RELEASE
MAD TARGETS:
CONT
PICH
HSV
AD12
VACC
VSV

14.4±1.7, and 47.9±1.3 for LVG and MHA hamsters respectively. Thus, Pichinde virus infection does not augment endogenous NK activity in LVG hamsters to the same extent that infection increases NK activity in MHA hamsters. In this respect, LVG hamsters resemble LSH hamsters, which show only a small increase in cytotoxic activity after Pichinde virus infection in comparison to the response of MHA hamsters.

Data from an experiment illustrating the inheritance of NK activity are shown in Table XIII. Spleens from five individual F_1(LSH X MHA) animals which had been infected with Pichinde virus 3 days previously were assayed for cytotoxicity against ^{51}Cr-labelled MAD targets. Results given by pooled spleens of infected MHA and LSH hamsters are included for comparison. Although the variation between individual F_1 animals was high, it is seen that all had levels of cytotoxicity which were lower than the MHA parent. Both female F_1(LSH X MHA) hamsters had lower per cent specific ^{51}Cr release than the spontaneous values. One male had levels that were very similar to its LSH parent, while the other two males had intermediate values. These data suggest that low NK activity behaves as a dominant characteristic in F_1(LSH X MHA) progeny.

3.3.4 Susceptibility of MHA and LSH hamsters to tumour formation

A considerable amount of evidence now supports the theory that NK cells are important in immunosurveillance against tumour formation. If this is the case in the Syrian hamster, then the genetic difference in NK activity in MHA and LSH hamsters would predict that a concomittant difference in tumour susceptibility would also be present. Therefore, neonatal hamsters were injected subcutaneously with $\log_{10}$ dilutions of

Table XIII

NK Activity in Parents and F_1 Progeny Infected with Pichinde Virus

Effector[a]	Percent specific ^{51}Cr release mean ± SEM		
	50:1[b]	25:1	12.5:1
MHA ♂	35.8±2.5	24.8±1.2	13.5±1.1
LSH ♂	8.4±1.7	6.0±1.7	<SR
F_1 ♀	<SR[c]	<SR	<SR
F_1 ♀	<SR	<SR	<SR
F_1 ♂	15.8±1.5	7.9±0.5	<SR
F_1 ♂	8.4±1.8	3.7±1.5	2.3±1.7
F_1 ♂	20.9±1.0	14.0±1.2	10.1±2.1

[a] Animals were infected with 2000 pfu Pichinde virus IP 3 days prior to assay. Spleens from 3 animals were pooled to obtain both MHA and LSH effectors. Individual F_1 spleens were assayed for cytotoxic activity.

[b] Spleen cells were assayed for cytotoxicity at effector-to-target cell ratios of 50:1, 25:1 and 12.5:1 using MAD cells as targets.

[c] Value less than spontaneous release.

adenovirus type 12 and observed daily for tumour development. Tumours appeared to occur more frequently among LSH hamsters injected with 10^8 virus particles (Figure 11); however, with the numbers studied the differences between the strains were not statistically significant ($\chi^2 = 1.29$; $0.2<p<0.3$). Cell lines were established in vitro from tumours and these were subsequently injected into weanling hamsters. As is evident from the data presented in Figure 12, MHA hamsters were more resistant than LSH hamsters to tumour induction with either syngeneic or allogeneic adenovirus type 12-induced tumour cells ($\chi^2 = 7.14$; $p<0.01$).

3.4 Studies on the Mechanism Underlying Susceptibility to Pichinde Virus Infection in Syrian Hamsters

The resistance of Syrian hamsters to fatal Pichinde virus infection and the ability to limit viremia are both dominant phenotypes which are controlled either by a single autosomal gene or closely linked genes. The limited information that is available on the genetics of NK activity suggests that low NK activity is also a dominant phenotype. These observations raise the possibility that a relationship may exist between the presence of high NK activity in MHA hamsters and their susceptibility to fatal Pichinde virus infections. In addition, analysis of viral antigen distribution in the spleens of infected animals suggest that certain spleen cells which have not been identified may be targets for virus growth (Murphy et al., 1977). Therefore, studies designed to examine the possibility that NK cells in MHA hamsters may represent a target cell for virus replication were carried out.

Figure 11

Induction of tumours by adenovirus type 12 in neonatal hamsters. Neonatal MHA or LSH hamsters received subcutaneous injections of $\log_{10}$ dilutions of adenovirus type 12 particles. The numbers of animals (n) receiving each dose are shown beside each line. Animals were observed weekly for the appearance of tumours beginning at day 20.

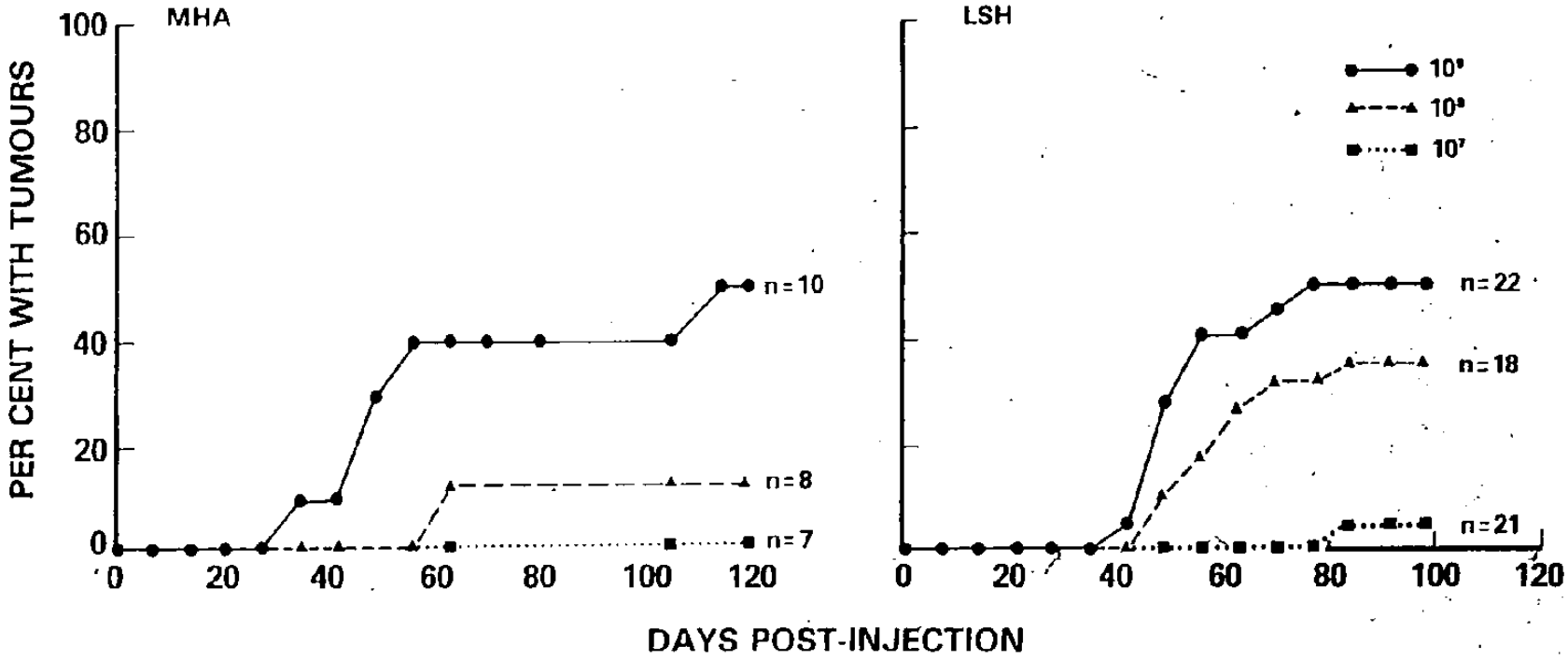
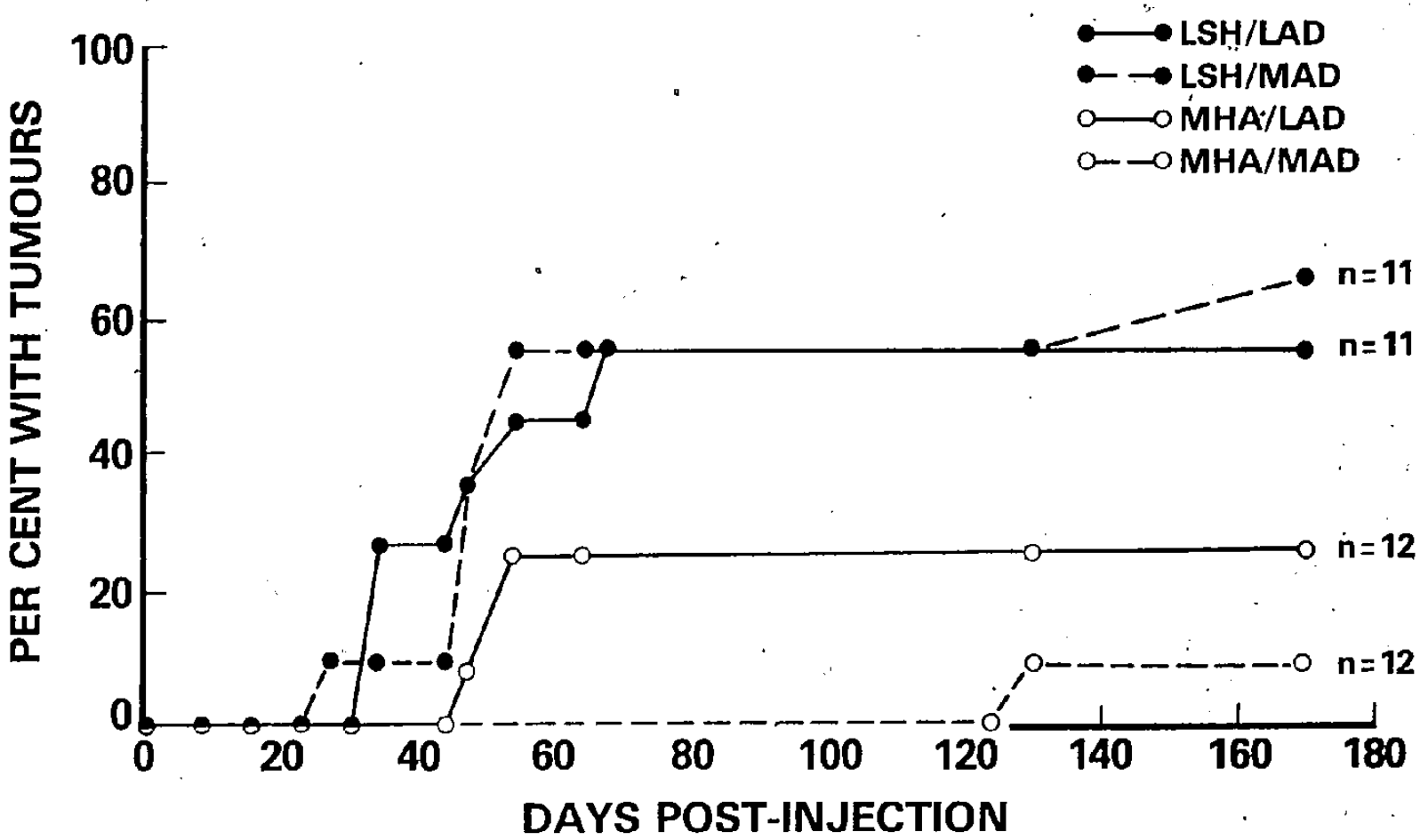

MHA
LSH
PER CENT WITH TUMOURS
100
80
60
40
20
0
0
20
40
60
80
100
120
n=10
n=8
n=7
n=22
n=18
n=21
10⁹
10⁸
10⁷
DAYS POST-INJECTION

Figure 12

Induction of tumours by injection of tumour cells. Hamsters aged 5-7 weeks were injected with 10^5 LAD or 10^5 MAD cells in a volume of 0.1 ml subcutaneously. The number, n, of MHA or LSH hamsters receiving either tumour cell line is indicated in the graph. Animals were observed weekly for the appearance of tumours.


LSH/LAD
LSH/MAD
MHA/LAD
MHA/MAD
n=11
n=11
n=12
n=12
PER CENT WITH TUMOURS
DAYS POST-INJECTION
0
20
40
60
80
100
120
140
160
180

3.4.1 Ability of Hamster Spleen Cells to Support Pichinde Virus Replication *In Vitro*

As an initial approach to determine whether MHA hamsters had a target cell for Pichinde virus replication which the LSH strain lacked, spleen cells from MHA and LSH hamsters were assayed for the ability to support Pichinde virus growth *in vitro*.

Preparations of whole spleen cells and peritoneal exudate cells from MHA and LSH hamsters were infected with Pichinde virus *in vitro*. Duplicate samples were harvested at various times after infection and assayed for infectious virus. The results of a typical experiment are shown in Figure 13. No significant difference between the LSH and MHA strains was noted in the ability of either peritoneal exudate cells or the whole spleen cell population to support Pichinde virus growth *in vitro*.

The failure to demonstrate a difference between LSH and MHA hamster spleen cells in the ability to support virus replication may occur because growth in a particular target is masked by the presence of other cells in the whole spleen cell population. Therefore, the population of whole spleen cells was fractionated according to the properties of resistance to carbonyl iron pretreatment or adherence to plastic. These fractions were then tested for the ability to support Pichinde virus replication (Figure 14). Once again, no difference in the ability of cell fractions derived from either the MHA or LSH hamster strains to support virus growth was observed.

Spleen cells which had been depleted of adherent and/or phagocytic cells by incubation with carbonyl iron showed an inferior ability to

Figure 13

Growth of Pichinde virus in hamster spleen and peritoneal exudate cells. Peritoneal exudate cells and a whole spleen cell preparation were obtained from MHA and LSH hamsters. The cells were then infected with Pichinde virus at a MOI of 1-3 pfu/cell. After adsorption for 90 min at 37°C, the cell suspensions were washed 3 times and adjusted to 2 X 10^6 live cells/ml; 1.0 ml was dispensed into small polypropylene tubes, and the infected cells were returned to the 37°C incubator. Duplicate samples were removed at 0, 2, 24 and 48 hrs after infection. These were frozen and thawed 3 times prior to assaying for plaque formation on monolayers of Vero cells. A sample of Pichinde virus alone was also incubated at 37°C to measure thermal inactivation.

o——o Whole spleen

□-----□ Peritoneal exudate

Δ—·—Δ Pichinde virus alone

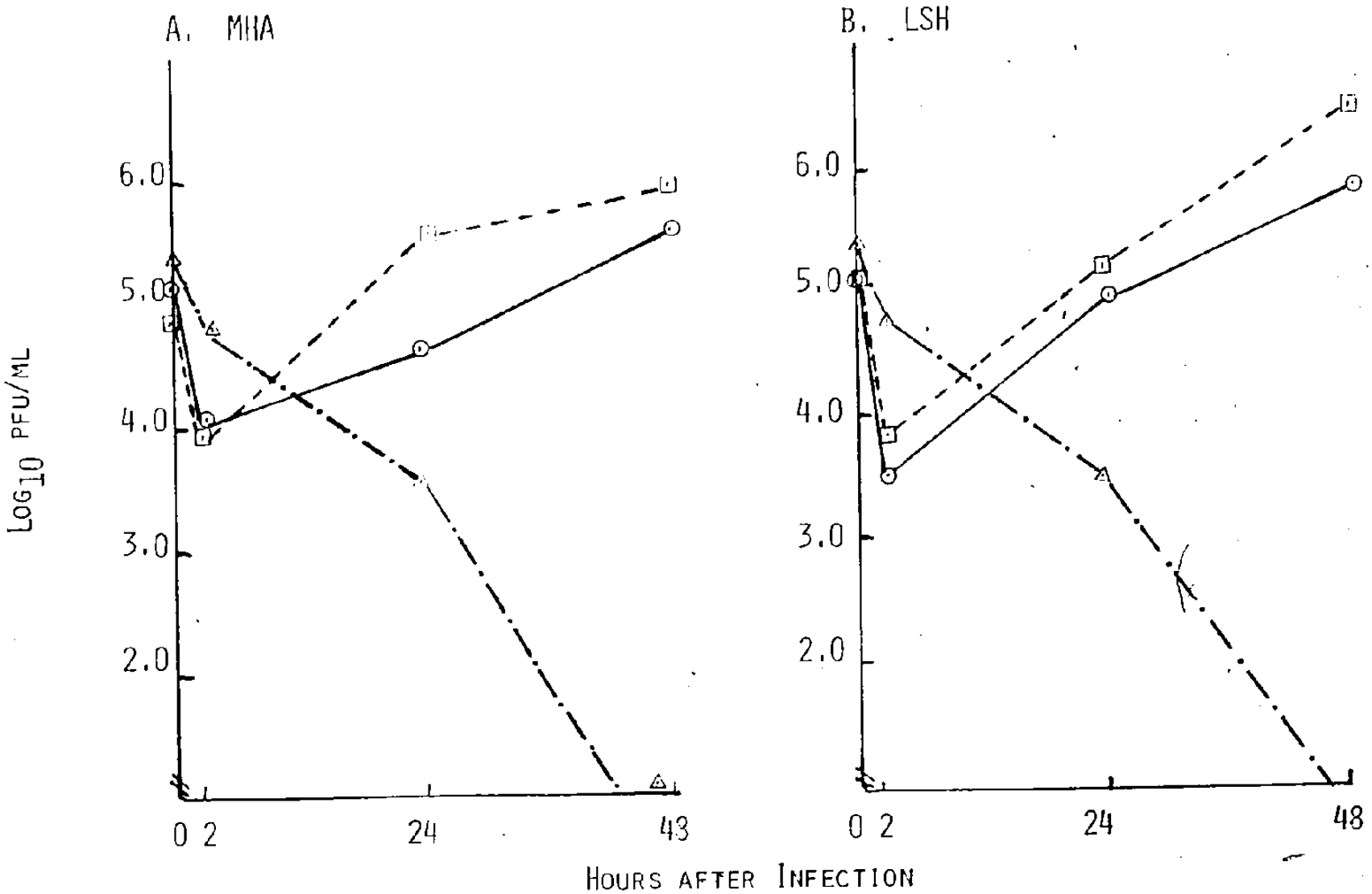

A. MHA
B. LSH
6.0
5.0
4.0
3.0
2.0
0 2
24
43
48
Log10 PFU/ML
Hours after Infection

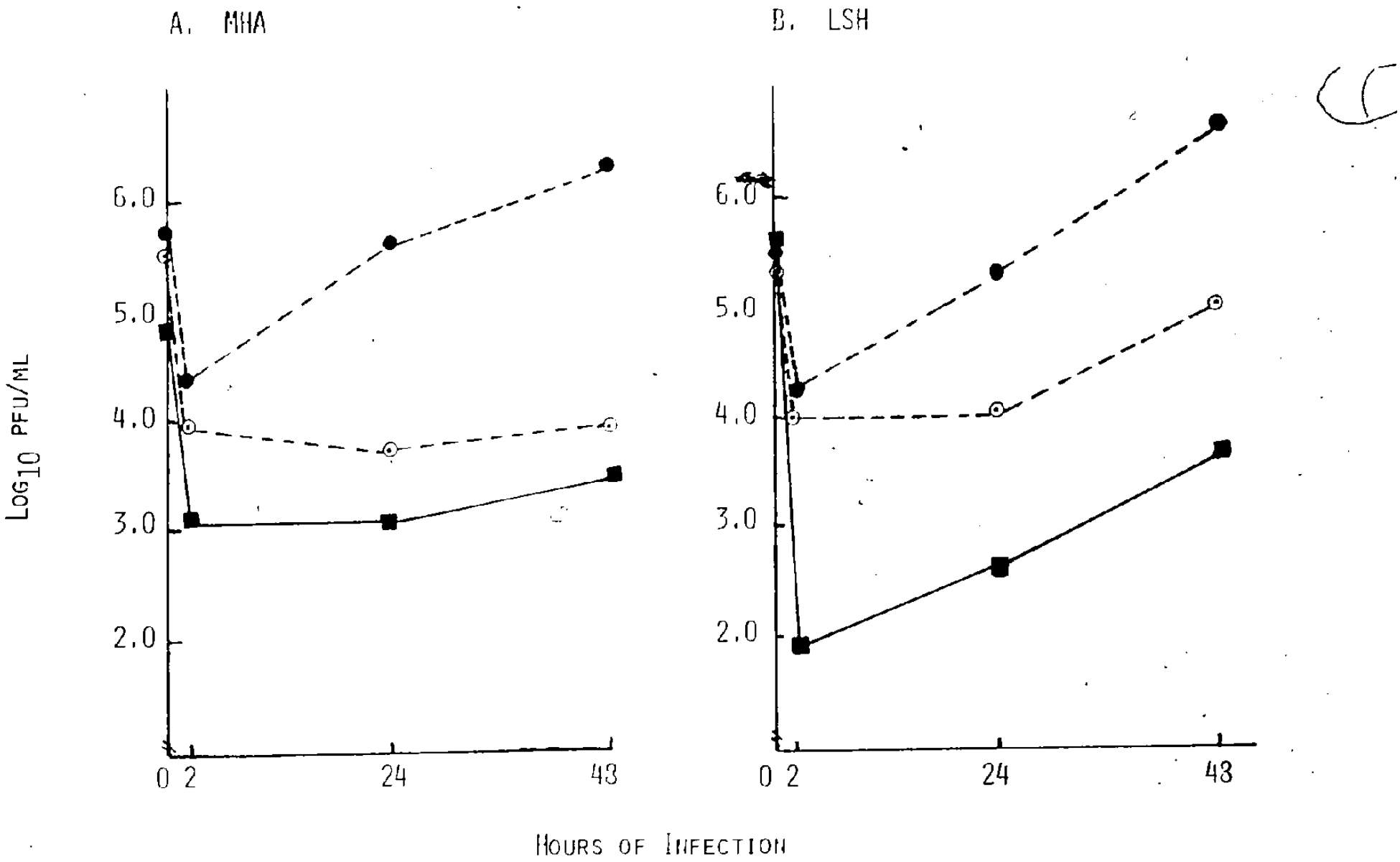
Figure 14

Growth of Pichinde virus in spleen cell fractions. Whole spleen cell preparations were obtained from MHA and LSH hamsters. Carbonyl iron-resistant cells were prepared by incubating spleen cells with carbonyl iron as described in Materials and Methods. An aliquot of spleen cells was incubated on plastic dishes. Non-adherent cells were obtained by decanting the supernate and washing the monolayer to retrieve any remaining free cells. Adherent cells were removed with the aid of a rubber policeman. The populations of cells were then infected and assayed for growth of Pichinde virus as described in the legend of Figure 14.

■——■ Carbonyl iron-resistant

o----o Adherent

●------● Non-adherent


A. MHA
B. LSH
6.0
5.0
4.0
3.0
2.0
0 2
24
43
Log10 PFU/ML
Hours of Infection

support virus replication when compared to the adherent or non-adherent populations which had been prepared by incubation on plastic dishes. This effect of carbonyl iron might occur if the treatment removed a population of cells which is required for virus growth. Alternatively, carbonyl iron itself may be toxic to the cells. To examine these possibilities, carbonyl iron was added to cultures of Pichinde virus-infected BHK cells or hamster peritoneal exudate cells. Duplicate samples were harvested at 24, 48 and 72 hrs after infection and assayed for infectious virus. It was observed that the addition of carbonyl iron to cell cultures reduced the virus yield by 1-2 $\log_{10}$ pfu/ml. The removal of carbonyl iron-containing cells with a magnet, either before or after infection with Pichinde virus, did not affect the inhibition of virus growth that was observed in carbonyl iron-treated BHK or peritoneal exudate cell cultures (data not presented). These observations indicate that carbonyl iron alone may exert an inhibitory effect on Pichinde virus replication.

The inability to demonstrate differences in growth or indeed even an appreciable increase in net yield of Pichinde virus pfu in whole spleen cells, adherent and non-adherent spleen cells, and in carbonyl iron-resistant cells of either LSH or MHA hamsters may have several explanations. Perhaps no difference actually exists between LSH and MHA hamster spleen cells in the ability to support virus replication in vitro. A second possibility is that the in vitro culture conditions do not reflect the in vivo situation; for example, NK cell activity is labile in vitro, and therefore NK cells would not be expected to support virus growth in vitro. Studies were therefore initiated on spleen cells from infected

animals.

3.4.2 Kinetics of Appearance of Infectious Centres in the Spleens of Pichinde Virus-infected Hamsters

Levels of viremia in Pichinde virus-infected MHA and LVG hamsters are parallel for the first 4 days of infection. After this time, a plateau is reached in the resistant LVG strain, but levels continue to rise in MHA hamsters until 8 days after infection (Buchmeier and Rawls, 1977).

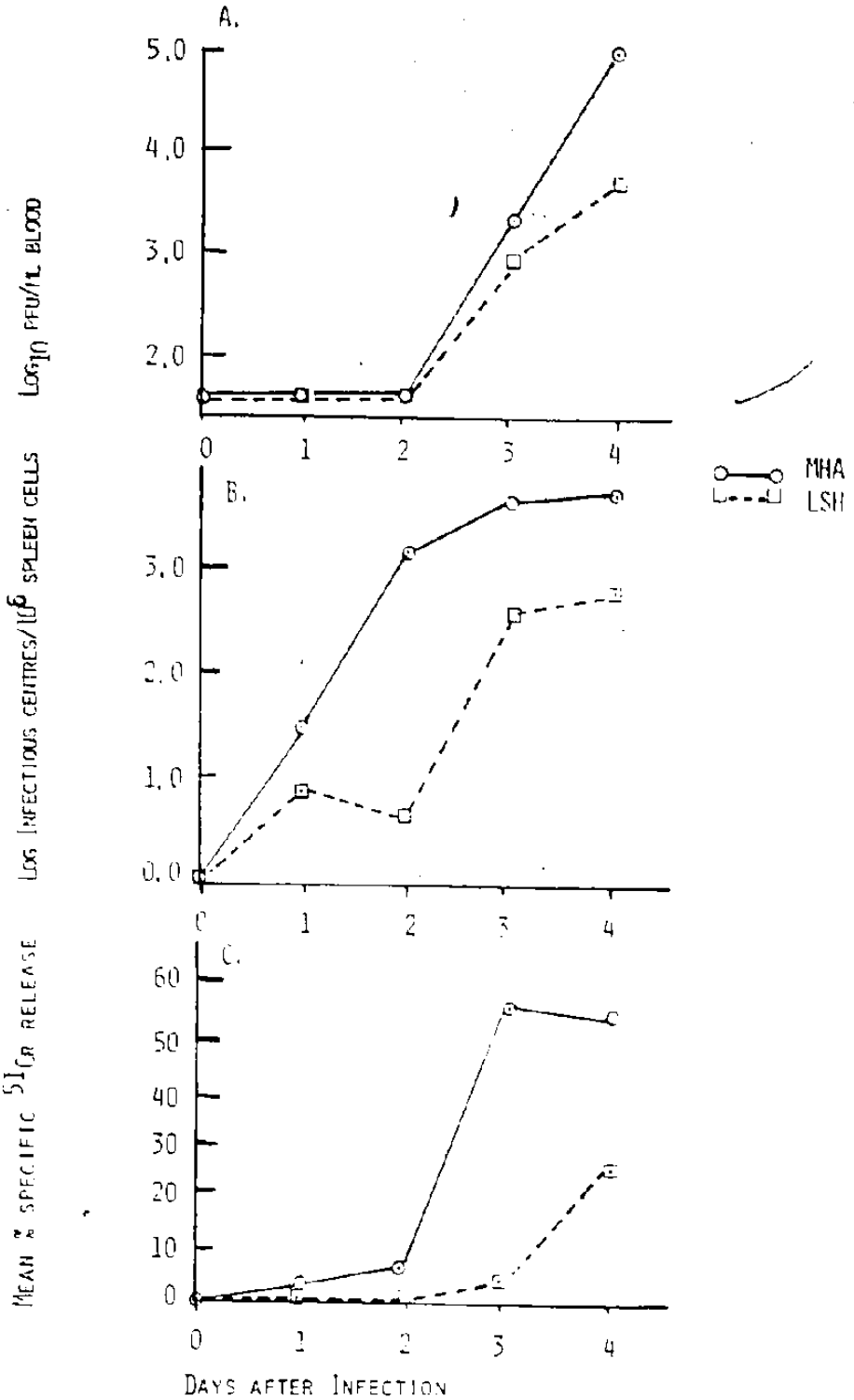
If the hypothesis is correct that activated NK cells support Pichinde virus replication in vivo, then the kinetics of appearance of infectious centres in the spleen should parallel the kinetics of NK activity, preceding the development of maximum viremia. Moreover, higher numbers of infectious centres would be expected in spleen cells derived from MHA hamsters than in spleen cells from LSH hamsters because spleens of MHA hamsters show higher levels of NK activity compared to LSH hamsters.

To test these predictions, animals were injected with virus on different days. Samples of blood were obtained by cardiac puncture for assay of infectious Pichinde virus, and the spleen cells were assayed for cytotoxic activity against ^{51}Cr-labelled MAD targets, and for infectious centres of virus by plating dilutions of the spleen cell population on monolayers of Vero cells. The results of a typical experiment are shown in Figure 15.

No virus was detectable in the blood until 3 days after infection; by day 4, MHA hamsters had higher virus titres than did LSH hamsters. Thus, the kinetics of viremia early after Pichinde virus infection of LSH hamsters were comparable to that described in the LVG strain (Buchmeier

Figure 15

Kinetics of viremia, and appearance of infectious centres and NK activity in the spleens of Pichinde virus-infected MHA and LSH hamsters. Groups of 2 hamsters were infected with 2000 pfu Pichinde virus IP. Hamsters were bled by cardiac puncture at various times after infection, and the blood was assayed for infectious virus (Figure 15A). Spleen cell suspensions were tested for infectious centres by plating 10-fold dilutions on monolayers of Vero cells (Figure 15B) and for cytotoxic activity against ^{51}Cr-labelled MAD targets (Figure 15C). The data for the NK assay was obtained using an effector: target cell ratio of 50:1; similar data were obtained using ratios of 100:1 or 25:1.


A.
5.0
4.0
3.0
2.0
0
1
2
3
4
Log10 PFU/mL BLOOD
MHA
LSH
B.
3.0
2.0
1.0
0.0
Log Infectious centres/10^6 spleen cells
C.
60
50
40
30
20
10
0
Mean % specific 51Cr release
Days after Infection

and Rawls, 1977).

Detectable infectious centres were present in the spleen as soon as 1 day of infection. By day 2, MHA hamsters had 100-fold more infectious centres than did LSH hamsters. The number of infectious centres reached a plateau by day 3-4, at which time the MHA animals had approximately 10 times the number of infectious centres than did LSH hamsters. The total difference in the number of infectious centres on a per spleen basis was even greater, since Pichinde virus-infected MHA hamsters usually had more cells per spleen than did infected LSH animals of the same age (data not shown).

NK activity peaked at 3 days after Pichinde virus infection in MHA hamsters, but it was still rising at day 4 in the LSH strain. These observations suggested that MHA spleen cells were subject to an early productive infection with virus, and that more MHA spleen cells on a per spleen basis were actively supporting virus replication than in the case of LSH spleen cells. This rapid growth of virus in the spleen preceded the development of significant differences in viremia by 2 days, and the appearance of NK activity by 1 day. These observations are consistant with the hypothesis that MHA hamsters have an extra target cell for Pichinde virus replication that is lacking in LSH hamsters, and that possibly the NK cell itself is this target.

3.4.3 Demonstration of Target Cells for Pichinde virus Replication in vivo

Experiments were initiated to test the hypothesis that an extra target cell for Pichinde virus replication existed in the MHA hamster. Spleen cells from Pichinde virus-infected MHA and LSH hamsters were

separated by several methods and assayed for infectious centres, and NK activity.

3.4.3.1 Separation of Spleen Cells by Adherence

As an initial approach, spleen cells from hamsters which had been infected with Pichinde virus for various times were incubated with carbonyl iron, and then assayed for NK activity against ^{51}Cr-labelled MAD targets and for infectious centres (Table XIV). As usual, MHA hamsters showed higher levels of endogenous NK activity than did LSH animals, and Pichinde virus infection enhanced this activity to a greater extent. Carbonyl iron pretreatment of these cells enriched the cytotoxic activity in all spleen cell suspensions, confirming the data shown in Figure 7.

By 3 days after infection, MHA hamster spleens contained 1 $\log_{10}$ more infectious centres per 10^5 cells than LSH hamster spleens. Carbonyl iron-pretreatment of the spleen cells resulted in a reduction of infectious centres that seemed to be roughly proportional to the starting number. If this is true, the data suggest that Pichinde virus is not preferentially replicating in the carbonyl iron-resistant fraction that is enriched in cytotoxic activity. However, it has already been pointed out that carbonyl iron appears to exert an inhibitory effect on Pichinde virus replication. Also, overnight incubation of carbonyl iron-treated spleen cells failed to abrogate cytotoxic activity against fresh ^{51}Cr-labelled MAD targets (data not presented), suggesting that some of the cytotoxic activity may have been due to a toxic effect of residual carbonyl iron on the targets, or that the carbonyl iron treatment might induce NK cell activation in vitro. Therefore, any conclusions regarding

TABLE XIV

Infectious Centres and NK Activity in Carbonyl Iron-treated Spleen Cells

Source of effectors		Log_{10} infectious centres per 10^5 cells[b].		Mean % specific ^{51}Cr release ± SEM[c].	
Strain	Infected with Pichinde virus[a].	before CAFE	after CAFE[d].	before CAFE	after CAFE[d].
LSH	no	0.0	0.0	3.1±0.3	45.7±5.8
	yes	2.54	1.15	8.5±0.8	41.9±1.6
MHA	no	0.0	0.0	12.2±2.5	52.4±1.9
	yes	3.43	2.11	24.1±2.8	64.0±3.1

[a] Groups of 4 animals were infected with 2000 pfu Pichinde virus IP and sacrificed after 3 days of infection. Suspensions of spleen cells were then made.

[b] Aliquots of cells were diluted 10-fold and then plated on monolayers of Vero cells to assay for infectious centres.

Table XIV (footnotes cont'd)

[c] Cytotoxic activity against ^{51}Cr-labelled MAD targets was assayed in the usual 16 hr assay. The data presented here were obtained using an effector-to-target cell ratio of 25:1; similar data were obtained using ratios of 100:1 and 50:1.

[d] An aliquot of 1 X 10^8 cells was incubated with 1g carbonyl iron (CAFE) for 45' at 37°C, and the carbonyl iron-containing cells were removed with a magnet.

carbonyl iron-pretreated cells must be viewed with caution.

The property of adherence to plastic was used as a second marker to locate the Pichinde virus-infected target cell(s). Spleen cells from LSH and MHA hamsters which had been infected 3 days previously with Pichinde virus were incubated in plastic dishes for 60' at 37°C. The adherent and non-adherent fractions were then assayed for cytotoxic activity and for infectious centres. The results of a representative experiment are shown in Table XV. An enrichment in cytotoxic activity was found in both the adherent and non-adherent fractions of MHA hamster spleen cells, and in the non-adherent fraction of LSH hamster spleen cells. In all spleen cell populations, MHA cells contained a greater number of infectious centres. The non-adherent population of MHA hamster spleen cells accounted for a slightly higher proportion (12.2%) of the splenic infectious centres than did the non-adherent cells in the LSH hamster spleen (9.2%).

These data suggest that whereas the non-adherent fractions of both MHA and LSH hamster spleen cells, are slightly enriched for NK activity, only a very modest increase in the relative numbers of infectious centres in MHA hamster cells had been attained. The separation of spleen cells by the property of plastic adherence does not appear to be efficient enough to demonstrate differences between the two strains of hamsters with respect to NK activity or infectious centres.

3.4.3.2 Separation of Spleen Cells According to Size by Velocity Sedimentation at Unit Gravity

As a second means of testing the hypothesis that NK cells in MHA hamsters may represent a target cell for Pichinde virus replication, spleen cells from infected MHA and LSH hamsters were separated according to size by velocity sedimentation and the fractions were assayed for NK

Table XV

Distribution of Infectious Centres and NK Activity in the Adherent and Non-adherent Spleen Cell Fractions

Effector[a]	Number of cells	% of input	Mean % specific ^{51}Cr release ± SEM[b]	Infectious centres per 10^5 cells[c]	Total number of infectious centres
LSH Whole	$16.80X10^7$	-	5.3±0.5	82.5	$1.39X10^5$
LSH Adherent	$5.26X10^7$	31.3%	-0.4±0.4	57.5	$3.02X10^4$ (21.7%)
LSH Non-Adherent	$4.21X10^7$	25.1%	21.4±4.0	30.4	$1.28X10^4$ (9.2%)
MHA Whole	$29.0X10^7$	-	35.3±0.7	411.6	$1.19X10^6$
MHA Adherent	$5.22X10^7$	18.0%	61.4±2.3	240.0	$1.25X10^5$ (10.5%)
MHA Non-Adherent	$8.51X10^7$	29.3%	68.7±3.1	170.0	$1.45X10^5$ (12.2%)

Table XV (Footnotes)

[a] Spleen cells from 6 LSH or 6 MHA hamsters, which had been infected 3 days previously with 2000 pfu Pichinde virus IP, were incubated in 60 mm^2 plastic petri dishes at a concentration of 1 X 10^7 cells/ml for 1 hr at 37°C. The supernate was decanted and then pooled with the remainder of the non-adherent cells, which were obtained by washing the cells off the plastic dishes with 3 washes of 5.0 ml medium. Adherent cells were removed by scraping the monolayer with a rubber policeman. All cells were washed once and resuspended in a medium at a concentration of 1 X 10^7 live cells/ml.

[b] Cells were assayed for cytotoxic activity by ^{51}Cr release from labelled MAD targets in the standard 16 hr assay. The above data represents values obtained using an effector-to-target cell ratio of 50:1; similar data were obtained using 100 or 25 effector cells per target cell.

[c] Log_{10} dilutions of spleen cells were plated on monolayers of Vero cells and allowed to settle for 60' at 37°C. The plates were then carefully overlaid with agar, and infectious centres were visualized by adding neutral red 3 days later.

activity and for infectious centres.

The results are shown in Figure 16. The sedimentation profile of spleen cells from LSH (Figure 16A) and MHA (Figure 16B) hamsters were similar; however, the distribution of infectious centres and cytotoxic activity differed. The greatest concentration of infectious centres in LSH hamsters was in the rapidly sedimenting cells (Figure 16C), whereas comparable concentrations of infectious centres were found throughout the gradient of cells from MHA hamsters (Figure 16D). The cytotoxic activity, expressed as per cent ^{51}Cr release per 10^6 cells using effector-to-target ratios of 100:1 and 25:1, is shown in Figures 16E and 16F. Greater lytic activity was obtained from MHA hamsters than from LSH hamsters and furthermore, in both strains there appeared to be two peaks of cytotoxic cells. One peak corresponded to a cell population which sedimented between 2.8 and 4.3 mm/hr, a rate which is typical of small lymphocytes. The second peak of killing was mediated by a population of cells with a sedimentation velocity of 5.3 to 6.4 mm/hr, which is typical of macrophages. In spleens from MHA hamsters a considerable proportion of the virus-producing cells co-sedimented with the peak of lytic activity corresponding to small lymphocytes. In contrast, few virus-producing cells in LSH spleens co-sedimented with NK activity associated with small lymphocytes.

3.4.3.3 Separation of Spleen Cells by the Combined Properties of Adherence and Size

In an effort to improve the resolution of the cell populations containing NK activity and infectious centres, studies on spleen cells which had been separated by both adherence to plastic and velocity

Figure 16

Separation of hamster spleen cells by velocity sedimentation. Pooled spleen cells from 10 MHA and 10 LSH hamsters, which had been infected 3 days previously with 2000 pfu Pichinde virus IP, were allowed to sediment in a STA-PUT apparatus for 3.5 hrs at 4°C. Fractions were then collected and the viable cells counted (A-LSH, B-MHA). The fractions were assayed for infectious centres (C-LSH, D-MHA) and for NK activity against MAD cells. NK activity at cytotoxic effector cell-to-target cell ratios of 100:1 (O) and 25:1 (□) are shown (E-LSH, F-MHA).

A
B
C
D
E
F
10^8
10^7
10^6
10^5
10^2
10^1
10^0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
SEDIMENTATION VELOCITY (mm/hr)
Live white cells per fraction
Infectious centers per 10^6 cells
Per cent specific ^{51}Cr release per 10^6 cells

sedimentation at unit gravity were performed. It has already been shown that the small MHA cytotoxic fraction has approximately 1 $\log_{10}$ more infectious centres than do LSH cells of the corresponding size (Figure 16). Spleen cells from MHA and LSH hamsters, which had been infected 3 days previously with Pichinde virus, were separated into adherent and non-adherent fractions by incubating on plastic. The four groups of cells were then further separated according to size by velocity sedimentation in a STA-PUT apparatus. The results of this separation are illustrated in Figure 17. Spleen cells from MHA hamsters which had been infected with Pichinde virus contained a higher proportion of small non-adherent cells than spleen cells obtained from infected LSH hamsters. In contrast, LSH spleen cells contained a higher number and proportion of both small and large adherent cells.

Fractions of cells were combined to give comparable pools of cells of various sizes as shown in Figure 17. These pools were then assayed for cytotoxic activity against ^{51}Cr-labelled MAD targets, and for infectious centres. These data, which confirm and extend the previous data, are presented in Table XVI.

A number of points can be made. All cell fractions exhibited cytotoxic activity. The non-adherent cell population was responsible for more cytotoxicity than the adherent cells, and the bulk of this activity in the non-adherent population was mediated by a cell sedimenting between 4.6 and 6.7 mm/hr. These data appear to differ from Figure 16, which showed two populations of cytolytic activity at 2.8–4.3 mm/hr and at 5.1–6.6 mm/hr. Several explanations may account for the anomalies in the profile shown in Figure 17. First, the given velocities represent

Figure 17

Separation of adherent and non-adherent spleen cells by velocity sedimentation at unit gravity. Suspensions of spleen cells were obtained from LSH and MHA hamsters which had been infected 3 days previously with 2000 pfu Pichinde virus IP. The cells were then incubated on plastic for 60' at 37°C, and adherent and non-adherent fractions were obtained as described in Materials and Methods. Each of the four fractions of cells was then further separated by velocity sedimentation at unit gravity in a STA-PUT apparatus (diameter, 12.0 cm). The cells were layered beneath PBS and allowed to sediment through a stacking region of 0.35% BSA in PBS, and then through a gradient of 1-2% BSA in PBS for 35 hrs at 4°C. Fractions of 25.0 ml were collected and the cells were pelleted at 1000 rpm for 10'. The cells were resuspended in 1.0 ml αMEM. After counting viable cells, fractions of cells were combined as indicated to give pools of comparable sedimentation rates.

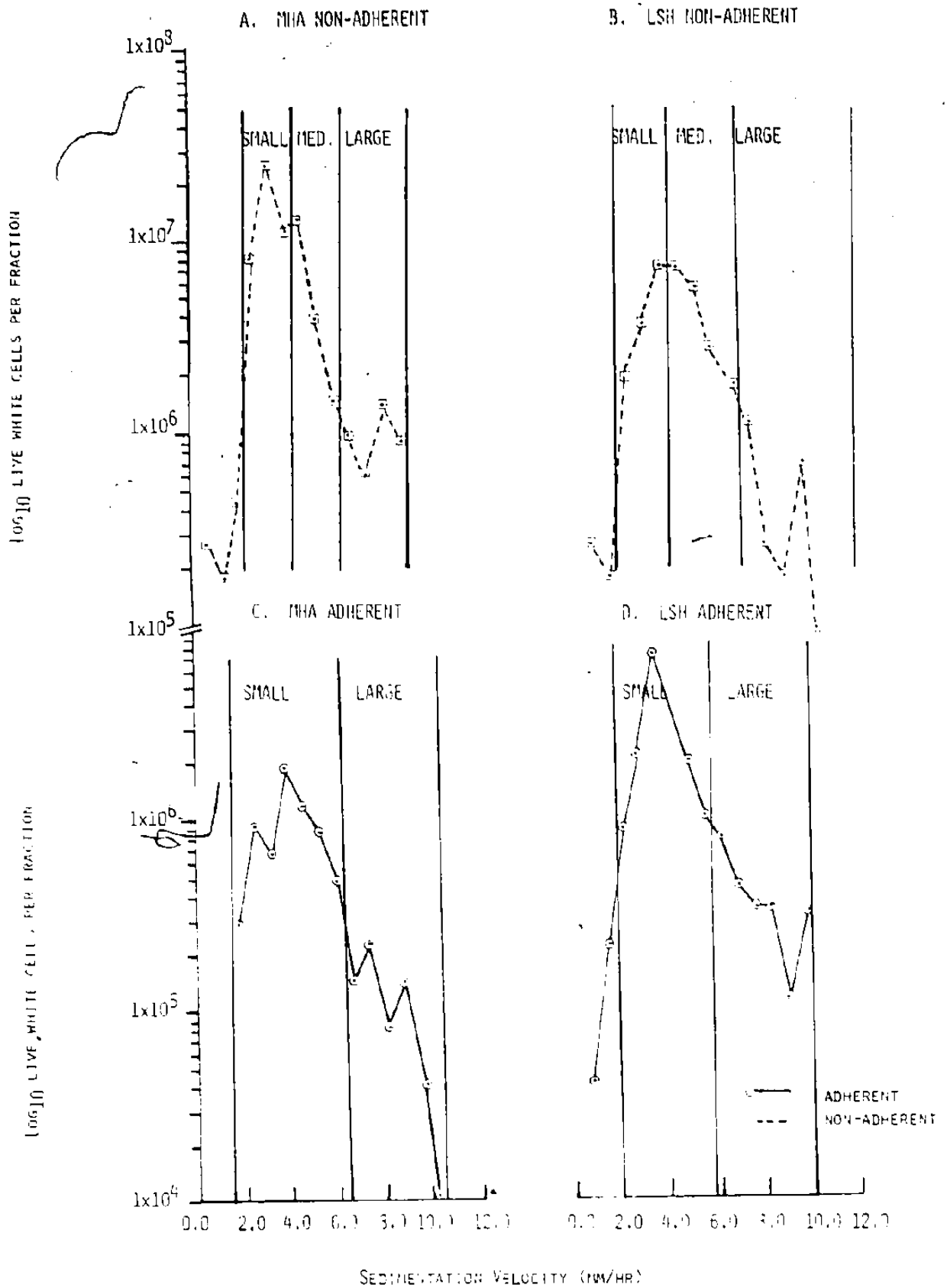

A. MHA NON-ADHERENT
B. LSH NON-ADHERENT
C. MHA ADHERENT
D. LSH ADHERENT
SMALL
MED.
LARGE
1x10^8
1x10^7
1x10^6
1x10^5
1x10^4
LOG10 LIVE WHITE CELLS PER FRACTION
0.0
2.0
4.0
6.0
8.0
10.0
12.0
ADHERENT
NON-ADHERENT
SEDIMENTATION VELOCITY (MM/HR)

Table XVI

Infectious Centres and NK Activity in Hamster Spleen Cells Separated by Adherence and Velocity Sedimentation at Unit Gravity

Sample [a.]	Sed. rate mm/hr	# cells per group	% of input ($\frac{\#\ cells/pool}{total\ \#}$)	NK:% ^{51}Cr release [b.] 50:1	25:1
LSH Adherent		$4.18X10^7$	34.3%	28.7±1.7	18.5±1.8
LSH Non-adherent		$5.24X10^7$	43.0%	50.0±4.0	34.0±1.3
MHA Adherent		$1.44X10^7$	12.0%	37.0±2.7	37.0±2.7
MHA Non-adherent		$1.08X10^8$	90.0%	74.0±2.9	46.9±2.2
LSH Adherent					
large	6.3-9.8	$2.88X10^6$	7.9%	NT [d.]	NT
small	2.2-5.6	$1.19X10^7$	32.6%	35.1±2.6	20.7±3.6
LSH Non-adherent					
large	7.5-12.0	$1.08X10^6$	2.3%	NT	NT
medium	4.6-6.7	$1.02X10^7$	21.5%	69.3±1.2	52.0±2.3
small	2.4-3.9	$1.35X10^7$	28.5%	34.5±2.4	18.9±2.2
MHA Adherent					
large	6.7-10.2	$5.40X10^5$	4.5%	NT	NT
small	1.9-6.0	$5.04X10^6$	42.0%	35.5±0.2	18.8±1.6
MHA Non-adherent					
large	6.8-8.9	$1.62X10^6$	1.8%	NT	NT
medium	4.7-6.1	$1.19X10^7$	12.9%	81.0±1.1	61.8±2.0
small	2.7-4.1	$4.43X10^7$	47.9%	43.7±1.8	21.8±2.3

Table XVI (cont'd)

Sample[a.]	Infectious centres per 10^5 cells[c.]	Total number of infectious centres
LSH Adherent	74.7	$3.12X10^4$
LSH Non-Adherent	108	$5.69X10^4$
MHA Adherent	341	$4.92X10^4$
MHA Non-adherent	355	$3.84X10^5$
LSH Adherent		
large	125	$3.60X10^3$
small	29.5	$3.51X10^3$
LSH Non-adherent		
large	153	$1.65X10^3$
medium	20.2	$2.06X10^3$
small	14.8	$2.01X10^3$
MHA Adherent		
large	171	$9.24X10^2$
small	39.7	$2.00X10^3$
MHA Non-adherent		
large	142	$2.32X10^3$
medium	91.3	$1.09X10^4$
small	147	$6.56X10^4$

Table XVI (Footnotes)

[a] Adherent and non-adherent cell populations were obtained from Pichinde virus-infected LSH and MHA hamsters and separated into fractions by velocity sedimentation at unit gravity as described in the legend of Figure 18. After counting in trypan blue, the fractions were combined to give pools of comparable sedimentation rates. These pools were then recounted and assayed for NK activity and infectious centres.

[b] Pools were assayed for cytotoxic activity against ^{51}Cr-labelled MAD targets, at ratios of 100:1 through to 6:1; only data using ratios of 50:1 and 25:1 are presented, but similar data were obtained using the other ratios.

[c] Log_{10} dilutions of spleen cells were allowed to settle out on monolayers of Vero cells for 60' at 37°C, and were then carefully overlaid with medium containing agar. Infectious centres were visualized by adding neutral red after 3 days of incubation at 37°C.

[d] NT, not tested.

the mean velocity for that fraction; hence; a pool of cells from fractions with velocities from 4.6-6.7 mm/hr would include cells sedimenting at 4.2-7.1 mm/hr. This pool thus includes a proportion of small (2.8-4.3 mm/hr) cells. Secondly, the adherence procedure may have removed a suppressor in the 4.6-6.7 mm/hr region, or may have activated interferon production in this population.

The cytotoxicity of fractionated spleen cells from MHA hamsters was greater than that seen in comparable fractions of spleen cells from LSH hamsters. As was seen previously, MHA spleen cells contained a greater number of infectious centres overall than did cells of LSH origin. The non-adherent fraction appeared richer in both infectious centres and NK activity than adherent cells. Velocity sedimentation separation revealed that large non-adherent cells from LSH and MHA hamsters contained similar numbers of infectious centres. However, MHA non-adherent cells of medium and small size had 91.3 and 147 infectious centres per 10^5 cells, respectively, while the corresponding LSH fractions contained only 20.2 and 14.8 infectious centres per 10^5 cells, respectively. The non-adherent, medium-sized cell was also responsible for enhanced cytotoxic activity in both strains, although to a greater degree in MHA than in LSH hamsters. These data are consistant with the idea that the MHA hamster spleen contains an additional population of non-adherent cells which may be a target for Pichinde virus replication early in infection. Furthermore, the presence of increased numbers of target cells appears to be associated with increased cytotoxic activity mediated by cells which had physical properties similar to the putative target cell.

3.4.4 Effect of the Footpad Route of Injection of Pichinde Virus on Development on NK Activity and Infectious Centres

The observation that footpad injection of Pichinde virus into normally susceptible MHA animals fails to result in a fatal outcome, and protects the hamsters against a subsequent IP challenge of virus suggests that the cell-mediated immune response in MHA animals against Pichinde virus is adequate. If the hypothesis is correct that MHA hamsters possess a target cell for Pichinde virus replication which the LSH strain lacks, and this target cell is indeed the NK cell, then the prediction is that NK cells in the spleens of footpad-inoculated MHA animals fail to become activated or are activated too late, so that the humoral and cell-mediated immune response can begin to clear the virus particles from extracellular and intracellular locations.

Groups of 3 MHA hamsters received footpad injections of virus at various times; uninfected hamsters and hamsters which had been injected IP with Pichinde virus 3 days previously were included as controls. Cells from the spleen and the popliteal lymph nodes draining the footpad were then tested for cytotoxic activity against ^{51}Cr-labelled MAD targets, and for infectious centres. The results are shown in Table XVII. The footpad route of inoculation of virus elicited an augmented NK response in the draining lymph node population which peaked between the third and fifth day of infection. Only a minimal increase in lytic activity was seen in the popliteal lymph node cells of IP inoculated hamsters, although the spleens showed the typical augmentation. An increase in splenic NK activity was observed in footpad-inoculated hamsters, but did not reach a peak until day 5, two days later than usual. Infectious centres were

present in the popliteal lymph nodes of footpad-inoculated hamsters; the peak occurred on day 5, and declined afterwards. No detectable infectious centres were present in the popliteal lymph nodes of IP-inoculated hamsters; virus-producing cells were present in the spleens as usual. In footpad-inoculated hamsters, no detectable infectious centres were found in the spleen at day 3; the development was delayed until day 5, and they were undectable by day 8. Thus the development of infectious centres paralleled the kinetics of development of NK activity in the popliteal lymph nodes and the subsequent spread to the spleen was delayed. These observations are consistant with the hypothesis that the delayed development of NK activity in the spleen results in a slower development of infectious centres, thereby retarding virus growth sufficiently so that the humoral and cell-mediated immune response can remove the virus particles from circulation.

Table XVII

Effect of Footpad Route of Inoculation of Pichinde Virus on Development of NK Activity and Infectious Centres

Effector Cells[a]		Source	Mean % specific ^{51}Cr release ± SEM[b]	Infectious centres per 10^5 cells[c]
Days post-infection	Route of injections			
cont	-	DLN	-0.1±3.4	-
3	IP		9.5±0.3	<.05
3	footpad		45.4±0.3	$9.61X10^1$
5	footpad		38.6±2.4	$2.78X10^2$
8	footpad		1.4±1.5	$4.58X10^1$
cont	-	spleen	2.9±3.5	-
3	IP		52.9±3.8	$1.85X10^2$
3	footpad		19.3±1.7	<.05
5	footpad		37.2±1.6	$1.50X10^2$
8	footpad		9.3±1.2	<.05

a Groups of 3 animals were injected with 2000 pfu Pichinde virus either IP or into both hind footpads. On the day of assay, the spleens, and the lymph nodes draining the footpad (DLN) were excised.

b Cell suspensions were assayed for cytotoxic activity against ^{51}Cr-labelled MAD targets using an effector-to-target cell ratio of 50:1. Similar data were obtained using ratios of 100:1, 25:1, 12.5:1 and 6.25:1.

Table XVII (Footnotes cont'd)

[c] Log_{10} dilutions of spleen cells and DLN cells were incubated on Vero monolayers, and then carefully overlaid with medium containing agar. Plaques were visualized by adding neutral red to the plates after 3 days of incubation.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

Pichinde virus has been shown to cause a lethal disease in the MHA strain of Syrian hamsters, but not in other hamster strains (Buchmeier and Rawls, 1977). The phenotype of survival correlated with the ability to limit virus replication; death was thought to be a direct consequence of the virus-induced cytopathic effect within the host's reticuloendothelial system (Murphy et al., 1977). The current studies were initiated in order to determine whether the resistance to fatal Pichinde virus infections was genetically acquired, and to establish the basis for the susceptibility of MHA hamsters to the fatal Pichinde virus infection.

4.1. Genetics of Susceptibility of Syrian Hamsters to Fatal Pichinde Virus Infection

The susceptibility of Syrian hamsters to a fatal Pichinde virus infection was shown to be under genetic control. Both survival and the ability to limit viremia were dominant phenotypes (Figure 1; Tables I and II), and were independent of virus dose over a range of 10^3 to 10^6 pfu (data not presented). The survival rates that were observed in back-cross progeny following infection with Pichinde virus are consistent with the results expected if a single gene or linked genes controlled this trait. Similarly, the phenotype of ability/inability to limit virus titres in the blood segregated in a 1:1 ratio in (F_1 X MHA) progeny, suggesting that a single gene or closely linked genes regulated this phenotype. It is not yet clear

whether the same gene controls both survival and the ability to limit viremia. However, if the viral cytopathic effect is actually the cause of death, as has been suggested by the work of Murphy et al. (1977), one would then predict that only one gene controls virus replication and ultimately is responsible for the death of the host. Furthermore, no influence of sex of the parent animal on survival or on the ability to limit viremia in F_1 and back-cross progeny was noted, implying that the genes involved are autosomal.

Although no genetic studies were carried out using the resistant LSH strain as a parent, the LSH hamsters were found to be similar to the LVG strain in their response to Pichinde virus infection. Adult LSH hamsters survived a challenge with 10^3 to 10^6 pfu Pichinde virus. These animals limited virus titres in the blood to low levels. Peritoneal exudate cells from LSH hamsters supported virus replication in vitro as well as did cells derived from MHA or LVG animals. Finally, the LSH strain responded to Pichinde virus infection with the production of complement-fixing antibodies. These observations suggest that LSH hamsters strongly resembled the LVG strain in their response to infection with Pichinde virus. However, the LSH hamsters, unlike LVH hamsters, are inbred and appear to share the same major histocompatability complex haplotype as the MHA strain (Duncan, 1976). It was hoped that these properties of the LSH strain would minimize variation among individual animals and facilitate comparisons with the MHA strain. Experiments designed to test the genetics of susceptibility to lethal Pichinde virus infection in LSH X MHA crosses have not yet been completed.

4.2. Mechanisms Underlying the Genetically Acquired Susceptibility of MHA Hamsters to Lethal Pichinde Virus Infections.

Studies were then initiated to determine the basis for susceptibility to fatal Pichinde virus infection in these strains of hamsters. In other examples of inherited resistance to viral infections, the presence of a target cell for virus replication has been shown to be genetically determined and is responsible for the outcome of the infection. The target cell defect can be ubiquitous, as is the case with the "tv"-controlled susceptibility of chickens to the avian leukosis viruses (Pani, 1976), or in the Fv-1-mediated restriction on replication of lymphoid leukosis virus in Friend disease of mice (Lilly and Pincus, 1973). These general defects were manifested in vitro; primary cultures of cells derived from resistant hosts were able to prevent or abort the virus growth cycle, while cells derived from susceptible strains allowed a productive infection (Vogt and Ishizaki, 1975; Jolicoeur and Baltimore, 1976). Alternatively, the host may be able to restrict virus replication in one specific target cell, but not in other cells. Although the virus may be able to replicate in other cells, the levels of virus replication may be such that the host is able to effectively recognize and eliminate the viral antigens before the virus-induced damage is insurmountable. Several instances of a specific target cell defect have been reported in the literature. Differences in the ability of macrophages, but not other somatic cells, to support virus replication are thought to account for the genetically acquired resistance of mice to in vivo infection with several flaviviruses

(Goodman and Koprowski, 1962), and influenza virus (Lindenmann et al., 1978).

The initial studies undertaken by Buchmeier and Rawls (1977) to establish the basis of the dichotomous response to Pichinde virus infection in MHA and LVG hamsters attempted to determine whether a general target cell defect or a specific macrophage defect could account for the differences in susceptibility between the two hamster strains. However, both primary kidney cells and adherent peritoneal exudate cells derived from the resistant LVG strain supported Pichinde virus replication in vitro to the same extent as did cells derived from susceptible MHA hamsters. In the present studies, it was observed that peritoneal exudate cells from resistant LSH hamsters also supported Pichinde virus growth in vitro (Figure 13). Thus, these studies were unable to detect a target cell difference in the ability of cells derived from resistant or susceptible hamsters to support Pichinde virus replication in vitro.

The inability to detect differences in target cells in kidney or peritoneal exudate cells did not rule out the possibility of a target cell difference in other locations. The studies of Murphy et al. (1977) had suggested that Pichinde virus replicated preferentially in reticuloendothelial cells . Because the spleen is a major reservoir for antigens inoculated intraperitoneally, a search for a target cell difference in the spleens of resistant and susceptible hamsters was begun. Again, however, it was not possible to demonstrate a difference in the ability of spleen cells derived from resistant or susceptible

hamster strains to support Pichinde virus replication in vitro (Figures 13 and 14). This observation may have several explanations. If more than one target cell for Pichinde virus replication is present in the hamster spleen cell population, then a very good separation of spleen cells could be required in order to demonstrate a difference in the ability of one particular target cell population to support Pichinde virus replication in vitro. Secondly, in vitro culture conditions may not sufficiently resemble the in vivo situation to support viral growth. A situation in which one might envision this event occurring is if the target cell was actually an activated NK cell. Since NK activity is labile under normal culture conditions (Table IX), the ability to support virus replication could theoretically be lost. A requirement for lipopolysaccharide activation of B cells has been shown necessary to demonstrate growth of herpes simplex virus in mouse spleen cells in vitro (Kirchner et al., 1976); VSV replicates in mouse spleen cell cultures only after pretreatment with the T cell mitogens phytohemagglutinin or concanavalin A (Nowakowski et al., 1973). These results are consistent with the idea that a target cell for virus growth must exist in an activated state in order to support virus replication. Further attempts to demonstrate a target cell difference in susceptibility to Pichinde virus replication were therefore directed towards the in vivo situation.

Studies on the kinetics of appearance of infectious centres in the spleens of infected hamsters revealed the presence of almost 100 times the number of infectious centres in MHA hamsters compared to LSH hamsters two days after infection (Figure 15). A ten-fold difference

in the number of infectious centres was maintained over the next two days of infection. These observations suggested that the MHA spleen cell population was undergoing an extensive and early productive infection with Pichinde virus. The appearance of infectious centres in the spleen preceded the development of viremia by several days; furthermore, the difference between MHA and LSH hamsters in the number of infectious centres was manifested immediately. This suggests that the high virus titres in the blood later in infection could be a consequence of this early rapid viral multiplication. These results are consistent with the hypothesis that MHA hamster spleen cells contained an additional population of cells, which actively supported Pichinde virus replication.

Other explanations for the apparent paucity of infectious centres in LSH spleens, as compared to MHA spleens could be that target cells in the LSH spleen undergo an abortive or prolonged growth cycle with Pichinde virus, or that the spread of virus to neighbouring cells is somehow limited very early after infection.

The observation that the development of natural killer (NK) cell-mediated cytotoxicity against ^{51}Cr-labelled tumour target cells paralleled the development of infectious centres (Figure 15) raised the possibility that the target cell for Pichinde virus growth was in fact the NK cell. To clarify the nature of the putative MHA spleen target cell for Pichinde virus replication, spleen cell populations were subjected to various fractionation procedures and assayed for infectious centres and NK activity.

As an initial approach, spleen cells from infected hamsters

were treated with carbonyl iron and a magnet, a treatment which removed phagocytic and/or adherent cells and, at the same time, enchanced cytotoxic activity (Figure 7). The carbonyl iron-resistant population of neither MHA nor LSH hamster spleen cells was enriched in infectious centres (Table XIV). However, this data cannot be regarded as conclusive, since carbonyl iron has been shown to exert an inhibitory effect on Pichinde virus replication in both BHK and peritoneal exudate cells (data not presented).

A second approach entailed the separation of spleen cells from Pichinde virus-infected hamsters into adherent and non-adherent cells on plastic dishes (Table XV). The non-adherent fraction accounted for a slightly higher proportion of infectious centres in cells derived from MHA hamsters than from LSH hamsters. Furthermore, considerable cytotoxic activity was manifested by both the adherent and non-adherent MHA fractions, suggesting that only a poor separation of spleen cells had been achieved.

The separation of spleen cells from infected MHA hamsters by velocity sedimentation at unit gravity revealed that two peaks of cytotoxic activity existed, and that these peaks of lytic activity coincided with the profile of infectious centres (Figure 16). LSH hamster spleen cells had markedly lower cytotoxic activity, and the virus was located predominatly in a population of cells which sedimented at a rate typical of macrophages. It is not clear whether the cytotoxicity mediated by the faster sedimenting population is due to macrophages or some other large cell, or rather due to the sticking of NK cells to an adherent population as Welsh has reported (personal communication).

It should be mentioned that no doublets were observed, but these could have been disrupted during the vigorous pipetting prior to counting. Whatever the nature of this cytotoxicity, it is apparent that at least two populations of target cells for Pichinde virus replication are found in MHA hamster spleens, while the target cell for Pichinde virus replication which cosedimented with the cytotoxic activity of smaller cells was reduced in resistant LSH hamsters.

In an effort to increase the resolution of purification of the putative target cell, two techniques were combined. Spleen cells from Pichinde virus-infected hamsters were fractionated into adherent and non-adherent populations and then subjected to velocity sedimentation at unit gravity to further separate cells on the basis of size. This experiment demonstrated the presence of a non-adherent small- to medium-sized cell in MHA hamster spleens which contained ten-fold more infectious centres than the comparable population in LSH spleens; all other fractions were very similar with respect to number of infectious centres (Figure 17, and Table XVI). The medium-sized non-adherent cell population also exhibited the highest levels of cytotoxic activity. These observations support the hypothesis that susceptible MHA hamster spleen cells contain an additional target cell for Pichinde virus replication which the resistant LSH strain lacks, and are consistent with the possibility that the target cell is the NK cell.

4.3. Natural Killer (NK) Cells in Syrian Hamsters

The finding that infectious centre development in the spleens of Pichinde virus-infected hamsters slightly preceded the development of, and

copurified with, a population of cytotoxic effector cells, stimulated interest in the nature of the cytotoxic cell population.

The hamster effector cell was a non-adherent, non-phagocytic, small- to medium-sized cell which did not express surface immunoglobulin (Figures 6,7 and 13; Table XI). Its cytotoxic activity was rapidly lost in vitro at 37°C (Table IX) but was not abrogated by pretreatment with ammonium chloride (Table X). In these parameters, then, the hamster effector cell closely resembled the mouse NK cell (Herberman and Holden, 1978) and seemed to be typical of NK cells described in other species. It is not known whether the hamster NK cell expresses a theta-like antigen or an Fc receptor on its surface, markers which are present in low levels in mouse and human NK cells (Herberman et al., 1977; Herberman et al., 1978; Saksela et al., 1979).

Since these studies were carried out, work has been published which confirms the existence of NK cells in hamsters (Datta et al., 1979a, b). Endogenous cytotoxicity in LSH and outbred hamsters against a simian adenovirus-induced LSH tumour cell line was mediated by a non-adherent, radiation- and ammonium chloride-resistant cell (Datta and Trentin, 1979; Datta et al., 1979a,b). This hamster cell differed from the mouse NK cell in that high levels of NK activity were apparent in both the bone marrow and the spleen; however, as in the mouse, intermediate activity was observed in the mesenteric lymph nodes, and the thymus demonstrated little reactivity. In the present studies, cytotoxic activity has also been demonstrated among peritoneal exudate cells and in cells from the popliteal lymph node (Table XII).

Some studies on the mechanism of hamster NK cell-mediated cytolysis were carried out. The interaction between hamster NK effector cells and targets was inhibited by the presence of EDTA (Table VI), suggesting that divalent cations were required for a productive interaction between the effector and the target. A requirement for divalent cations has also been demonstrated by Roder et al. (1978) and Kiessling and Wigzell (1979) in the mouse NK cell system. Unlike the rapid lysis observed with mouse NK effectors (Welsh, 1978a), hamster NK cell-mediated cytotoxicity continued to increase throughout the incubation period (Figure 4). This was comparable to human NK-mediated cytotoxicity, which was found to increase at a linear rate for at least 18-20 hrs (Santoli and Koprowski, 1979). Furthermore, the addition of EDTA to stop the interaction of hamster NK cells with targets, followed by a second incubation, did not increase the amount of radioactive label released (Table VII). This suggests that the need for a long incubation period did not reflect delayed release of the label from damaged target cells.

The amount of lysis was a function of the number of effector cells present; increasing effector-to-target cell ratios induced proportional increases in specific lysis up to a plateau maximum value. Furthermore, mixing spleen cell preparations containing high NK activity with spleen preparations of low NK activity gave an amount of lysis that was directly related to the number of NK cells present in the mixture (Figure 5). Datta et al. (1979b) have reported that lysis can be inhibited by the presence of unlabelled target cells, in a

dose-dependent fashion. These results have been confirmed using MAD targets (data not presented). The observations suggest that lysis of target cells is attributable to a cellular function.

The injection of Pichinde virus-immune antisera into control or Pichinde virus-infected hamsters did not increase splenic cytotoxic activity above levels observed in recipients of normal hamster serum (Table XI). Although antibody-dependent cell-mediated cytotoxicity (ADCC) can be easily demonstrated by hamster peritoneal exudate cells against herpes simplex virus-infected BHK target cells in the presence of anti-herpesvirus antibodies, no lysis of Pichinde virus-infected BHK targets in the presence of immune sera and the same peritoneal exudate cells has been observed (data not presented). In addition, the infection of target cells with Pichinde virus did not alter their susceptibility to lysis by spleen effectors from control or Pichinde virus-infected animals (Figure 3). These observations suggest that the lysis of target cells by Pichinde virus-induced cytotoxic cells is not attributable to an ADCC-like mechanism.

Hamster NK cells were able to kill syngeneic and allogeneic tumour target cells, continuous cell lines and embryo fibroblasts (Figure 9), although with differing efficiencies. Datta and Trentin (1979b) have also demonstrated lysis of a xenogeneic cell line, human chronic myelogenous leukemia K562 cells, by hamster effector cells. Thus, hamster NK cell activity was not restricted to histocompatible targets. Except in the case of VSV, virus infection of the target cells did not alter their susceptibility to lysis (Figure 10). These observations

are comparable to the findings of Welsh et al. (1979), who demonstrated that mouse NK cells induced by LCMV or vaccinia virus were able to kill syngeneic, allogeneic and xenogeneic continuous and early passage cell lines, and that LCMV-induced NK cells apparently did not recognize LCMV antigens on the surface of infected target cells.

The infection of MAD targets with VSV augmented the specific ^{51}Cr release values elicited by the presence of hamster NK cells; no other viruses had this effect (Figure 10). In this context, the suggestion that 70-90% of the NK activity seen in the usual 18 hr assay is due to the generation of interferon released into the supernate, which subsequently induces NK activity, may be relevant (Trinchieri and Santoli, 1978; Trinchieri et al., 1978; Santoli, 1978). VSV is a particularly potent inducer of interferon; thus, NK hamster cells may be undergoing activation during the assay. An alternative explanation is that VSV infection of the target cell may render it more susceptible to lysis.

As has been discussed in the introduction, many agents, including viruses, certain bacteria, tumour cells, interferon, and interferon-inducing agents, have been shown to augment mouse or rat NK activity in vivo (Welsh, 1978; Herberman and Holden, 1978; Herberman et al., 1979; Oehler et al., 1978). The present studies on hamster NK activity have shown that both Pichinde virus and herpes simplex virus are able to augment activity in vivo (Figure 8). No inducing effect could be attributed to vaccinia virus after 3 days of infection. In addition, Datta et al. (1979b) have pointed out that the challenge of inbred LSH

or random-bred hamsters with syngeneic or allogeneic tumour cells elicited an augmented NK response soon after the challenge. Thus, tumour cells, Pichinde virus, and herpes simplex virus are agents which are able to augment NK activity in Syrian hamsters.

High NK activity is a dominant trait in mice (Kiessling et al., 1976) and appears to be controlled by multiple genes, including an H-2 linked component (Kiessling et al., 1975; Petranyi et al., 1976). However, high NK activity in the rat acts as a recessive phenotype (Oehler et al., 1978b), and no linkage of genes controlling NK activity to loci within the major histocompabibility complex of the rat could be demonstrated. In a preliminary experiment, individual F (LVG X MHA) progeny hamsters were assayed for NK cytotoxic activity following Pichinde virus infection (Table XI). The results suggested that low levels of NK activity were expressed in these F_1 (LSH X MHA) progeny; however, the experiment must be repeated using larger numbers, and the values obtained from F_1 spleens should be compared to a range given by spleens of individual parents rather than data obtained from pooled spleen cells. If the results are correct, then the inheritance of NK activity in the hamster resembles the situation in rats rather than in mice. The observation that both F_1 females had lower NK activity than their male littermates may be related to the observation that β-estradiol suppresses NK activity (Seaman et al., 1978).

NK cells have been alleged to play a role in immunosurveillance against tumour formation (Baldwin, 1977). The observation that Pichinde virus infection augmented NK activity to a greater extent in

MHA hamsters than in LSH hamsters was construed as an indication that MHA hamsters might actually be more resistant to tumour induction than the LSH strain. This appeared to be true; although no significant difference was observed in the induction of tumours in neonates by injection of adenovirus type 12 (Figure 11), a difference was demonstrable in the increased resistance of MHA adults to challenge with syngeneic or allogeneic adenovirus-induced tumour cells compared to LSH hamsters (Figure 12). The inability to demonstrate a difference in induction of tumours by adenovirus type 12 in neonates may be because the numbers tested were so small. An alternative possibility is that NK cell activity is inadequately expressed in neonates; NK activity in hamsters appears to increase with age (Datta et al., 1979a,b). It has been reported that the challenge of adult hamsters with syngeneic or allogeneic tumour cells induced an increase in NK activity early after injection of the tumour cells (Datta et al., 1979b). Once the tumours had attained a large mass in their hamster recipients, the NK activity appeared to be suppressed. This reduction in activity may be directly related to growth of the tumour (Datta et al., 1979b).

4.4. The Role of NK Cells in the Susceptibility of Syrian Hamsters To Lethal Pichinde Virus Infections

The demonstration that the spleen cell population which contained infectious centres of Pichinde virus increased and co-purified with NK activity raised the possibility that a target cell for Pichinde virus replication was the NK cell. Other

evidence presented in this thesis supports this conjecture. Endogenous NK activity was usually higher in the MHA strain of hamsters than in LSH animals. Pichinde virus infection augmented NK activity in both strains, but to a greater extent in MHA than in LSH hamsters. Kirchner et al. (1976) have reported that herpes simplex virus, like other members of the herpesvirus group, induces B cell proliferation. Since treatment of mouse spleen cells with B cell mitogens is required to render the cells susceptible to herpes simplex virus growth in vitro, the observation suggested that the virus was able to induce a target cell for its growth. A similar situation may underlie the preferential Pichinde virus-induced augmentation of NK activity in MHA spleen cells, and the subsequent rapid virus growth.

The current studies have provided evidence that survival and the ability to limit viremia are each dominant phenotypes controlled by either a single or linked autosomal gene(s) (Tables I and II). If the NK cell is actually a target cell for virus growth, then the genetics of susceptibility to the fatal virus infection predicts that low NK activity will be a dominant trait. This prediction has been borne out in preliminary experiments on the genetic control of NK activity in Syrian hamsters (Table XIII).

Data from several other studies are consistent with the hypothesis that the hamster NK cell may be a target for Pichinde virus replication. Stephen et al. (1977) have studied the effect of poly(ICLC) treatment on the infection of Rhesus monkeys by Machupo virus. Like Pichinde virus, Machupo virus is a member of the arenaviridiae. Monkeys treated

early in infection with the interferon inducer poly(I)·poly(C) stabilized with carboxymethyl cellulose and poly-l-lysine [poly(ICLC)] developed significantly higher titres of virus in the blood than animals not given poly(ICLC). Since interferon can induce NK activity both in vivo (Gidlund et al., 1978; Oehler et al., 1978) and in vitro (Santoli et al., 1978; Trinchieri and Santoni, 1978; Einhorn et al., 1978), the effect of poly(ICLC) on Machupo virus infection of monkeys is consistent with the hypothesis that new targets for arenavirus replication are elicited by the drug and that these new targest may be NK cells.

In a similar vein, nude mice have been shown to have higher titres of interferon in their spleens and serum following infection with the arenavirus, lymphocytic choriomeningitis virus (LCMV) than do normal mice; this was accompanied by higher titres of virus in the tissues of nude mice (Merigan et al., 1977). Furthermore, the administration of anti-interferon antibodies inhibited the liver cell necrosis and death that is observed in suckling mice which have been infected with LCMV (Riviere et al., 1977). Mice which have been congenitally infected with LCMV, a condition which converts the mice into chronic carriers of the virus, have been shown to have reduced interferon titres (Holtermann and Havell, 1970). Finally, Popescu et al. (1979) have shown that these carrier mice have infectious centres of LCMV in splenic and peripheral blood lymphocytes. It is interesting that murine lymphocytes are relatively resistant to infection with LCMV in vitro (quoted in Popescu et al., 1979);

activation induced in vitro with T cell mitogen PHA or by incubation with allogeneic cells in mixed lymphocyte culture reversed this resistance, resulting in high levels of LCMV replication (Eustatia and van der Veen, 1971). NK cell activity has been demonstrated to arise in mixed lymphocyte cultures (Jondal and Targan, 1978). Taken together, these observations are consistent with the possibility that natural killer cells represent a target cell for the replication of certain arenaviruses.

4.5. Influence of the Foot-pad Route of Injection of Pichinde Virus on Survival of MHA Hamsters

The observation that the usually susceptible MHA hamsters are able to limit Pichinde virus replication and consequently survive the injection when the virus is given by the foot-pad route (Table IV) requires explanation. It is well known that the route of virus injection can be very important in determining the outcome of the infection (Fenner, 1973). Several explanations for this effect may be considered. The rapid development of the host's immune responses may be crucial to survival when the animal is dealing with a replicating agent such as a virus. If the virus has initial access to a cell which supports its growth, the heavy virus load could render the host's defense mechanisms inadequate. Alternatively, an inappropriate host response can develop as a consequence of the route of exposure (Ogra et al., 1968; Tomasi and Bienenstock, 1968), leading to an inability to effectively clear the virus. The sequestering of a replicating antigen

at a distance from organs involved in the generation of either non-specific defenses or the specific immune response could also delay, or reduce, the manifestations or immunity (Webster, 1965, 1968).

The basis for survival of MHA hamsters following foot-pad injection of Pichinde virus may be understood in the context of the hypothesis that a spleen cell, possibly the NK cell , is a primary target for Pichinde virus replication. Foot-pad injected MHA hamsters showed a delayed rise in splenic NK activity (Table XVI) compared to hamsters inoculated by the intraperitoneal route (Figure 3). Most importantly, the development of infectious centres in the spleen was also delayed (Table XVI; Figure 15), and no infectious centres were detectable eight days after a foot-pad injection of Pichinde virus. Thus, this route of injection appeared to delay the spread of virus sufficiently to permit the hamster's immune defense mechanisms to clear the relatively low virus load. In contrast, when Pichinde virus is inoculated intraperitoneally, the spleen is seeded with virus early in infection. The hamster spleen appears to be a major reservoir of NK cells (Table XII; Datta et al., 1979b). The route of injection of agents which augment NK activity is known to be important in determining the magnitude and location of the increase in NK activity (Ojo et al., 1978); thus, intraperitoneal administration of Pichinde virus may preferentially induce splenic NK activity. If the hypothesis is correct that Pichinde virus replicates in NK cells, then the early proliferation of Pichinde virus within the numerous spleen NK cells may result in an overwhelming amount of virus against which the host's

defense mechanisms are inadequate. When, however, Pichinde virus in inoculated into the foot-pad, the retarded virus growth that is a consequence of the paucity of NK cells in this location (Datta et al., 1979b) may allow the host's immune defenses to win the race.

This conjecture rests on the implicit assumption that the MHA hamster is able to mount an appropriate immune response against Pichinde virus, provided that the virus is administered in such a way as to delay its early replication. Some justification for this viewpoint is provided by the work of Buchmeier and Rawls (1976), and by the current studies. Antibody production against Pichinde virus antigens appeared to be similar in both the resistant and susceptible strains of hamsters (Buchmeier and Rawls, 1976) and passive transfer of antibody did not alter the development of infectious centres or NK activity in the spleen (Table VIII). The fact that resistance to lethal Pichinde virus infection in LVG hamsters developed postnatally, together with the finding that cyclophosphamide abrogates their age-acquired resistance (Buchmeier and Rawls, 1976), suggested that immunity does have a protective role in Pichinde virus infection. In addition to the observation that MHA hamsters were able to limit Pichinde virus replication and survive the infection when the virus is inoculated into the foot-pad, (Table IV) it was also shown that a foot-pad injection of virus protected MHA hamsters against a normally lethal intraperitoneal challenge of Pichinde virus (Table V). Taken together, these observations suggest that the normally susceptible MHA strain of hamsters is capable of generating a protective immune response.

Both LSH and LVG hamsters responded to a foot-pad inoculation of Pichinde virus with swelling at 8 days after injection. These results are in agreement with those reported by Zinkernagel et al. (1978), who demonstrated that a foot-pad inoculation of vaccinia virus or lymphocytic choriomeningitis virus was capable of eliciting a swelling response in hamster foot-pads. The observed infiltration of lymphocytes and macrophages into the foot-pads of LVG and LSH hamsters which had received a foot-pad injection of virus (Table III) is typical of delayed-type hypersensitivity, a manifestation of cell-mediated immunity. In contrast to these observations, the MHA strain failed to show foot-pad swelling after a foot-pad injection of Pichinde virus (Figure 2), and lacked evidence of a mononuclear cell infiltrate at the site of injection (Table III).

The development of the foot-pad swelling response requires both the immune recognition of the injected antigen and the production of soluble factors and sensitized cells which induce the mononuclear cell infiltrate and edema. One explanation for the lack of foot-pad swelling in MHA hamsters, then, is that this strain is defective in the recognition of Pichinde virus antigens. However, the observation that MHA hamsters which received a foot-pad inoculation of Pichinde virus did produce complement-fixing anti-viral antibodies (Table IV), and furthermore, were protected against a subsequent intraperitoneal challenge of Pichinde virus (data not presented), argues that recognition of Pichinde virus antigens has occurred. In addition, MHA hamsters did not respond to a foot-pad challenge of herpes simplex virus (data

not presented). Thus, the defect in the foot-pad swelling response of MHA hamsters did not appear to be virus-specific, and as such, was unlikely to be at the level of the T lymphocyte. Nevertheless, the possibility that a defect exists in T lymphocyte recognition of a specific Pichinde virus antigen has not been excluded.

An alternate possibility which accounts for the lack of foot-pad swelling in the MHA strain is that these hamsters are unable to generate the effector mechanisms which are responsible for the components of the foot-pad swelling response. Thus, MHA hamsters may be unable to produce a lymphokine or monokine which is necessary for the infiltration of mononuclear cells and/or edema. An alternate explanation was suggested by the observation that the popliteal lymph nodes draining the foot-pad became enlarged in MHA hamsters within 3 to 5 days of a foot-pad injection of Pichinde virus (data not presented), and the lymph node cells demonstrated cytotoxic activity against MAD targets (Table XVII). De Maeyer (1976) has reported that interferon, an inducer of NK activity, strongly inhibited the foot-pad swelling associated with the delayed-type hypersensitivity response to Newcastle disease virus in mice. Thus, interferon production in the draining lymph node of MHA hamsters may account for the lack of foot-pad swelling. If this suggestion is true, then it is predicted that LSH hamsters will not show as marked an elevation of NK activity as do MHA hamsters in the popliteal lymph nodes subsequent to a foot-pad injection of Pichinde virus.

The cellular component of the footpad swelling response must also be considered. For example, a defect in trafficking of lymphocytes

to the foot-pad could account for the observed lack of swelling in the MHA strain. An alternate hypothesis which explains the difference in the foot-pad swelling response to Pichinde virus is that foot-pad swelling in LSH hamsters is due to an infiltration of mononuclear cells which suppress NK activity. Some support for this idea of a suppressor cell comes from the observation that carbonyl iron treatment of LSH spleen cells markedly enhanced cytotoxic activity (Figure 7). This suggested that the LSH spleen cell population did contain NK cells, but that the NK activity was suppressed by a carbonyl iron-sensitive cell, possibly a macrophage. This possibility is consistent with reports from other laboratories that a phagocytic and/or adherent suppressor cell inhibits NK activity. Spina and Hofman (1979) have shown that an adherent esterase-positive cell suppressed human NK activity in vitro. A mouse suppressor cell, which was induced by carrageenan administered in vivo and in vitro, has been shown to inhibit NK activity; the suppressor cell was removed by carbonyl iron pretreatment, and less efficiently by adherence to plastic (Cudkowicz and Hochman, 1979). These studies thus support the concept of an adherent, macrophage-like cell, which is able to suppress NK activity, and raise the possibility that the lack of foot-pad swelling in MHA hamsters may be due to a lack of suppressor cells in this strain. At the present time, it is not possible to distinguish among these alternate explanations for the lack of swelling that is observed in MHA hamsters after a foot-pad injection of Pichinde virus. Further work is required to resolve the problem.

4.6. Statement of the Model and Proposals for Further Work

The available data are consistent with the hypothesis that a target cell, defined by its NK activity, provides an additional target cell for Pichinde virus replication in MHA hamsters, leading to an overwhelming virus load and subsequent host death. In contrast, the relative absence of this target cell in LSH hamsters results in low levels of virus replication, which the host's immune defense mechanisms are able to clear.

It has already been pointed out that the presence of a suppressor cell, which inhibits NK activity in LSH hamsters but not in MHA hamsters, could account for the observed differences in foot-pad swelling. Such a cell could also be responsible for the relatively low NK activity observed in LSH hamster spleens, thereby functionally removing a potential target cell for Pichinde virus replication. This theory imposes a restriction on the hypothesis; if the target cell for Pichinde virus replication is indeed the NK cell, then the hypothesis must be extended to say that the target is the activated NK cell. This hypothesis would be compatible with the observed difficulty in demonstrating a spleen target cell difference *in vitro* (Figures 13 and 14), and is consistent with the effects of an interferon or interferon inducers on arenavirus replication in monkeys (Stephen *et al.*, 1978) and in mice (Riviere *et al.*, 1977). The absence of suppressor cells, a trait which could be acquired genetically or through inactivation by Pichinde virus infection, would then lead to the phenotype of augmented NK activity. When the site of primary replication is the

spleen, the numerous NK cells that reside at this location are subjected to an early and overwhelming virus proliferation.

Other mechanisms which may account for the relative lack of LSH splenic NK activity can also be postulated. LSH hamsters may lack a T helper cell for NK activity. Alternatively, the LSH strain may be defective in production of a monokine or lymphokine, such as interferon, which is required for the activation of NK cells. Mechanisms for trafficking of the NK cell could differ in the two strains of hamsters. At the present time, no information is available which allows us to distinguish between these possibilities.

Further support for the hypothesis that the hamster NK cell represents a target cell for Pichinde virus replication awaits experimentation. Genetic studies could determine whether a correlation exists in individual test-cross progeny between viremia and infectious centres of Pichinde virus and levels of NK activity; a lack of correlation would negate the hypothesis. The deletion of NK activity in vivo by agents such as ^{89}Sr or anti-NK cell antiserum would be expected to result in a loss of the putative target cell for Pichinde virus replication, thereby reducing viremia and ultimately resulting in host survival. Alternatively, agents which increase NK activity such as interferon or interferon inducers should render the animals more susceptible to the lethal Pichinde virus infection. The adoptive transfers of spleen cells or bone marrow cells into irradiated recipients should confer the donor's phenotype of NK activity/survival after Pichinde virus infection on the recipient. The survival of MHA

hamsters that is observed following a foot-pad injection of Pichinde virus suggests that the spleen, as a major source of NK cells, acts as a reservoir for infectious centres of Pichinde virus. Therefore, splenectomy of MHA hamsters, followed by an immediate intraperitoneal injection of Pichinde virus, should permit the MHA hamsters to survive the normally lethal challenge of virus.

Alternate explanations for the susceptibility of MHA hamsters to fatal Pichinde virus infections must also be considered. The NK cell activity may be simply a marker for other perturbations in the network of host responses to the virus infection. For example, further studies on cell-mediated immunity in MHA hamsters could reveal defects in this branch of their immune response. Non-specific defenses, such as interferon, may also be different in the two strains of hamsters. An early target for Pichinde virus replication in LSH hamsters which is able to abort or delay the virus growth cycle, could tip the balance sufficiently so that the immune response is adequate. Another possibility is that the kinetics of the immune response is under genetic control, and is delayed in MHA hamsters. When a replicating antigen such as a virus is challenging the host, a delay of one or two days in generating the host's defense mechanisms could mean the difference between survival and death.

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APPENDIX

Data from 35 separate experiments in which spleen cells from control or Pichinde virus-infected MHA and LSH hamsters were assayed for cytotoxic activity against ^{51}Cr-labelled MAD targets were analyzed statistically. A mean percent specific ^{51}Cr release value was estimated for each group by summing the actual percent release values obtained for effector-to-target cell ratios of 100:1, 50:1 and 25:1 in all 35 experiments, and dividing by the total number (n) of values. These mean values are summarized below:

Strain	Pichinde virus	Mean % specific ^{51}Cr release	n	SD	SEM $(\frac{SD}{\sqrt{n}})$
LSH	no	4.23	63	9.38	1.18
LSH	yes	17.13	66	15.07	1.85
MHA	no	12.04	80	9.95	1.11
MHA	yes	43.05	85	19.97	2.17

The difference in endogenous levels of cytotoxic activity in uninfected MHA and LSH hamsters was statistically significant by Student's t test ($T' = 3.77$; $p<.0005$). Similarily, Pichinde virus infection induced an augmented response in both MHA hamsters ($T' = 12.51$; $p<.0005$) and LSH hamsters ($T' = 5.80$; $p<.0005$). The magnitude of the augmented response in MHA hamsters was significantly greater than that observed in LSH hamsters ($T' = 7.49$; $p<.0005$).