THE RELATIONSHIP BETWEEN LYMPHOCYTE RESPONSIVENESS TO LYMPHOKINES AND SUSCEPTIBILITY TO VIRAL INFECTION IN SYRIAN HAMSTERS

by

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ABSTRACT

Two inbred strains of Syrian hamster have been shown to display genetically determined differences in resistance to infections with the arenavirus, Pichinde virus (PV). After intraperitoneal injection, the virus grows to higher titres in the spleens of the susceptible strain, MHA, than in the spleens of the resistant strain, LSH. Preliminary studies examining the basis of susceptibility demonstrated that resistance or susceptibility to the virus did not lie in an inherent difference in target cells to become infected, but suggested that there was a quantitative difference in target cells between the two strains of hamster. The following experiments were conducted in attempts to verify the hypothesis that MHA hamsters are susceptible to infection with Pichinde virus because they possess larger numbers of a splenic lymphocyte that serves as a target cell for virus replication and that also functions as an effector cell of nonspecific cytotoxicity.

The spleens and thymi of the high NK strain were found to display greater cellularity than those of the low NK strain. Additionally, thymocytes from MHA hamsters were found to proliferate to a greater extent than those of LSH hamsters in response to ConA-induced conditioned medium or purified interleukin 2 plus

mitogen. As well, splenocytes from MHA hamsters showed high levels of lymphokine-activated killer cell (LAK) activity after culture in conditioned medium or interleukin 2. In both the thymus and the spleen, this difference in responsiveness was due to increased numbers of precursor cells responding to lymphokines in MHA organs . When lymphokine production was assessed, it was found that cells from the the high responder, MHA, synthesized less interleukin 2 than cells from LSH hamsters. Interleukin 1 production was equal in the two strains. These results led to the hypothesis that the susceptible hamsters contain immature lymphocytes, possibly because of the reduced interleukin 2 production. Increased numbers of relatively immature cells could account for the increased cellularity of lymphoid organs in these animals, and in the spleen, these cells may be responsible for increased NK activity, increased numbers of LAK precursors, and serve as target cells for PV replication.

Splenic cytotoxic cells in the hamster were characterized. Endogenous NK cells, virus-induced NK cells and LAK were all plastic nonadherent cells, as were the precursors for LAK. All populations expressed an antigen homologous to the murine Thy 1.2. Endogenous NK cells and virus-induced NK cells were similar in the expression of an asialo GM1 homologue; both were reduced

by 50% by treatment with this antiserum plus complement. LAK precursors and LAK effectors were negative for this marker, suggesting that LAK arise from the asialo GM1 negative component of endogenous NK activity. Treatment of hamsters with anti-asialo GM1 serum also reduced splenic NK activity in normal and virus-infected hamsters by 50%.

Cells infected with virus were characterized using the same criteria. A preferential infection of nonadherent cells was not evident before day 3 of infection, although MHA spleens already contained twice as much virus as LSH spleens. Both asialo GM1 negative and positive cells were infected. Culture of infected splenocytes in interleukin 2 induced high LAK, but failed to select out a population enriched for infectious centres compared to culture in medium alone, where no cytotoxic activity was evident. However, susceptible MHA hamsters with reduced NK activity after treatment with anti-asialo GM1 serum did display less virus at day 1 after infection with PV, but by day 3, virus loads were at control levels. Treatment with anti-asialo GM1 serum had no effect on the mortality of either strain of hamster. Treatment with purified interleukin 2 slowed mortality of MHA hamsters, although it did not do so by reducing viral replication.

In total, these data indicate that cells expressing an antigen detected by anti-asialo GM1 serum that mediate some NK activity can serve as target cells for PV replication, but these cells alone are not responsible for the susceptibility of MHA hamsters. Cytotoxic activity and infected centres could be dissociated, also suggesting that the initial hypothesis was incorrect. It could be that some other cell is the relevant target, that MHA hamsters may be susceptible because of increased numbers of all splenocytes, or that some factor other than the presence or absence of a target cell accounts for their susceptibility.

THE HAMSTER

There is not much about the hamster
To stimulate the epigramster.
The essence of his simple story,
He populates the laboratory,
Then leaves his offspring in the lurch,
Martyrs to medical research.
Was he as bright as people am,
New York would be New Hamsterdam.

Ogden Nash

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AHF	Argentinian hemorrhagic fever
B cell	bone marrow-derived lymphocyte
BHF	Bolivian hemorrhagic fever
BM	bone marrow
BHK	baby hamster kidney cells
C'	complement
CM	conditioned medium
CMV	cytomegalovirus
ConA	Concanavalin A
cpm	counts per minute
CTL	cytotoxic T lymphocyte
DTH	delayed type hypersensitivity
F-15 MEM	F-15 formula of Eagles' Minimal Essential Medium supplemented as described in Materials and Methods
FBS	fetal bovine serum
HBSS	Hanks' balanced salts solution
HEPES	N-2-hydroxyethyl piperazine-N-2'-ethane sulfonic acid
HSV	herpes simplex virus
IC	infectious centre
IL1	interleukin 1
IL2	interleukin 2
i.p.	intraperitoneal(ly)
i.v.	intravenously

JV	Junin virus
LAK	lymhokine activated killer cells
LCMV	lymphocytic choriomeningitis virus
LN	lymph node
LSH	inbred strain of Syrian hamster, originally bred at the London School of Hygiene
MCMV	murine cytomegalovirus
MDV	Marek's disease virus
MHA	inbred strain of Syrian hamster originally bred at Mill Hill (Mill Hill Albino)
MHC	major histocompatiability complex
MHV	murine hepatitis virus
MOI	multiplicity of infection
NDV	Newcastle disease virus
NK	natural killer cell
PEC	peritoneal exudate cells
pfu	plaque forming unit
PV	Pichinde virus
SD	standard deviation
SEM	standard error of the mean
SFFV	spleen focus forming virus
T cell	thymus-derived lymphocyte
VSV	vesicular stomatitis virus

CHAPTER ONE

INTRODUCTION

I. Viral Pathogenesis

A. General features of viral pathogenesis

To date, over 500 viruses have been described that infect warm-blooded vertebrates (Fenner et al 1974), but only a small proportion of these are pathogenic to humans. The development of disease proceeds through several stages and at each, multiple viral and host factors may facilitate or prevent the establishment of infection or the progression to disease.

The first step in the process is entry of the virus into the host. The epithelium of the skin is impenetrable to most viruses, but entry can be achieved through cuts, insect bites or hair follicles. The local environment may also be inhibitory to the virus, for example, many viruses can not withstand the low temperature of nasal mucosal surfaces, or the acidity of the stomach. Most viruses have specific attachment sites to target cells and many gain entry through binding to receptors on respiratory or intestinal epithelial cells. In some cases, infectivity requires the presence of specific host enzymes which act on viral glycoproteins to allow binding (Scheid and Choppin 1984).

The second stage of infection is replication of the virus at the site of entry. This depends on the presence

of appropriate target cells. Once initial replication has occurred, the organism spreads either locally or to distal sites. Viruses spread by three routes; through the extracellular fluid, through cell-to-cell contact or passage from parent to daughter cells. Dissemination to distant sites occurs through the lymphatic system, or blood. Infected cells, particularly monocytes and lymphocytes, may be important in the spread of viruses throughout the body (Mims 1964, Sharpe and Fields 1985).

At all stages, nonspecific and later, specific, defenses are encountered. First-line defense mechanisms prevent entry into deeper tissues or prevent colonization of body surfaces, and these include factors such as low pH, proteolytic and digestive enzymes, mucus, cross-reactive IgA in secretions and competition by normal flora (Sherris and Ray 1984). Second-line defenses are those encountered once the virus has penetrated into the host and include polymorphonuclear neutrophils, blood monocytes and fixed tissue macrophages. The antiviral activity of these cells has been divided into intrinsic activity, which concerns the permissiveness or nonpermissiveness of cells, usually macrophages, for virus replication, and extrinsic resistance, which is defined as the ability of cells to inactivate extracellular virus or to reduce replication in adjacent cells (Morahan et al 1985). Most nonpathogenic viruses are phagocytosed, destroyed intracellularly and

cleared in the liver (Mims 1964). The ability to replicate within macrophages is a mechanism whereby many viruses evade host resistance, and disseminate within the host (Mims 1964, Morahan et al 1985). Examples of this are ectromelia virus in mice (Mims 1964), MHV in some strains of mice (Bang and Warwick 1960), lentiviruses in sheep (Gendelman et al 1984), certain of the flaviviruses (van de Groen et al 1976) and arenaviruses (Murphy et al 1977). Mechanisms of extrinsic resistance include phagocytosis, release of proteolytic enzymes or reactive oxygen metabolites (MacGregor et al 1980, Morahan et al 1985), and synthesis of regulatory molecules that induce inflammatory reactions or specific immune responses (Morahan et al 1985). Macrophages may also interfere with viral attachment to target cells, deplete nutrients required for viral replication, or lyse virus-infected targets (Morahan et al 1985, Mak et al 1982, Hirsch et al 1979). These activities inhibit infection or disease in several animals models; murine hepatitis virus (MHV) (Stohlman et al 1982), murine cytomegalovirus (MCMV) (Selgrade and Osborne 1974), influenza and Sendai viruses (Mak et al 1982), herpes simplex virus (HSV) (Mogensen 1977, Hirsch et al 1979) and vaccinia virus (Chapes and Tompkins 1979).

Extracellular molecules can also reduce viral replication. The interferons are one of the earliest elicited responses in the host. Not all viruses induce

interferon production to the same degree, and not all viruses are equally sensitive to the antiviral effects of interferons (Morahan et al 1985, Rager-Zisman and Bloom 1985). Interferons are very potent inducers of an antiviral state, so even circulating interferon may be protective to cells distant from the site of initial virus replication (Preble and Friedman 1983). Interferons have effects other than direct antiviral activity. They induce the release of lymphokines and reactive metabolites from macrophages, increase opsonization (Rager-Zisman and Bloom 1985), and increase macrophage and natural killer cell (NK) lysis of infected targets in vitro (Stanwick et al 1980, Trinchieri and Santoli 1978), and increase NK activity in vivo (Welsh 1978a, Gidlund et al 1978). As well, interferons potentiate specific immune responses through their effects on antigen presentation and antibody production (Rager-Zisman and Bloom 1985). Interferon production correlates with and is thought to be responsible for a decrease in virus titres or resistance to disease in murine infections with herpes simplex virus type 1 (HSV-1) (Zawatsky et al 1979, 1981), MCMV (Grundy(Chalmer) et al 1982), picornaviruses and rhabdoviruses (Gresser et al 1976).

Other serum factors also participate in viral infections. Complement can be activated in the absence of antibody by viruses (retroviruses, Sindbis and Newcastle

disease virus (NDV)) or by viral proteins on the surface of infected cells (Cooper 1984), resulting in the lysis of enveloped viruses and infected cells, increased phagocytosis, and release of biologically active molecules that promote inflammatory reactions. Systemic effects of inflammatory reactions, such as fever, may also influence viral replication (Sherris and Ray 1984). Coating of virus particles with complement proteins may also prevent attachment to target cells.

Natural killer cells, a heterogeneous population of lymphoid cells that lyse some tumour target cells and virally infected targets without prior sensitization, may also play a role in initial resistance. In mice, infection with many viruses, including lymphocytic choriomeningitis virus (LCMV), MCMV, adenovirus, MHV, NDV and other viruses boosts endogenous splenic NK activity at a time when specific T cell responses are not yet detectable (reviewed by Welsh 1981). In many instances, this increased activity is associated with serum interferon produced as a result of infection (Welsh 1978a, 1978b, Gidlund et al 1978). NK activity can also be rapidly boosted by exposure of lymphocytes to viral glycoproteins (Biron and Welsh 1982, Casali et al 1981). Recently, an interferon-independent NK-type activity that is detected in the lung only 4 hours after injection of infected target cells has also been described (Biron et al 1984).

Specific immune responses develop only after infection has been established, and may or may not be responsible for limiting further virus replication, although they are clearly responsible for protection against subsequent infections with the same virus. Cellular responses are demonstrable earlier than humoral responses (Zinkernagel and Doherty 1979, Greenspan et al 1983, Sharpe and Fields 1985). The significance of T lymphocyte responses in primary infections varies from virus to virus. These responses are thought to be more significant in infections with viruses that spread cell-to-cell or insert their antigens into the cellular membrane (Doherty 1985). T cell deficient nude mice survive initial infections with some viruses (Sharpe and Fields 1985, Zawatsky et al 1979) but not others (Bancroft et al 1981). For those viruses where T cell responses appear to be important, two responses have been implicated. These include cytotoxic responses (CTL) restricted by the major histocompatability complex (MHC) and reactions mediated by delayed-type hypersensitivity cells (DTH). More than one subset of T cells is likely to participate in viral resistance, either by direct lysis of virally infected cells, by induction of DTH and activation of macrophages, or by release of other antiviral factors, such as interferon (Greenspan et al 1983).

Humoral antibody develops later than cell-mediated immunity and is probably most effective against viruses that spread through extracellular fluid. Neutralizing antibodies of IgM, IgA and IgG classes are thought to be most significant in limiting virus. IgG is best induced if viraemia occurs during infection (Dimmock 1984), and is the predominant serum antibody. IgA is locally produced at mucosal surfaces (Mandel 1984). There are many mechanisms whereby antibodies can neutralize virus. They can prevent attachment of virus to target cells through steric hindrance (Della-Porter and Westaway 1978), prevent penetration of the cell, or uncoating of the virion once in the cell (Mandel 1984, Dimmock 1984). Neutralizing antibody bound to viral envelopes or protein shells may also inactivate viral enzymes essential for replication through transmembrane signals (Dimmock 1984). Neutralizing antibodies are induced by every virus examined to date with the exception of African swine fever virus, Aleutian mink disease virus and some of the arenaviruses, and in almost every case, presence of the antibody is protective against further challenge with the virus (Dimmock 1984). Non-neutralizing antibody also contributes to antiviral responses. Sensitizing antibodies, that can enhance neutralization, can be present (Greenspan et al 1983, Lachmann 1985). Antibody and complement might result in lysis of enveloped viruses and infected cells.

Non-neutralizing and/or complement-fixing antibodies also increase phagocytosis and participate in antibody-dependent-cell-mediated cytotoxicity (ADCC) where lymphoid killer cells (K) or macrophages lyse targets coated with specific antibody. This type of response has been demonstrated in HSV-infected mice (Kohl and Soo 1982) and in Coxsackie B infections (Rager-Zisman and Allison 1973).

Although one usually conceives of the immune response facilitating the elimination of the infectious agent, there are many viruses that use the host response to their advantage, or block the response. Some of the viruses that preferentially replicate in cells of the immune system have already been mentioned. Specific antiviral antibody can enhance the entry of viruses into Fc receptor(FcR)-bearing target cells (Halstead and O'Rourke 1977, Porterfield and Cardosa 1984), can modulate expression of viral antigens on the surface of infected cells so that cells are not recognized by the immune system, or cause antigenic shift in viruses, the best example being influenza virus.

Virus induced immunosuppression is a well-studied phenomenon and occurs through several mechanisms. Both general and specific responses can be suppressed. Many viral infections deplete lymphocytes (Bro-Jorgensen and Volkert 1972, McFarland 1974), or induce suppressor cells

that prevent specific responses to the infecting virus (Nash et al 1981, Sharpe and Fields 1985) or induce nonspecific suppressor cells (Honen et al 1980). More recently, viruses have been shown to abrogate lymphocyte responses by inducing macrophages to produce prostaglandins (Wainberg and Mills 1985), or by interfering with the production of interleukin 2 (Wainberg et al 1983, Wainberg and Mills 1985).

The final outcome of infection depends on the interplay of all the above factors, plus the influence of virus virulence genes, host genes and other broader determinants such as the general health, nutritional and psychological status of the host. The possible outcomes of infection are many: 1) asymptomatic infection with no disease, 2) acute infection with tissue damage, 3) persistent infection with or without symptoms or, 4) latency where intermittent episodes of disease occur. In acute infections, tissue damage occurs by direct lysis of the cell as a result of virus replication, or by inflammatory responses elicited by necrosis, by destruction of virus infected tissues by specific immune responses or by the deposition of antigen-antibody complexes. In many infections, particularly persistent infections, chronic inflammatory responses occur, and antigen-antibody complexes can be deposited in blood vessels, the brain and kidney (Oldstone 1984). Both acute and chronic infections

can induce antibodies reacting with multiple organs in the body (Notkins et al 1984). Viruses, particularly LCMV in mice, can alter the functions of cells without causing cell death, and this too could lead to disease (Oldstone et al 1982, 1984).

B. Mechanisms of genetic resistance to viral infection

The first observation of host genetic factors influencing the outcome of viral infection was made by Webster in 1937 in his studies of flavivirus infection in mice. Most extensive genetic studies of resistance have been carried out in inbred mice, although virus diseases in other species have been examined. In most, death is the outcome measured in susceptible strains. Resistance mechanisms do not prevent infection, but do prevent progression to disease. Some generalizations can be made about the genes that have been described, especially those in the murine system (Brinton and Nathanson 1981). In some instances, a single locus has been identified, but in many 2 or more loci are involved. The dominant allele may encode either resistance or susceptibility, although resistance is more often dominant. Each locus identified to date only affects a particular virus or group of viruses, and each gene is probably unique. Only a few genes map to the major histocompatability complex (MHC), and when they do, are one of many genes involved.

The exact mechanisms for most instances of genetic resistance or susceptibility have not been delineated, but are generally operable at the cellular level. In many virus systems, infection and disease are limited because of a failure of the virus to replicate successfully in target cells. Resistance to flaviviruses in mice is controlled by a single autosomal dominant gene (Sabin 1952, Goodman and Koprowski 1962a), to murine hepatitis virus type 2 (MHV2) by a recessive autosomal gene (Bang and Warwick 1960), and to MHV3 by several genes controlling either acute disease or chronic disease (Levy-Leblond et al 1979). In all three infections resistance correlated with limited virus replication and a slower spread of virus (Goodman and Koprowski 1962b, Jacoby and Bhatt 1976, Bang and Cody 1980, LePrevost et al 1975). The differences in virus titres between resistant and susceptible animals were evident even at 1 day post infection suggesting that resistance could not be due to specific immune responses (Goodman and Koprowski 1962b, Dupuy et al 1980). When tissues from susceptible and resistant mice were infected with virus in vitro, the yields of virus parallelled those found in vivo, i.e. susceptible cells yielded more virus than resistant. For flaviviruses, this was true for macrophages, brain tissue and embryo fibroblasts (Goodman and Koprowski 1962a, 1962b, Darnell and Koprowski 1974, Brinton and Nathanson 1981); for MHV2, macrophages appeared to be the crucial

targets (Bang and Warwick 1960), and for MHV3, both macrophages and hepatocytes showed this differential infectivity (Virelizier and Allison 1976, Arnheiter et al 1982).

In all cases, there did not appear to be a difference in the number of receptors for virus since equal amounts of virus bound to both susceptible and resistant cells (Darnell and Koprowski 1974, Shif and Bang 1970, Arnheiter et al 1982). Viral RNA synthesis was limited in cells resistant to flaviviruses (Brinton and Nathanson 1981), and increased numbers of defective interfering particles (DIP) were observed (Darnell and Koprowski 1974, Brinton 1983). As well, there is evidence that the DIP produced in resistant cells were more effective in their capacity to inhibit virus replication (Brinton 1983). The mechanisms of resistance are less clear for the hepatitis viruses. For both MHV2 and MHV3, lymphokines could convert susceptible macrophages to resistant (Bang and Cody 1980, Virelizier et al 1977) or shift resistant cells to susceptible to MHV2 (Weiser and Bang 1976). These data suggest that the initial permissiveness of a cell for viral replication may depend on exogenous factors, although there is evidence for further resistance mechanisms in MHV3 (Virelizier and Gresser 1978). Differences in target cell replication have also been reported for MHV4 (Stohlmann et al 1983), in HSV1 (Stevens and Cook 1971, Kirchner et al

1978) and HSV2 infections (Mogensen 1977) and for MCMV infections (Harnett and Shellam 1982, Nedrud et al 1979) However, others have not found a correlation between infectivity in vitro and resistance for these viruses, so other mechanisms have been invoked (Selgrade and Osborn 1974, Brautigan et al 1979).

Quantitative rather than qualitative differences in target cells for a virus have rarely been described. Marek's disease virus (MDV) causes a malignant lymphoproliferative disease in susceptible chickens. Resistance is controlled by at least 3 genes, one of them linked to the MHC, that are thought to operate both at the level of infection and the level of transformation or tumour progression (Gallatin and Longenecker 1981, Lee et al 1981). The spleens and thymi of susceptible chickens were shown to contain more cells than those from resistant birds, and cells from susceptible birds adsorbed and produced more virus (Gallatin and Longenecker 1979, Lee et al 1981). The phenotype of lymphoid cells appeared to be dependent on the thymic microenvironment, leading these workers to hypothesize that the thymus confers permissiveness either through virus receptor expression on cells or by expanding the number of cells in the susceptible category.

There seems to be a quantitative difference in bone marrow (BM) target cells for murine spleen focus forming

virus (SFFV) arising from Friend leukaemia virus. This virus induces erythroleukaemia in mice, an event controlled by several genes, both H-2 linked and not (Brinton et al 1983). One of these, Fv-2, controls both the numbers of infectious centres after infection and the proportion of erythroid progenitor cells that are thought to be targets for virus (Steeves et al 1980). Resistant mice have fewer of these cells. It has also been suggested that target cells for SFFV-type viruses are limited not in number, but because virus receptors are blocked by the presence of endogenous viral proteins on the surface of cells (Ruscetti 1985).

The second group of mechanisms that may be responsible for genetic resistance encompasses nonspecific aspects of the host response, such as interferon production and NK and macrophage antiviral activities. Interferon has already been discussed as a possible means of inducing an antiviral state in target cells. The evidence for a role in influenza infection of mice is strong. Resistance to lethal influenza infection is due to a single autosomal dominant gene, the Mx gene, and again is correlated with lower virus titres observed at 2 days after infection (Haller and Lindemann 1974). Interferon had no effect on the phenotype of susceptible cells, but anti-interferon serum converted resistant cells to susceptible (Haller et al 1979). It has since been demonstrated that interferon

induces a protein encoded by the Mx gene in the nucleus of resistant cells (Horisberger et al 1983). This protein appears to interfere with the primary transcription of viral RNA into mRNA (Haller 1985), but is active only against influenza viruses (Haller et al 1980).

Interferon is suspected to play a key role in resistance to other viruses, particularly the herpesviruses. Resistance to fatal infection with HSV-1 is controlled by 2 non-H-2 genes (Lopez 1975). Susceptible mice do not differ in humoral responses after infection, or in the activation of macrophages (Zawatsky et al 1981), but they do fail to produce interferon early after infection (Zawatsky et al 1981, Engler et al 1982). Low production of interferon in vitro is also correlated with susceptibility (Zawatsky et al 1981). The levels of natural killer cell activity after infection also correlated with resistance, but this may have been a consequence of interferon production.

Decreased early interferon production is thought to be the reason for susceptibility to MCMV (Grundy (Chalmer) et al 1982), which is controlled by an H-2 gene with non H-2 influence (Chalmer et al 1977). The gene controlling early interferon production is also non H-2 linked (Grundy (Chalmer) et al 1982). Treatment of some resistant strains of mice with anti-interferon serum reduced resistance but in others it had no effect, indicating that other factors,

perhaps encoded by the H-2 liked gene, are involved (Grundy(Chalmer) et al 1982). More recent data suggest a difference in sensitivity to interferon may explain resistance and susceptibility (Harnett and Shellam 1985).

Examination of NK levels in resistant and susceptible strains of mice has also revealed a positive correlation between higher, more prolonged NK and resistance to CMV (Bancroft et al 1981). Nude mice with high NK levels were still susceptible, but died later than susceptible strains suggesting that early mechanisms are essential but not sufficient for resistance. NK activity has also been presented as the resistance mechanism in HSV-1 infection. Resistance was sensitive to marrow depletion, as is NK activity (Lopez et al 1980), and correlated with NK-type activity against HSV-infected targets (Lopez et al 1983).

Other nonspecific responses have been postulated to account for genetic resistance but have not been examined in detail. The magnitude of the inflammatory response has been both positively (Bartholomaeus et al 1983) and negatively (Selgrade and Osborne 1974) correlated with hepatitis in mice infected with CMV, and there is some evidence for activated macrophages as the crucial element in resistance to HSV2 (Rosenstreich et al 1982). Infiltration of lymphocytes into infected airways is

genetically determined in Sendai virus infection of mice (Brownstein et al 1981, Brownstein 1983).

There are no clear examples of the specific immune response controlling resistance to viral disease, with the exception of the tumour viruses. In these instances, resistance to the tumour caused by the virus is mediated by the immune response, although resistance to infection is not. This has been demonstrated for MDV in chickens (Lee et al 1981) and for the murine leukaemia viruses, where recovery from splenomegaly caused by SFFV is controlled by a gene in the H-2 region (Britt and Cheseboro 1980). In the other virus systems, specific responses appear to be essential for full resistance, and act synergistically with the described resistance factors.

A genetic basis has been described for many other viruses, reviewed in Brinton and Nathanson (1983), including ectromelia (Rager-Zisman et al 1980), and the agent responsible for scrapie (Kingsbury et al 1983). There are also many examples of differences in nonspecific and specific immune responses to viral antigens, presumably encoded by immune response (Ir) genes, but these have not been correlated with disease resistance or susceptibility.

C. Pathogenesis of arenavirus infections

Within the family Arenaviridae are 13 viruses characterized by segmented single-stranded RNA genomes, pleomorphic virions containing electron dense granules, and

envelopes with regular peplomers (Fenner et al 1974, Howard and Young 1984, Casals 1984). Based on geographical location and serological relationships, the arenaviruses can be subdivided into Old and New World groups (Rowe et al 1970, Howard and Young 1984). The prototype virus, lymphocytic choriomeningitis virus (LCMV), Lassa fever virus, Mopeia virus and a virus from the Central African Republic, make up the Old World group. LCMV is found worldwide, the others in Africa only. The New World arenaviruses include Tamiami, found in southern Florida, Tacaribe, from Trinidad, and Junin, Machupo, Latino, Flexal, Amapari, Parani and Pichinde, all from South America (Howard and Young 1984, Casals 1984, Fenner et al 1974).

Four of these viruses, 2 from each group, cause disease in man; LCMV, Lassa fever virus, Junin and Machupo. These viruses, like all arenaviruses, establish persistent infections in their rodent hosts, and are probably transmitted to man through the respiratory or gastrointestinal tracts, or through abrasions in the skin from contaminated soil, water, or food (Ray 1984, Casals 1984). LCMV is contracted from the house mouse, pet mice or hamsters, or laboratory animals (Ackerman 1973, Casals 1984). Infections are rare, usually mild, and almost never fatal.

The other three viruses, Lassa fever virus, Junin and Machupo, cause more serious diseases. Argentinian hemorrhagic fever (AHF) is caused by Junin virus, and Machupo is the agent of Bolivian hemorrhagic fever (BHF). The diseases are virtually the same, with symptoms of a high unremitting fever, malaise, headache, muscular pains, nausea and leukopenia, appearing 7 to 14 days after exposure. In severe cases the symptoms become more pronounced with hemorrhagic manifestations; petechiae, bleeding from the gums, nose, stomach, intestine and uterus. There may also be signs of CNS involvement. Acute disease can last for up to 3 weeks. Death is due to uremic coma, hypotension or shock due to plasma loss. The mortality rate for AHF is 3 to 15% and for BHF up to 30%.

Lassa fever is encountered in Western Africa. Early symptoms are similar to those for the South American hemorrhagic fevers, but with marked pharyngitis. Petechiae and subcutaneous hemorrhages may occur later and death, occurring in 30 to 60% of hospitalized cases during the second week, is due to cardiovascular collapse.

The mechanisms whereby these viruses cause disease in man is not known. During acute illness, virus can be recovered from the blood, throat and sometimes in the urine. In hemorrhagic fever victims, the spleen and lymph nodes are most often positive for virus (Johnson et al 1973, Laguens et al 1984). The most consistent lesion

reported is swelling of the endothelium of capillaries and arterioles in all organs in the absence of any inflammatory reaction (Casals 1984). A role for complement has been hypothesized early in infection (de Bracco et al 1978), and other workers have suggested that high levels of interferons produced as a result of lymphocyte destruction may damage phagocytic cells and activate complement, leading to the observed damage (Laguens et al 1984). In Lassa fever, a direct cytopathic effect is responsible for major lesions in the liver, lungs, spleen, lymph nodes and intestinal mucosa (Casals and Buckley 1973). In survivors, neutralizing antibody, complement-fixing antibody and antibody detected in a fluoresence test develop. The latter can be detected by the 10th day in Lassa fever, the other antibodies by day 18. Antibodies appear late in the hemorrhagic fevers, and peak titres are not observed until 30 to 60 days after infection. Neutralizing antibodies persist for years (Casals 1984). Antibodies arising so late after infection are unlikely to play a role in eliminating the primary infection, and favourable results after transfer of immune sera have been reported only for AHF (Casals 1984).

D. Animal models of arenavirus pathogenesis

1. Lymphocytic Choriomeningitis Virus

Lymphocytic choriomeningitis virus is the prototype arenavirus. This agent is found in wild mice which appear

to be persistently infected (Murphy 1978). In the laboratory mouse, LCMV produces a wide spectrum of disease depending on the strain of virus, dose and route of inoculation, and the age of the host.

When virus is given by any route to neonatal mice, all organs become infected, although the primary targets are cells of the lymphoreticular system (Murphy 1978, Lehmann-Grube et al 1983a, Löhler and Lehmann-Grube 1981). Animals survive and are persistently infected. The animals are healthy, although they may undergo a transient runting (Hotchin 1962, Mims 1973) caused by alterations in growth hormone synthesis by infected cells in the anterior pituitary (Oldstone et al 1982, 1984). These carrier mice do not have detectable circulating antibody (Hotchin 1962), although antibody is produced that participates in immune complex formation (Oldstone and Dixon 1967). A proportion of carrier mice develop a 'late-onset' disease due to deposition of immune complexes in the kidney (Mims 1973, Oldstone et al 1985). These carrier mice resist infection by the intracranial route as adults and appear to be T cell tolerant to LCMV antigen (Lehmann-Grube et al 1983b). This disease pattern probably reflects the natural course of infection in neonatal feral mice (Murphy 1978).

When adult mice are infected intracranially with LCMV, mortality is virtually 100% within 8 to 10 days, and the major finding is widespread lymphocytic infiltration in

most organs including the CNS (Hotchin 1962). Because similar infiltration was absent in immunologically immature mice that survived infection, Hotchin proposed that disease was due, not to direct viral effects, but to the lymphocyte infiltration. Since then, several investigators have shown that immunosuppression prevented disease without altering viral replication (Rowe 1956, Hotchin 1962, Gilden et al 1972a, Lundstedt 1973, Gledhill 1967). Adoptive transfer of LCMV immune splenic T lymphocytes, but not immune serum, into immunosuppressed mice could induce disease (Gilden et al 1972b, Doherty and Zinkernagel 1975).

The actual mechanism of disease development is not clear. The pathological changes in the CNS are not severe enough in themselves to cause death, suggesting a generalized mechanism (Lehmann-Grube 1984a). However, some believe CTL action is responsible for death. T cells with cytotoxic activity have been isolated from the cerebrospinal fluid of mice infected with LCMV (Zinkernagel and Doherty 1973), and transfer of fatal disease with immune T lymphocytes was restricted by class I MHC antigens, and was positively correlated with in vitro killing activity (Doherty and Zinkernagel 1975, Doherty et al 1976). Others have found that the presence of high CTL activity did not necessarily correlate with disease (Pfau et al 1982a,1982b,1985) whereas DTH type reactions did (Thomsen et al 1983). Some reports have suggested that

interferon may contribute to disease (Riviere et al 1977, Riviere et al 1980, Jacobson et al 1981). Interferon may induce disease by activating macrophages and CTL in the infiltrate, or by direct action on epithelial and endothelial cells (Ronco et al 1980). T cells may participate in this aspect of pathogenesis by production of interferons.

The outcome of LCMV infection when the virus is administered extraneurally varies with the strain of mouse and the virus used. Generally, intraperitoneal (i.p.) or intravenous (i.v.) inoculation of LCMV does not produce disease (Buchmeier et al 1980). The virus replicates in many organs including spleen, thymus and lymph nodes, liver, kidney and brain, and can be demonstrated in macrophages, B cells and T cells (Popescu et al 1979, Lohler and Lehmann-Grube 1981, Doyle and Oldstone 1973). Titres in the spleen and blood peak at day 5 after infection and decline rapidly (Doyle and Oldstone 1978). Interferon production parallels increase in virus, as does splenic NK activity, peaking at days 3 to 5 (Welsh 1978a, 1978b). CTL can be demonstrated by day 4, with maximal activity occurring 3 to 5 days later (Marker and Volkert 1973). Activated macrophages are present in the tissues and peritoneal cavity slightly later than CTL activity (Buchmeier et al 1980). Primary footpad responses develop on the 8th day after footpad inoculation (Tosolini and Mims

1971, Lehmann-Grube and Lohler 1981). Reports of humoral responses have been conflicting, but this appears to be due to the use of different strains of virus. Neutralizing and sensitizing antibodies can be detected after 2 to 3 weeks when mice are infected with a viscerotropic strain (WE strain) of virus, but cannot be detected after infection with a neurotropic strain (Armstrong strain) (Kimmig and Lehmann-Grube 1979). Complement fixing antibodies are readily induced by all strains of virus, appearing early and peaking at 2 to 3 weeks. Both types of antibody persist for several months (Kimmig and Lehmann-Grube 1979, Buchmeier et al 1980).

These mice are protected from intracranial challenge with LCMV and it is believed that the protective immunity that develops by day 4 is mediated by the same responses that clear virus (Hotchin 1962, Marker and Volkert 1973). Most investigators believe the major factor responsible for virus clearance is CTL (Mims 1973, Zinkernagel and Doherty 1979, Zinkernagel and Welsh 1976, Mims and Blanden 1972, Marker and Volkert 1973, Anderson et al 1985, Byrne and Oldstone 1984). Others have found that CTL activity in the spleens of infected mice did not correlate either with viral clearance or with the ability to transfer protection to other mice (Lehmann-Grube et al 1985, Pfau et al 1982a, 1985) and T cell production of an antiviral lymphokine such as interferon (Greenspan et al

1984) or another, unidentified, factor (Lehmann-Grube et al 1985) has been proposed as instrumental in clearing virus.

The data indicate that immune T cells can both protect and induce disease. It has been proposed that when the antigen load is light (prior to 3 days) or widely dispersed throughout the body (later than 6 days), the immune reponse is protective, but when a significant amount of antigen is largely in the brain, the immune response concentrates there, and death ensues (Hotchin 1962, Thomsen et al 1979). Others have differentiated the T cell populations responsible for transfer of the two activities, and hypothesize that cells inducing disease do so because of their ability to recruit macrophages (Johnson and Cole 1975).

The role of interferon in primary extraneural infections is unclear, and not much studied, but the capacity of cloned CTL to reduce virus titres after transfer to infected mice is not correlated with their interferon production (Byrne and Oldstone 1984). As well, NK cells appear to be of little significance in LCMV infections. Beige mice, which are deficient in some NK-type activities, are as resistant to i.p. infection as other mice (Welsh and Kiessling 1980), and depletion of NK activity in vivo by anti-asialo GM1 serum does not alter the course of infection (Bukowski et al 1983). A protective role for macrophages has also been suggested, as

treatments that deplete macrophages without altering NK or T cell responses delay clearance of virus after i.p. inoculation (Thomsen and Volkert 1983).

Extraneural inoculation of LCMV, particularly the viscerotropic strain, does induce disease, often fatal, in certain strains of mice, and this disease, like the neurological manifestations, is immune-mediated (Rowe 1956, Lehmann-Grube and Lohler 1981). One of the major observations is that of destruction in lymphatic organs which appears to be the result of the immune response (Lehmann-Grube and Lohler 1981), although others have claimed that the observed damage is the result of viral infection of the cells of the immune system (Hanaoka et al 1969). Even surviving mice show evidence of alterations in lymphoid tissues (Lehmann-Grube and Lohler 1981), and these alterations have been implicated in the transient immunosuppression observed in infected mice (Mims and Wainwright 1968, Guttler et al 1975).

Very little has been done with LCMV in animal models other than the mouse. In the rat, intracranial inoculation at 4 days of age, but not earlier or later, induced changes in the CNS that were mediated by the immune system (Monjan et al 1973). Peripheral inoculation of neonatal hamsters resulted in a chronic infection with persistent viraemia and viruria which cleared eventually or led to late-onset wasting disease as in mice. Infection at

three weeks of age induced an inapparent persistent infection, and older hamsters totally cleared the infection (Parker et al 1976). Complement-fixing antibodies, antibodies active in immunofluorescence assays and neutralizing antibodies could be demonstrated (Smadel and Wall 1942, Thacker et al 1982), although the latter only appeared 6 weeks after infection (Smadel and Wall 1942).

2. New World arenaviruses

The New World arenaviruses have been studied less extensively than LCMV, but the few studies that have been carried out reveal similarities to the pathogenesis of LCMV. In their natural hosts, after neonatal exposure, these viruses clear slowly or establish persistence, whereas older animals tend to undergo transient infection only (Murphy et al 1976, Murphy 1978, Johnson et al 1973, Webb et al 1973, Howard and Young 1984). In other rodents, the pattern may be different (Coto et al 1980, Johnson et al 1973, Barrios et al 1982, Winn et al 1973, Borden et al 1971). The viruses preferentially replicate in cells of the reticuloendothelial system and lymphoid tissues (Murphy et al 1976, Johnson et al 1973, Carballal et al 1977), with the exception of Tacaribe virus (Borden and Nathanson 1974, Borden et al 1971). Both Tamiami and Junin viruses were present in megakaryocytes in the bone marrow (Murphy et al 1976, Carballal et al 1977), Junin was present in macrophages in the peritoneal cavity (Laguens et al 1983a),

and an attenuated strain of Junin virus was found chiefly in plastic adherent cells identified as dendritic cells (Laguens et al 1983a).

Complement-fixing, immunofluorescence and neutralizing antibodies are readily induced by most of these viruses (Murphy and Walker 1978, Winn et al 1973, Johnson et al 1965, 1973, Coto et al 1980, Blejer et al 1983, Barrios et al 1982), and in nonfatal infections, an increase in antibody titres often correlated with a decrease in virus titres (Murphy 1978, Winn et al 1973, Blejer et al 1983). Transfer of specific antibody protected guinea pigs from Junin virus infection (Weissenbacher et al 1975a), as did prior infection with Tacaribe virus (Coto et al 1980, Weissenbacher et al 1975b) or Machupo virus (Weissenbacher et al 1975b). It is not clear whether or not neutralizing antibody was the responsible factor (Weissenbacher et al 1975b). Immune serum could also transfer protection from Junin virus infection in rats (Blejer et al 1984). Much less is known about T cell responses. Adult mice surviving Junin virus infection developed CTL, but never displayed DTH, and treatment with cyclophosphamide abrogated resistance (Barrios et al 1982, 1984). Transfer of either T cells or serum protected these animals from death, but only T cells reduced virus titres in the brain (Barrios et al 1982).

Immmunologically mediated disease occurs when rodents other than the natural host are infected. Tamiami, Tacaribe and Junin viruses cause fatal neurological disease in neonatal or suckling mice (Gilden et al 1973, Borden et al 1971, Weissenbacher et al 1975a, Barrios et al 1982, Winn et al 1973). Nude mice were resistant to lethal infection with Junin virus, whereas immunocompetent controls were not (Weissenbacher et al 1983), and thymectomized mice resisted infection with Machupo, Tacaribe and Junin viruses (Besuschio et al 1973). A disease caused by an attenuated strain of Junin virus, but not wild type virus, in guinea pigs also appeared to be immunologically induced (Laguens et al 1983b).

Infection with wild type Junin virus in guinea pigs induced an acute disease with fever, leukopenia, hemorrhagic lesions and necrosis of bone marrow and lymphoid tissue. Virus was found in most tissues of the body with increasing titres until death, but there was no evidence of infiltration of inflammatory cells (Weissenbacher et al 1975a). Infection of infant and sometimes adult Calomys with Machupo produced a syndrome similar to late-onset wasting disease described for LCMV (Johnson et al 1973).

E. Pichinde virus

Pichinde virus (PV) was isolated in 1971 from the rodent Calomys albigularis in Colombia, South America

(Trapido and Sanmartin 1971). The host was found to be persistently infected, with virus in the blood, liver, spleen, heart and brain. Complement-fixing but not neutralizing antibody could be demonstrated in these animals.

In neonatal mice, infection is fatal (Trapido and Sanmartin 1971). In adult mice, PV infection proceeds much like extraneural infection with LCMV. Animals survive infection, with development of elevated NK responses at 3 days (Welsh 1978b, Walker et al 1984) and CTL at days 5 to 7 (Walker et al 1984). Complement-fixing antibodies are elicited, but neutralizing antibodies are not (Trapido and Sanmartin 1971). PV has also been adapted to the guinea pig (Jahrling et al 1981). Outbred guinea pigs showed delayed viraemia after infection, with low titres, and survived. The virus was fatal for inbred guinea pigs. Viraemia occurred only 2 days after infection and titres rose until death at 16 days. Virus spread throughout the body, and could be isolated from the spleen, particularly in large cells between the white and red pulp, and in lung, brain, kidney, pancreas, adrenals and thymus. The major finding was of extensive lesions in the liver. Histologically, there was no evidence of inflammation, and the disease pattern was reminiscent of Lassa fever in man.

Pathogenesis of PV infection has been studied most in the Syrian hamster, a rodent from the same family as the

host in nature. Intracranial or intraperitoneal infection of neonates was fatal (Trapido and Sanmartin 1971, Buchmeier 1976, Buchmeier and Rawls 1977). Death was associated with high virus titres, and renal damage, with some involvement of brain and liver (Buchmeier 1976). Resistance to infection developed with age, so that by day 8 LVG outbred and LSH inbred hamsters were no longer susceptible (Buchmeier and Rawls 1977). Resistance was positively correlated with the ability to limit viral replication. In surviving LVG and LSH animals, virus could still be isolated from the spleen, liver, kidney and blood, but titres reached a peak, then declined after day 8. Complement-fixing antibodies and antibody directed to surface antigen were found in the serum by day 10, reaching peak titres at 3 weeks after infection. Neutralizing antibodies could not be demonstrated (Buchmeier and Rawls 1977).

One strain of inbred hamster, MHA failed to acquire resistance to PV, so that 7 week old MHA animals succumbed to infection with the same rapidity as 4 day old LVG hamsters (Buchmeier and Rawls 1977). Failure to survive appeared to be due to an inability to limit viral infection as in neonatal hamsters. A comparison of MHA and either LVG or LSH hamsters showed that virus titres in the spleen, blood, liver and kidney were the same at day 4 post infection, but later reached higher levels in MHA animals

and continued to rise until death at 2 to 3 weeks after infection (Buchmeier and Rawls 1977). When splenic infectious centres (IC) were examined, differences were detected as early as 2 days after infection (Gee et al 1981a). The major lesions were in the spleen and liver, and antigen was first detected in the cells of the reticuloendothelial system within these organs, with macrophages and Kupffer cells being the prime targets (Murphy et al 1977). Lymph nodes did contain viral antigen, again in macrophages, but lesions did not progress here, and other lymphoid tissues were spared, contrary to infection with the other arenaviruses (Murphy et al 1976). There was no evidence of lymphocyte infection. In LVG hamsters lesions were resolved after the fourth day of infection, but in MHA animals progressed until death. There was no inflammation associated with these lesions (Murphy et al 1977). This, plus the association of death with increasing virus load, the loss of susceptibility to PV as the immune system matured, and the ability of treatment with cyclophosphamide to abrogate resistance indicated that death was due to virus-induced changes rather than immunopathological damage.

Primary kidney cells and peritoneal exudate macrophages from both strains of hamster supported PV replication to the same extent, (Buchmeier and Rawls 1977), as did plastic adherent and nonadherent spleen cells (Gee

1979), suggesting that the differences observed in resistance were not due to intrinsic differences in target cell permissiveness but perhaps to host responses to the virus. Various parameters of the response to PV were then examined. The footpad response to primary inoculation was assessed as a measure of cell-mediated immunity (Gee et al 1980). The reaction was measured by two methods, footpad swelling and by influx of radiolabelled protein, and by both, a significant response developed in resistant strains (LVG and LSH), but not in the susceptible strain. On the other hand, footpad inoculated MHA hamsters did not succumb to infection, and were protected against subsequent intraperitoneal challenge.

The production of antibody was assessed in these surviving animals. Previously, titres of complement-fixing antibody and antibody binding to surface antigen had been determined in MHA hamsters surviving intraperitoneal infection at day 10, and there was no difference from production of antibody in LSH hamsters (Buchmeier and Rawls 1977). This was confirmed in footpad inoculated hamsters (Gee et al 1980). It would seem from these results that the susceptiblity of MHA hamsters was not due to an inability to mount an immune response, as protective immunity could develop under the right conditions. Antibody was not responsible for this protection, as passive transfer of immune serum did not alter infection in

MHA animals (Gee, unpublished). The fact that differences in infectious centres could be detected by day 2 after infection indicated that crucial events limiting viral replication were occurring very early.

Further study of cell-mediated responses in the spleen were unable to reveal virus-specific CTL activity in either strain of hamster (Gee et al 1979). In mice, NK activity is boosted after infection with LCMV and PV (Welsh 1978a, 1978b). Nonspecific NK activity, lysing syngeneic and allogeneic tumour targets, was present in the spleens of both strains of hamster, and was elevated after virus infection peaking at day 3 (Gee et al 1979). Normal and infected MHA hamsters had significantly higher NK activity than LSH. In contrast to work in mice, high NK activity was correlated with susceptibility and not resistance to virus infection in hamsters. Examination of the kinetics of viral replication after both intraperitoneal and footpad inoculation confirmed the correlation of high NK activity with higher numbers of infectious centres in MHA hamsters (Gee et al 1981b). Genetic analysis of survival, the ability to limit viraemia and NK activity showed that resistance, limited viral growth and low NK activity were all controlled by single dominant autosomal genes.

II. Nonspecific Cytotoxic Cells in the Hamster

Cells expressing cytotoxic activity against tumour or virally infected targets in the absence of immunization

have been described in the hamster by several investigators. In the mouse, such cells are heterogeneous, and subpopulations of cells can be differentiated on the basis of morphology, surface antigen phenotype, target cell spectrum, sensitivity to lymphokines and assay conditions necessary to detect killing. The same is true in the hamster.

Endogenous NK-type activity against virus transformed hamster tumour targets or virally infected targets has been observed in the spleen and lymph nodes (Datta et al 1979, Gee et al 1979, Rees et al 1980, Lausch et al 1981, Cook et al 1982, Haddada et al 1982), and bone marrow (Datta et al 1979). Insignificant activity was found in the thymus (Datta et al 1979, 1982, Lausch et al 1981). In the spleen, this activity could be detected as early as 1 week of age and persisted for up to 1 1/2 years, was resistant to irradiation 5 hours prior to assay, but sensitive to cyclophosphamide and carageenan (Datta et al 1979), and was present in nude hamsters (Haddada et al 1982). In most studies, maximal lytic activity was observed in an assay after 16 to 18 hours (Datta et al 1979, Gee et al 1979, Lausch et al 1981, Cook et al 1982). Splenic NK cells were generally plastic or nylon wool nonadherent, sensitive to incubation at 37 for 18 hours, resistant to treatment with NH_4Cl, and trypsin resistant (Gee et al 1979, Trentin and Datta 1979). They were

nonphagocytic, negative for surface Ig (Gee et al 1979) and for an antigen homologous to the murine theta antigen (Yang et al 1982), and were positive for Fc receptors (Trentin and Datta 1979). A second cytotoxic cell from the spleen has been described that lysed both lymphoma and sarcoma targets, was not dependent on the age of the host, and was active in a 4 hour assay (Rees et al 1980). This cell, which the authors classified as a natural cytotoxic (NC) cell rather than an NK cell, was glass and nylon wool adherent and phagocytic.

Endogenous NK activity can also be detected using human tumour targets. Tsang and colleagues (1981) have described activity against Raji cells that was present in peripheral blood, was not observable in animals less than 1 month of age, and was active in a 6 hour assay. Peripheral blood and splenic effectors that lysed K562 targets in a 4 hour assay have also been demonstrated (Teale et al 1983). These effectors were enriched by nylon wool separation, were sensitive to an 18 hour incubation and to trypsin. On density gradients, the lytic activity separated with large granular lymphocytes (LGL), which are the cells believed to mediate human, rat and murine NK activities (Reynolds et al 1981, Timonen and Saskela 1980, Luini et al 1981).

NK activity in the hamster can be boosted by several treatments; infection with virus (Gee et al 1979, Yang et al 1983, Cremer et al 1982), with stimulants that

activate macrophages (Chapes and Tompkins 1979) and with tumour grafts (Datta et al 1982). In vitro, splenic NK killing can be augmented by culture with ConA (Singh and Tevethia 1973, Stewart et al 1985), and a cytotoxic cell can be induced in bone marrow cells by culture with immune peritoneal macrophages (Chapes et al 1981).

All these cells are plastic or nylon wool nonadherent (Gee et al 1979, Chapes and Tompkins 1979, Chapes et al 1981). These splenic effectors have been reported as resistant to NH_4Cl (Gee et al 1979), whereas cells from the peritoneal cavity were sensitive (Chapes and Tompkins 1979). Boosted splenic effector cells were negative for the theta homologue (Nelles and Streilein 1980a, Yang et al 1982), bone marrow cells were both positive and negative, and nonadherent effectors from the peritoneal cavity expressed the antigen (Yang et al 1983, Yang and Tompkins 1983).

In summary, splenic NK cells, either endogenous or induced, are plastic or nylon wool nonadherent, nonphagocytic cells that do not express surface Ig or the theta homologue. As well, a splenic adherent, phagocytic cell has been identified that lyses various tumour targets, and may be a macrophage. These cells may be further differentiated on the basis of target cell sensitivity and the kinetics of lysis. Effector cells in the peritoneal

cavity and bone marrow differ from splenic effectors in the expression of the theta homologue.

III. Hypothesis

The mechanism of genetic resistance to PV infection did not appear to function at the level of target cell permissiveness, nor at the level of specific immune responses. The genetic linkage of low splenic NK activity, the ability to limit viral replication and resistance, plus the correlation of splenic infectious centres with splenic NK activity after i.p. infection suggested that cells with NK activity may serve as target cells for PV replication in MHA spleens, but not LSH spleens. The kinetics of augmented NK activity and IC development were also correlated in the popliteal lymph node after footpad inoculation (Gee et al 1981b). As well, no augmented NK activity was observable in the spleens of these protected animals after i.p. challenge, and this low NK activity correlated with limited virus growth in the spleen (Gee et al 1981b).

Attempts were made to characterize the cells responsible for NK activity and viral replication at day 3 after a primary i.p. inoculation. Separation of spleen cells on the basis of plastic adherence demonstrated that for both adherent and nonadherent populations, MHA spleens contained more infectious centres per million cells than LSH, and the total number of infectious centres in the

nonadherent fraction was 7 times that in LSH spleens (Gee et al 1981a). Further separation of nonadherent cells by sedimentation at unit gravity showed that cytotoxic activity had two peaks, one corresponding to a large cell, and one corresponding to a small lymphocyte. The activity in both peaks was greater in MHA hamsters (Gee et al 1979). Infectious centres per million cells were equivalent for all fractions in MHA spleens, but for LSH, high numbers of infectious centres were only present in large cells.

From these data the following hypothesis was developed; small lymphocytes become infected in MHA spleens which are spared or are minimally infected in LSH spleens, and these small lymphocytes are those responsible for the increased NK activity, in MHA spleens. Because no intrinsic difference in permissiveness of splenocytes for virus replication had been demonstrated, it was suspected that the observed differences were due to quantitative differences in this target cell, rather than qualitative differences in the ability of the cells to become infected. The following experiments were conducted to further characterize the NK effectors cells and lymphocyte target cells in MHA spleens, in an attempt to verify the above hypothesis.

CHAPTER TWO

MATERIALS AND METHODS

I. Animals and Cell Preparations

A. Animals

Male inbred Syrian hamsters of two strains, MHA and LSH, were purchased from Charles River Laboratories, Lakeview, NJ. The hamsters had access to Purina chow and water *ad libitum*. For all experiments, animals were used at 6 to 12 weeks of age.

B. Preparation of lymphoid cell suspensions

Single cell suspensions of lymphoid cells from hamsters were prepared as follows. Organs were removed aseptically from animals sacrificed by etherization, minced with scissors and the fragments were forced through 60-gauge sterile wire mesh. The cells were resuspended in MEM F-15 medium, and large debris was removed by underlying the cell suspension with 2-3 ml of fetal bovine serum (FBS) for 10 min at room temperature. The supernatant was recovered, centrifuged at 150 x g for 10 min and resuspended in medium. Viable cells were counted by trypan blue exclusion in a haemocytometer.

II. Cell Culture and Virus Growth

A. Cell lines and cell culture

Vero cells were grown in Eagle's minimum essential medium (MEM F-15), Flow Laboratories, Mississauga, Ont) containing 10% v/v heat inactivated fetal bovine serum

(FBS:Grand Island Biological Co.(GIBCO), Grand Island, NY), 0.75 g/l sodium bicarbonate, 1% v/v 1M hydroxyethyl-piperazine-N'-2-ethanol sulfonic acid pH 7.24 (HEPES) buffer (Sigma, St. Louis, MO), 2.0 mM L-glutamine (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO).

Baby hamster kidney cells (BHK) were grown in Dulbecco's modified Eagle medium (GIBCO) supplemented as described for MEM F-15 except for 5% v/v FBS.

K562 cells, provided by Dr. H. Pross (Queen's University, Kingston, Ont) were maintained in RPMI 1640 (GIBCO), supplemented as described for MEM F-15.

B. Virus growth and assay

Pichinde virus, strain AN 3739, was originally isolated from the blood of the South American rodent Oryzmys albigularis by Trapido and Sanmartin (1971). Virus stocks were made by infecting BHK monolayers with 3 plaque-forming units (pfu) of virus per cell. The virus was allowed to adsorb for 1 hr at 37°C before the cells were refed with Dulbecco's medium. Cells were incubated for 48 hr at 37°C at which time virus was harvested by pouring off supernatant, which was aliquoted and stored at -90 C.

Pichinde virus was assayed by plating 0.2 ml of 10-fold dilutions of virus preparation on Vero monolayers in 60 mm tissue culture dishes (Corning Glass Works, Corning, NY). After 1 hr adsorption at 37°C, the cells

were overlaid with 1.75% w/v Bactoagar (Difco Laboratories, Detroit, MI) diluted 1/2 with Hank's Balanced Salt Solution (HBSS) 2X supplemented with 20% v/v heat-inactivated FBS, 2% v/v 100X BME amino acids (GIBCO), 2% v/v 100X BME vitamins (GIBCO), 4.5 g/l sodium bicarbonate, 2% v/v 1M HEPES buffer, 200 U/ml penicillin, 200 μg/ml streptomycin. Plates were incubated at 37°C for 3 days before a second overlay containing 0.1 g/l neutral red (GIBCO) was added. Plaques were counted after a further 24 hr incubation.

C. Infectious centre assay

Lymphoid cell suspensions were prepared from spleens of infected hamsters by the method described above, and ten-fold dilutions of cells were plated on Vero monolayers and overlaid as described above for assay of virus.

D. Infection of cells in vitro

Spleen cells from normal hamsters or from cultures of splenocytes were incubated with Pichinde virus on ice for 60 min, then assayed for virus adsorption or cultured further before assay for infectious centres as described above. In most experiments, cells were infected with MOI=0.5 or 1.0.

III. Preparation of Lymphokines

A. Conditioned medium

Conditioned medium (CM) was prepared from hamster splenocytes by preparing single cells suspensions, and

culturing cells in tissue culture flasks (Corning) for 24 hr at a density of 2 to 5×10^6/ml in RPMI 1640 supplemented as above containing 3.3 µg/ml of Concanavalin A (ConA:Pharmacia Fine Chemicals, Uppsala, Sweden). Rat CM was prepared by culturing splenocytes at a concentration of 5×10^6 /ml in RPMI 1640 containing 5 µg/ml ConA for 48 hr (Gillis et al 1978). Supernatants were collected after centrifugation at 300 x g to remove cell debris, filtered through a 0.2 micron disposable filter unit (Nalgene, Rochester, NY) and stored at -20°C. All batches of rat CM were kindly assayed for interleukin 2 (IL2) activity on a murine IL2 dependent cell line, HY-1, by Dr. K. L. Rosenthal.

B. Interleukin 1

Supernatants containing interleukin 1 (IL1) were prepared according to the method of Hoffenbach et al 1984. One ml of splenic or lymph node cell suspensions at 1×10^7 /ml were adsorbed to 60 mm plastic tissue culture plates (Corning) for 90 min at 37°C. Nonadherent cells were removed, and adherent cells were cultured for 48 hr in culture medium supplemented as above, except for 2% FBS, and 50 µg/ml LPS W, E. coli (Difco Labs, Detroit, MI). Supernatants were assayed for IL1 activity by C. D. Richards.

C. Purified interleukins

Purified murine IL2 was the gift of Dr. D.

Harnish. Human IL2 was purchased from Collaborative Research Inc. (Lexington, MA) or Amgen Biologicals (Thousand Oaks, CA). The units of human IL2 are half-maximal units assayed on murine T cell lines as described by Gillis and Smith (1977). One unit of murine IL2 is the amount of IL2 in 1 ml that causes 30% of maximal proliferation of murine T cells obtained in the same expriment, as described by Reindeau et al 1983.

IV. Culture of spleen cells in vitro

Suspensions of splenocytes were prepared as described and cultured in medium containing various concentrations of rat CM, purified murine IL2 or purified human IL2. Cells were cultured at densities of 2 to 4 x 10^6 /ml in 24-well tissue culture plates (Linbro, MacLean, VA) at 37 °C in 5% CO . Cultures were fed fresh medium every 3 days. After various incubation periods, the cells were collected, washed twice, and counted before use as effectors in a cytotoxicity assay. In some experiments, splenocytes were cultured in 96-well microtitre plates (Flow Laboratories). One hundred microlitres of a 5 x 10^6 /ml cell suspension were placed in each well with an equal volume of CM or IL2. After various incubation periods, 0.1 ml of medium was removed from each well, and target cells were directly added for the cytotoxicity assay. All wells were set up in triplicate.

V. Biological Assays

A. Thymocyte proliferation assay

The procedure of Shaw et al (1978) was followed with slight modifications. Thymocytes were prepared in single cell suspensions, and were adjusted to 2.5×10^4 cells/ml in culture medium to which 1 µg/ml ConA was added. Under these conditions, no proliferation of thymocytes was observed in the absence of CM. Cells were dispensed into 96-well, flat-bottomed microtitre plates (Flow Laboratories, McLean, VA) in volumes of 0.1 ml. Fifty microlitres of CM were added to each well. Control wells received 50 µl of medium containing ConA at a concentration used to prepare the CM. All assays were conducted in quadruplicte. Plates were incubated for 48 hr at 37°C in 5% CO_2. Cultures were pulsed for 6 hr with 1 µCi [3H]thymidine (New England Nuclear, Boston, MA) before harvesting ruptured cells onto glass fibre filter papers (M.A. Bioproducts, Walkersville, MD), using a Mini-Mash harvester (M.A. Bioproducts). [3H]thymidine uptake was determined in a liquid scintillation system, and counted in a Beckman beta counter (Beckman Instruments Inc., Palo Alto, CA).

B. Limiting dilution assay

To determine the frequency of cytotoxic cells in spleen, limiting dilution assays (LDA) were performed

according to the protocol of Riccardi et al (1982). Spleen cells were cultured at cell concentrations ranging from 5×10^5 to 160 cells/well, 16 replicate wells for each cell concentration, in 96-well, round-bottomed microtitre plates (Flow Laboratories) in a final volume of 0.2 ml culture medium supplemented with 30% CM. In some experiments, a feeder layer of 5×10^3 irradiated (3000 R) peritoneal exudate cells (PEC) was added to each well. PEC were collected from the peritoneal cavity of hamsters 3 days after i.p. administration of 5 ml sterile paraffin oil. Cells were washed three times with culture medium before use. After 7 days, 0.1 ml of medium was removed from each well and an equal volume containing 5×10^3 ^{51}Cr-labelled target cells was added. Plates were incubated for 16 hr before removing 0.1 ml supernatant for counting. Spontaneous Cr release was determined by incubating target cells in medium alone for the 16-hr period. Wells were considered positive for cytotoxicity when the counts were greater than or equal to 3 standard deviations (SD) above spontaneous release. The limiting frequency was determined by the method of maximum liklihood as described by Porter and Berry (1964).

C. Cytotoxicity assay

K562 cells were labelled in 400 μCi sodium chromate (New England Nuclear) for 90 min at 37°C, and were then washed three times before use. Effector cells at

various concentrations were added to 5 x 10^3 ^{51}Cr-labelled target cells in 96-well, flat-bottomed microtitre plates (NUNC, Roskilde, Denmark) to give effector to target ratios of 100:1, 50:1, 25:1 and 12.5:1. Spontaneous and maximal release of label was determined by the addition of medium alone or 1% Nonidet P-40 to target cells. All combinations were performed in quadruplicate. Plates were incubated at 37°C in 5% CO_2 for 16 hr. One hundred microlitres of supernatant were removed and total radioactivity over a 5-min period was ascertained in a Beckman gamma counter (Beckman Instruments Inc.). The data are expressed as percent specific release, calculated by using the following formula:

$$\frac{\text{(Experimental counts-spontaneous counts)}}{\text{(Maximal counts-spontaneous counts)}}$$

VI. Characterization of cytotoxic cells

A. Adherence

Plastic adherence properties were determined by incubating 1 x 10^7 cells in 1 ml medium in 100 mm sterile plastic tissue culture dishes (Corning) for 90 min at 37°C. The supernate, containing the plastic nonadherent cells, was decanted. The monolayer was washed twice with PBS lacking Ca++ or Mg++ to remove any remaining nonadherent cells. Adherent cells were removed by scraping the plates

with a rubber policeman.

Plastic nonadherent cells were further characterized by passage through nylon wool as described by Tabor et al (1984). Plastic syringes (10 ml) were packed with 0.7 g (dry weight) of nylon wool (Leukopak, Fenwal Laboratories, Morton Grove, IL) and autoclaved. The columns were washed with 20 ml PBS, then with 50 ml of RPMI 1640 supplemented with 1% FBS and then incubated at 37°C for 1 hr before rinsing again with 20 ml RPMI-1% FBS. Up to 5×10^7 cells in 1 ml RPMI-1% FBS were loaded and washed into the column by the addition of 1 ml RPMI-1% FBS. The column was then incubated at 37°C for 30 min in 5% CO_2 before adding another 1 ml medium, then incubated for another 15 min. Effluent cells were collected by washing the column slowly with 20 ml warm RPMI-1% FBS. Adhering cells were harvested by gently teasing apart the nylon wool with sterile forceps. All cells were washed twice before adjusting to the appropriate concentration.

B. Treatment of cells with antisera and complement

Monoclonal anti-Thy 1.2 (New England Nuclear) and anti-asialo GM1 (WAKO Chemicals, Dallas, TX) were diluted as specified and were incubated with 5×10^6 cells in 0.5 ml for 45 min at 4°C. Cells were centrifuged and resuspended in a 1/10 dilution of rabbit complement (C') (Cedarlane Laboratories, Hornby, Ontario) or in a 1/5 dilution of guinea pig C' (Cappel Scientific, Malvern, PA),

previously adsorbed with hamster spleen. After incubation at 37 °C for 30 min, cells were washed three times before counting. Control cell suspensions were treated with complement alone. The specificities of the antisera were accepted to be those determined by the suppliers.

VI. Statistical analyses

Where indicated, Student's T test was used to determine levels of significance in comparisons of means. Survival curves were compared using a Mandel-Haenszel Chi-square test of significance.

CHAPTER THREE

DIFFERENCES IN LYMPHOCYTE RESPONSIVENESS TO LYMPHOKINES

I. Spleens and Thymi Contain More Leukocytes in MHA Hamsters than in LSH Hamsters

During the course of these experiments, it was noticed that MHA spleens consistently yielded more cells than LSH spleens. This phenomenon was further investigated and confirmed. The data in Table I show that MHA spleens contained significantly more cells per gram body weight than LSH spleens ($p<0.005$). The same was true for the thymus, although the difference was less marked ($p<0.05$).

II. Strain Differences in Thymocyte Responsiveness to Lymphokines

A. Responsiveness to hamster lymphokines

Because the two strains differed in spleen and thymus cellularity, it was of interest to determine if the lymphocytes differed in their capacity to produce or respond to lymphokines. Thus, ConA CM prepared from either MHA or LSH spleen cells was assayed on homologous thymocytes for mitogenic activity in the presence of ConA (Paetkau *et al* 1976, Shaw *et al* 1978). The proliferation of MHA thymocytes induced by MHA CM was greater than that of LSH thymocytes stimulated with LSH CM (Figure 1) The differences observed were significant in 8 of 9 experiments ($p<0.025$), assaying 9 separate batches of CM on homologous thymocytes.

TABLE I

Number of Cells in Spleens and Thymi of MHA and LSH Hamsters

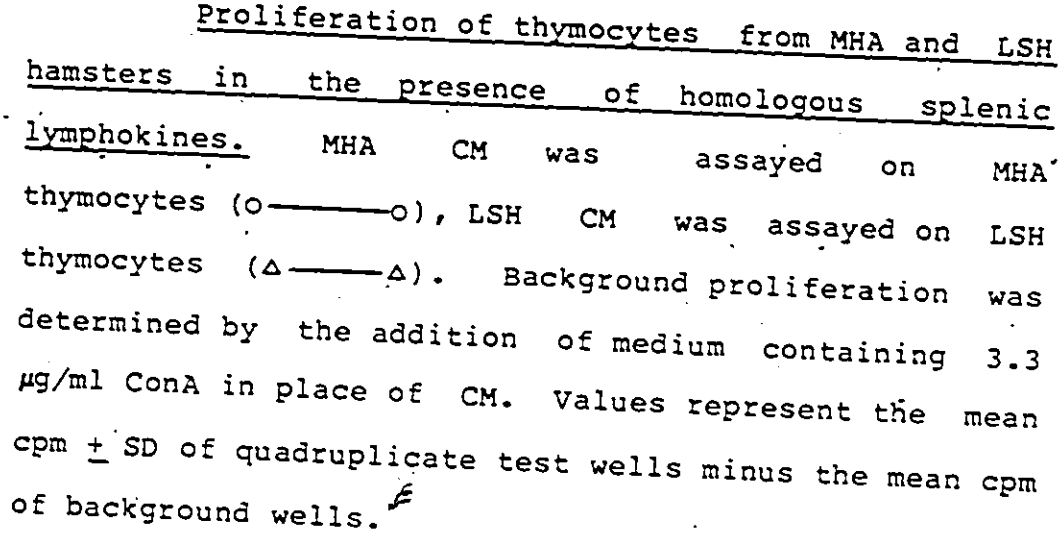
	Number of lymphocytes ($\times 10^5$) per gram body weight[a]	
	MHA	LSH
Spleen (n=12)	3.04 ± 0.89[b]	1.94 ± 0.77
Thymus (n=15)	10.80 ± 4.31[c]	8.20 ± 2.87

[a] Hamsters were sacrificed and weighed. Cell suspensions were prepared in culture medium as described in Materials and Methods. Cells were counted in a haemocytometer by trypan blue exclusion. Viability in thymocyte preparations from both strains was 90%, in splenocyte preparations, 80%. Values represent means ± standard deviation (SD).

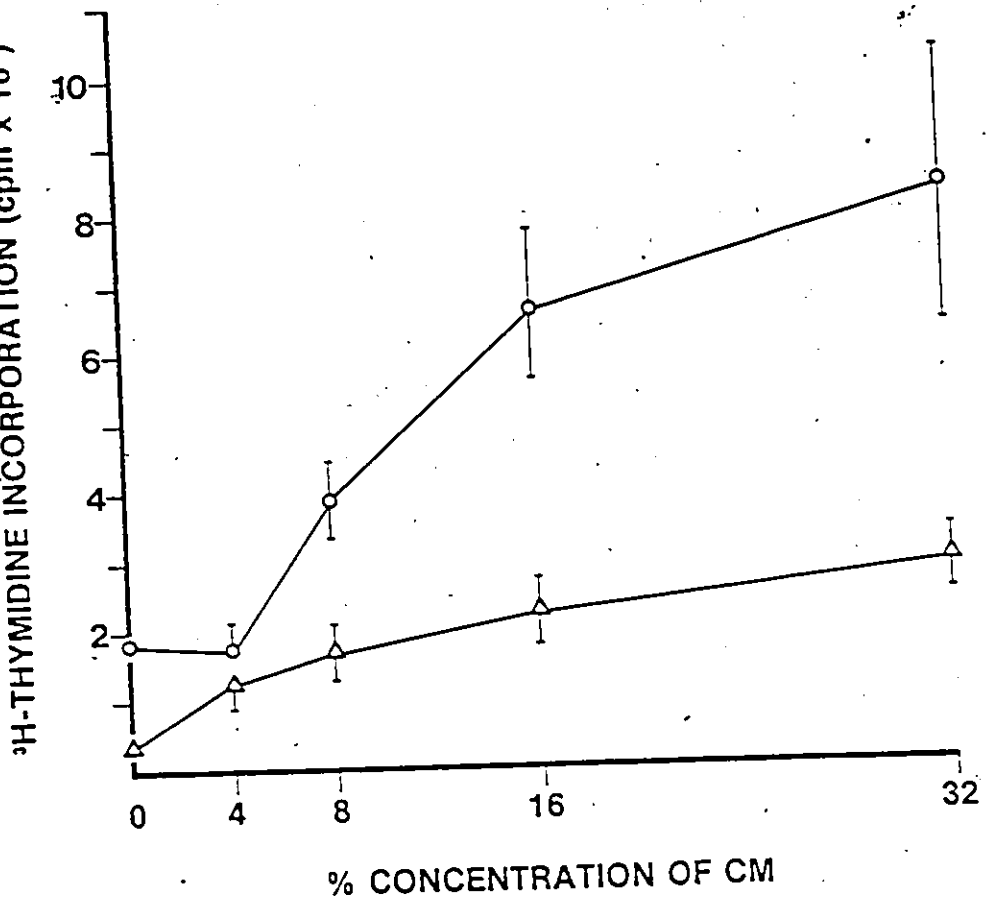
[b] $p<0.005$

[c] $p<0.05$

Figure 1



Proliferation of thymocytes from MHA and LSH hamsters in the presence of homologous splenic lymphokines. MHA CM was assayed on MHA thymocytes (o——o), LSH CM was assayed on LSH thymocytes (Δ——Δ). Background proliferation was determined by the addition of medium containing 3.3 μg/ml ConA in place of CM. Values represent the mean cpm ± SD of quadruplicate test wells minus the mean cpm of background wells.

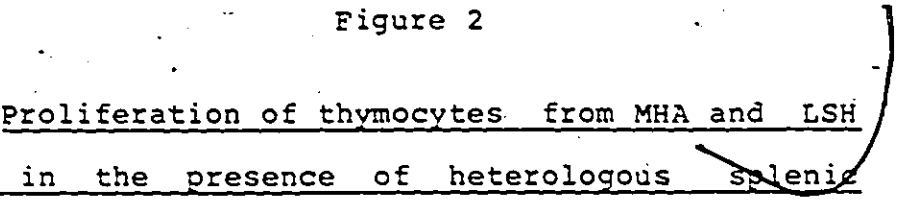

^{3}H-THYMIDINE INCORPORATION (cpm x 10^3)
10
8
6
4
2
0
4
8
16
32
% CONCENTRATION OF CM

B. Production of lymphokines

The differences in proliferation of thymocytes could represent the difference in lymphokine production or differences in responsiveness of the thymocytes to the lymphokines. To differentiate between these possibilities, CM prepared from the spleens of both strains were assayed on both MHA and LSH thymocytes. The data in Figure 2A indicate that both LSH and MHA CM produced a dose-dependent augmentation of proliferation of MHA thymocytes. In contrast, less mitogenic activation was seen in LSH cultures (Figure 2B). In experiments comparing the activity of 12 preparations of CM on either MHA or LSH thymocytes, analysis of variance showed no difference between MHA and LSH CM in the capacity to stimulate growth, and in 9 of 12 comparisons, MHA thymocytes proliferated to a greater extent than did LSH thymocytes ($p<0.025$), regardless of the source of CM. These results indicate that the production of lymphokines was similar in the two strains, but that MHA and LSH thymocyte populations differed in their capacity to respond to lymphokine(s).

CM prepared from lymph nodes (LN) of both strains also appeared to contain equal amounts of activity as assessed by thymocyte proliferation (Figure 3A and 3B). As well, LN CM, contrary to splenic CM, were able to induce proliferation of a murine IL2-dependent cell line. It was

Figure 2



Proliferation of thymocytes from MHA and LSH hamsters in the presence of heterologous splenic lymphokines. A. Proliferation of MHA thymocytes in the presence of MHA CM (open symbols) and LSH CM (closed symbols). B. Proliferation of LSH thymocytes in the presence of MHA CM (open symbols) and LSH CM (closed symbols). Background proliferation was determined by the addition of medium containing 3.3 μg/ml ConA in place of CM. Values represent the mean cpm ± SD of quadruplicate test wells minus the mean cpm of background wells.

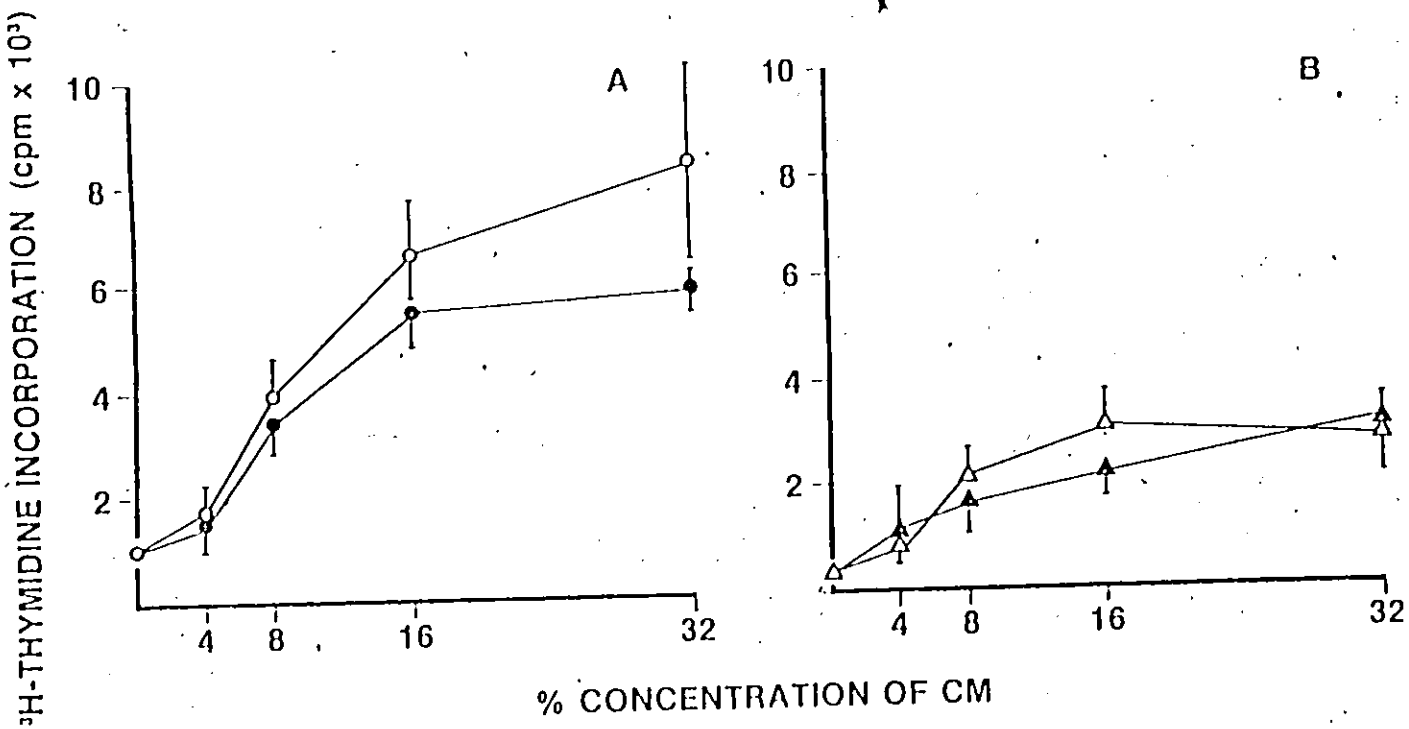
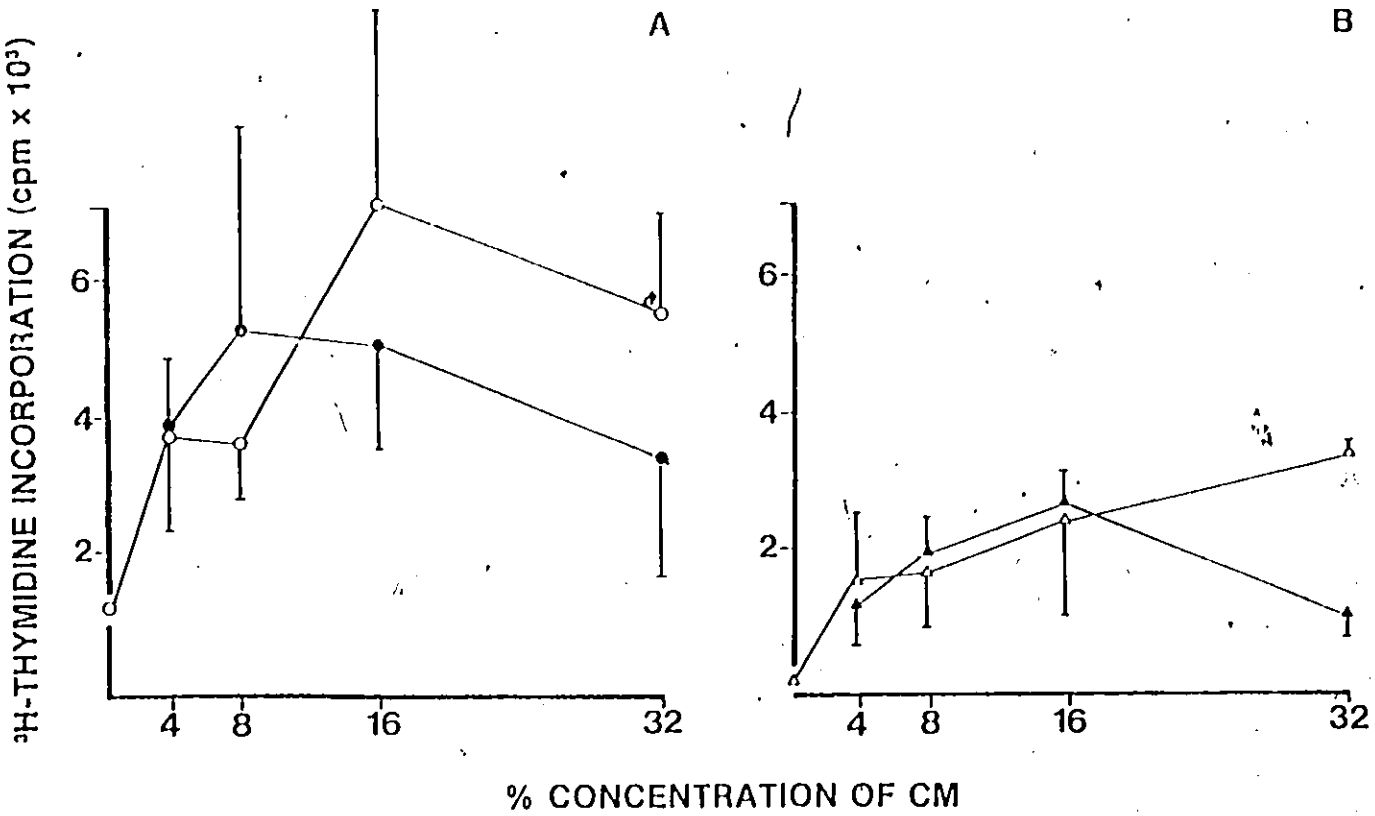

³H-THYMIDINE INCORPORATION (cpm x 10³)
10
8
6
4
2
A
4
8
16
32
B
10
8
6
4
2
4
8
16
32
% CONCENTRATION OF CM

Figure 3

Proliferation of thymocytes in response to lymphokines from hamster lymph node. A. Proliferation of MHA thymocytes in the presence of MHA CM (open symbols) and LSH CM (closed symbols). B. Proliferation of LSH thymocytes in the presence of MHA CM (open symbols) and LSH CM (closed symbols). Background proliferation was determined by the addition of medium containing 3.3 μg/ml ConA in place of CM. Values represent the mean cpm ± SD of quadruplicate wells minus the mean cpm of background wells.


A
B
³H-THYMIDINE INCORPORATION (cpm x 10³)
6
4
2
4
8
16
32
% CONCENTRATION OF CM

found that preparations of LN CM from LSH hamsters were significantly more active than MHA CM in 3 of 3 experiments when assayed on such a cell line in the laboratory of Dr. K. Rosenthal ($p< 0.05$) (Figure 4).

The production of IL1 by adherent splenic and lymph node cells was also assayed, and there was no significant difference in the synthesis of this lymphokine (Figure 5). There was also no difference in the production of epidermal T-cell activating factor (ETAF) by keratinocytes from the two strains, as tested by Dr. D. Sauder (data not shown).

C. Responsiveness to lymphokines from other species

Further evidence that thymocyte populations from the two strains differ in their response to lymphokines was provided by experiments in which a standard preparation of rat CM was tested (Figure 6). Again, the proliferation of MHA thymocytes was significantly greater than that of LSH thymocytes, and this difference in response was evident in testing 5 of 7 different rat CM preparations ($p<0.025$). Because rat CM proved to be more active in these assays than hamster CM, further experiments were performed only with rat CM.

III. Frequency of Thymocytes Proliferating in Response to Lymphokines

The difference in proliferation in thymocyte populations could be due to differences in sensitivity to

Figure 4

Activity of hamster lymph node lymphokines on a murine IL2-dependent cell line. CM prepared from MHA lymph node (circles) and LSH lymph node (triangles) were assayed at various concentrations for their ability to induce proliferation of the murine cells HY-1. Points represent the mean cpm of duplicate test wells; open symbols represent experiment 1, closed symbols, experiment 2. The assay was conducted in the laboratory of Dr. K. L. Rosenthal, McMaster University.

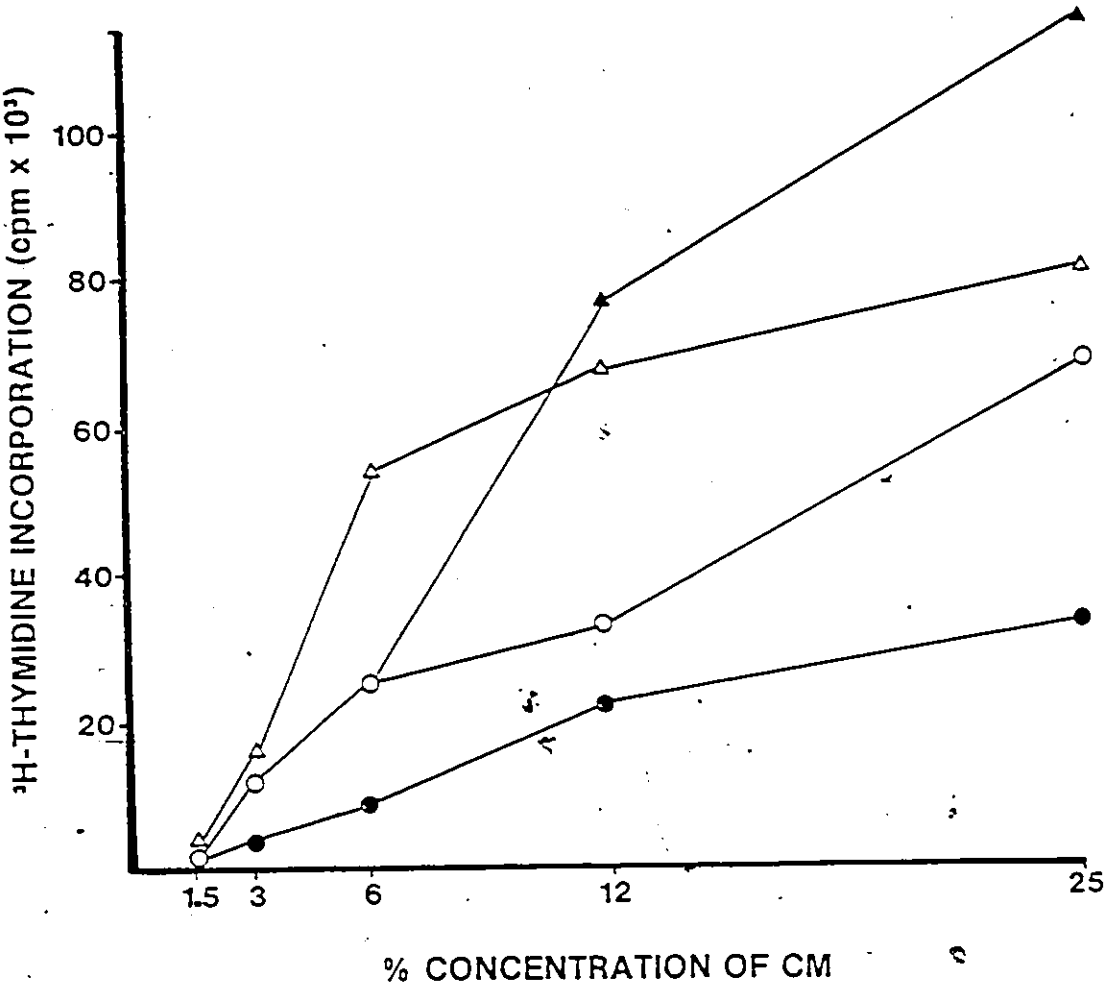

^{3}H-THYMIDINE INCORPORATION (cpm x 10^3)
100
80
60
40
20
1.5
3
6
12
25
% CONCENTRATION OF CM

Figure 5

Production of interleukin 1 by hamster adherent lymph node cells. Supernatants prepared from adherent cells from MHA lymph node (O—O) and LSH lymph node (Δ—Δ) were assayed for IL1 activity in a murine thymocyte proliferation assay. Points represent the mean cpm of duplicate wells minus the background proliferation of thymocytes in the presence of control medium. The assay was conducted by C.D. Richards, McMaster University.

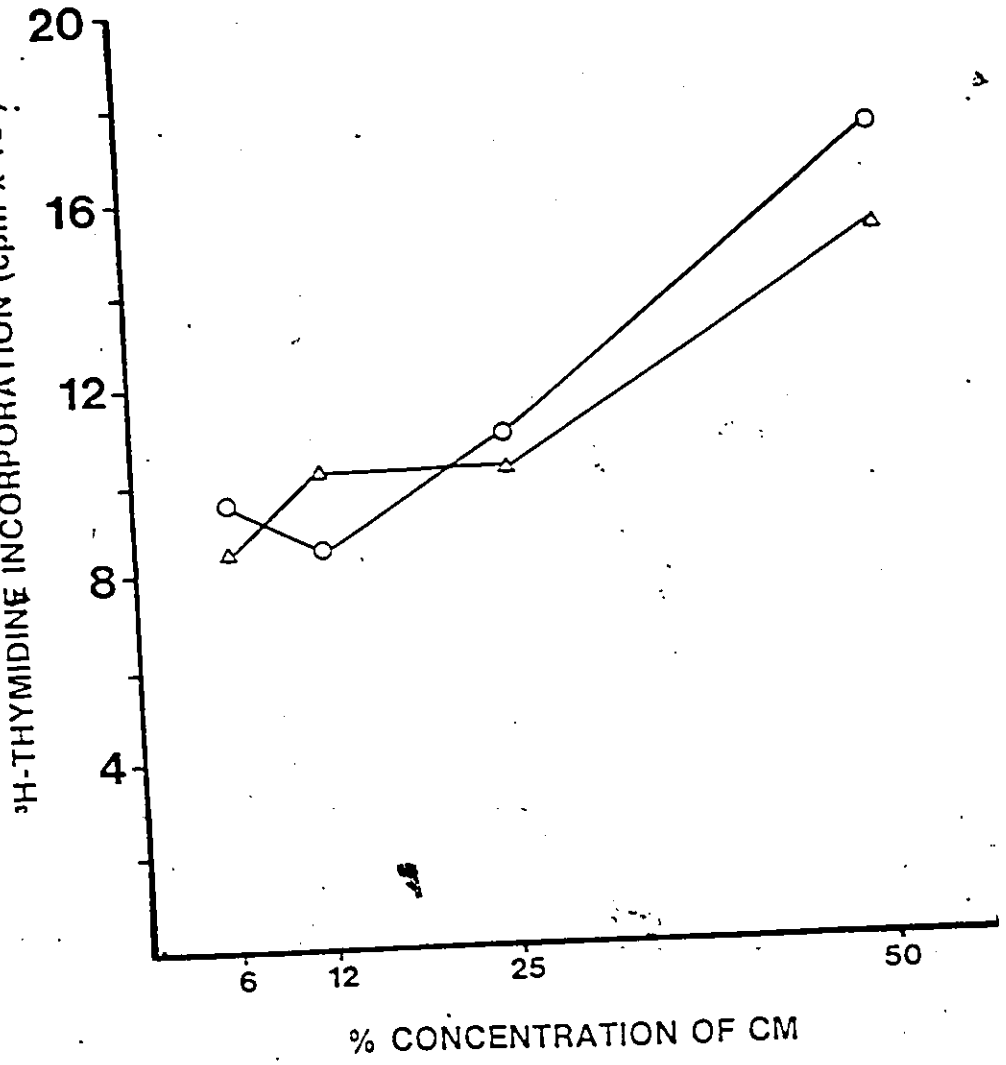
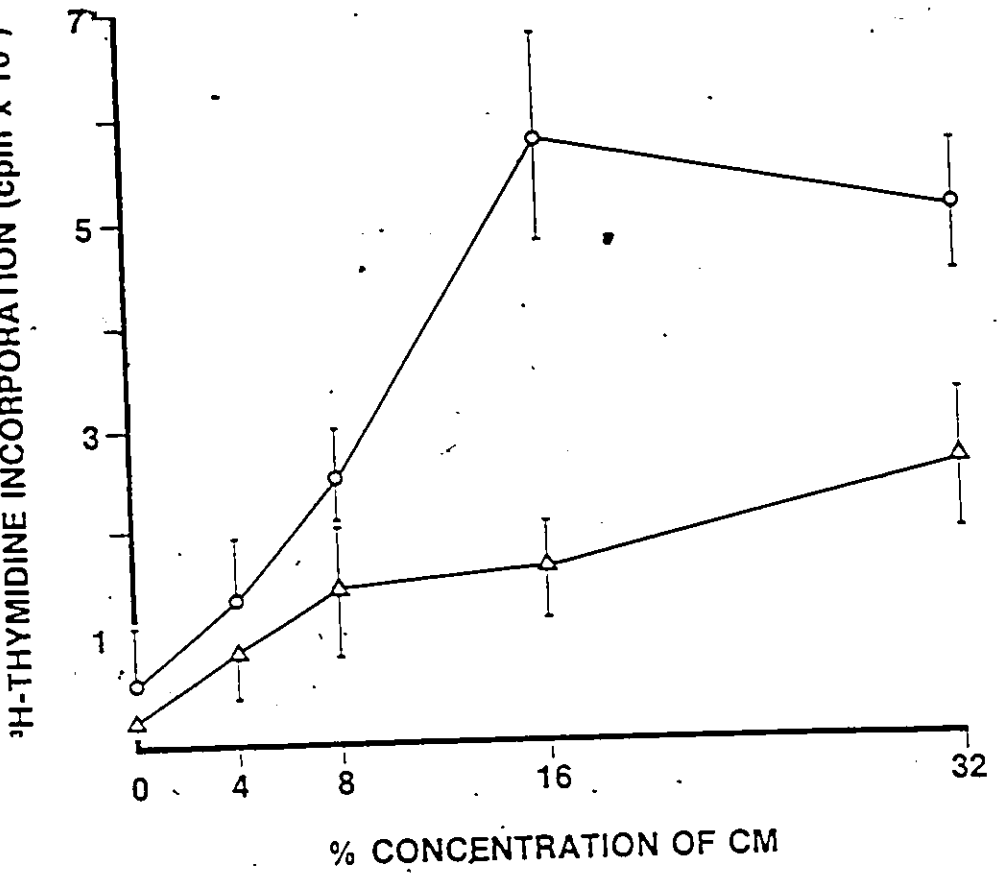

20
16
12
8
4
^{3}H-THYMIDINE INCORPORATION (cpm x 10^3)
6
12
25
50
% CONCENTRATION OF CM

Figure 6

Proliferation of hamster thymocytes in the presence of rat splenic CM. Rat CM was added to MHA thymocytes (O———O) or LSH thymocytes (Δ———Δ). Background proliferation was determined by the addition of medium containing 5 μg/ml ConA in place of CM. Values represent the mean cpm ± SD of quadruplicate test wells minus the mean cpm of background wells.


^{3}H-THYMIDINE INCORPORATION (cpm x 10^3)
7
5
3
1
0
4
8
16
32
% CONCENTRATION OF CM

CM or to a difference in the numbers of thymocytes responding to CM. To determine which of these possibilities was the case, limiting dilution assays were performed to evaluate the frequency of cells proliferating when cultured in the presence of 30% rat CM plus ConA. This concentration of CM was chosen as it was optimal in inducing proliferation in LSH thymocytes. The data in Table II show that there are more cells in MHA thymi proliferating in response to CM than in LSH thymi. The mean frequency of cells proliferating in MHA thymocyte populations was twice that of LSH thymocytes in 3 of 4 experiments.

IV. Growth and Cytotoxicity of Hamster Splenocytes in Rat Conditioned Medium

A. Growth of splenocytes in rat conditioned medium

Lymphokines in ConA-induced CM, particularly IL2, are known to be growth factors for both T cells and NK cells in human and murine systems (Gillis and Smith 1974, Morgan et al 1976, Luger et al 1983, Timonen et al 1983). Accordingly, the effects of CM on LSH and MHA splenocytes were examined. Normal splenocytes from MHA and LSH hamsters were cultured in medium alone, or in medium supplemented with 15% or 30% crude rat CM. The growth curves of splenocytes from both strains of hamster were similar. In the absence of CM, cell numbers decreased and remained low throughout the culture period (Figure 7A). In the presence

TABLE II

Limiting Dilution Analysis of Thymocytes Proliferating in Response to Lymphokines

Cells/well	Wells positive	Predicted wells positive	Frequency[a]
MHA			
50,000	16/16	16/16	
10,000	16/16	16/16	1/1860
2,000	9/16	11/16	
400	5/16	3/16	(1117-3093)
		X^2=2.190	
LSH			
50,000	16/16	16/16	
10,000	15/16	15/16	1/3485
2,000	0/16	7/16	
400	2/16	2/16	(1868-6501)
		X^2=0.057	

[a] The frequency of thymocytes proliferating in response to 30% rat CM was calculated as described in Materials and Methods. Parentheses enclose 95% confidence limits. Using calculated frequency of proliferating cells, the expected proportion of positive wells was calculated for each dilution of test wells using the following formula: Probability of no response, $P(0) = e^{-Nf}$, where N is the number of cells/well, and f is the calculated frequency of proliferating cells.

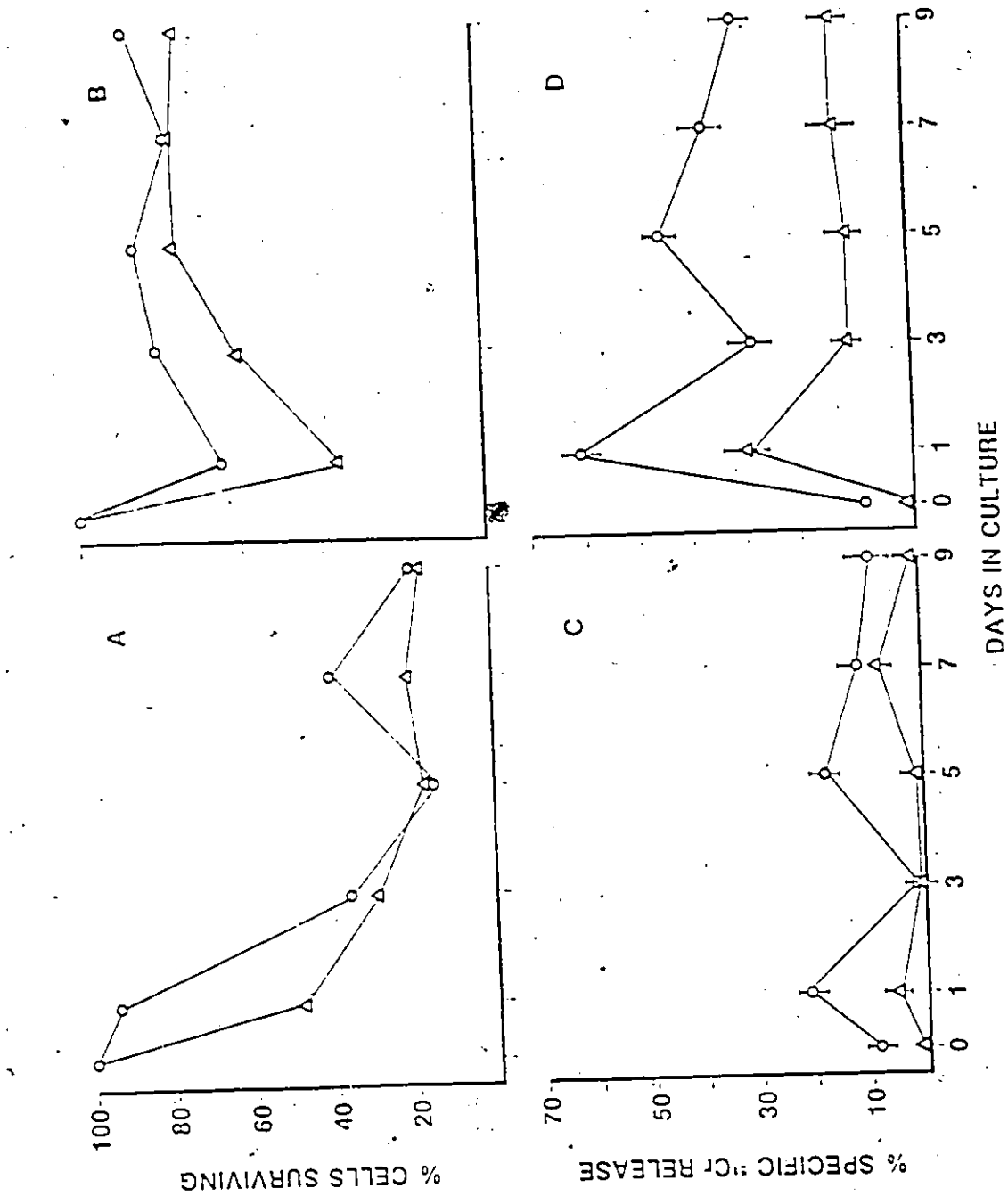
of CM, cell numbers initially dropped, then increased at days 3 to 5, and remained constant to day 9 (Figure 7B).

B. Cytotoxic activity of splenocytes cultured in rat conditioned medium

Lymphokines have been shown to augment human and murine NK-type activity after short term exposure in vitro (Kuribayashi et al 1981, Henney et al 1981, Minato et al 1981, Miyasaki et al 1984) and after longer culture (Minato et al. 1981, Grimm et al 1982,1983a). Splenocytes from both strains of hamsters were cultured in the presence or absence of rat CM and were then assayed for cytotoxic activity against K562 cells. Cytotoxic activity was induced in cultures of splenocytes from both strains of hamster, and appeared to be independent of growth of cells in culture. In the absence of CM, significant cytotoxic activity was not detected in cultured LSH splenocytes over the duration of the experiment (Figure 7C). MHA splenocytes cultured without CM were found to be cytotoxic after 24 hr in culture; the cytotoxic activity then declined, but increased at days 5 to 7. This pattern of a peak of cytotoxic activity after 24 hr in culture, followed by a decrease and subsequent increase in activity at 5 to 7 days, was observed when either LSH or MHA splenocytes were cultured in the presence of CM (Figure 7D). The peak at 24 hr was consistently higher than the later peak in LSH cultures, but at no time did the cytotoxic activity match

Figure 7

Growth and cytotoxicity of hamster splenocytes in rat CM. Normal splenocytes from MHA (O——O) and LSH (Δ——Δ) hamsters were cultured at 2 to 4 x 10^6 cells/ml in culture medium (A) or in culture medium supplemented with 30% rat CM (B). Growth was determined by counting viable cells by trypan blue exclusion. Cytotoxic activity of MHA (O——O) or LSH (Δ——Δ) spleen cells cultured in culture medium (C) or in culture medium supplemented with 30% rat CM (D) was determined. Cytotoxic activity was assayed on ^{51}Cr-labelled K562 target cells. Points represent the mean ^{51}Cr release of quadruplicate wells ± SD, at an effector to target ratios of 50:1.


A
B
C
D
% CELLS SURVIVING
% SPECIFIC 51Cr RELEASE
DAYS IN CULTURE
100
80
60
40
20
70
50
30
10
0
1
3
5
7
9

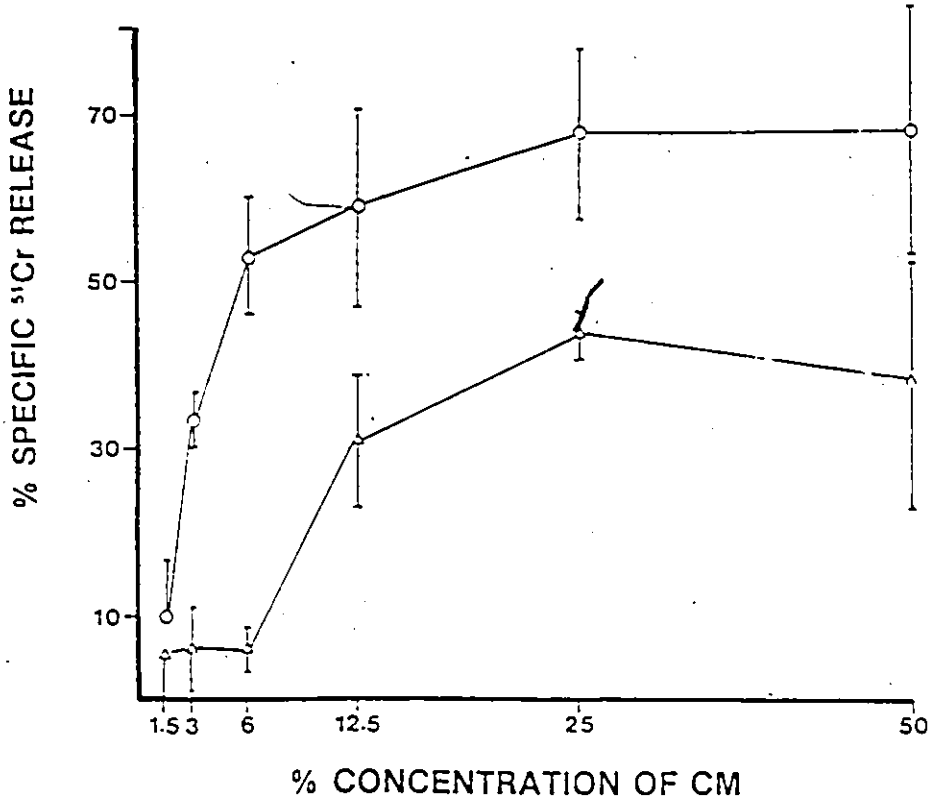
that in MHA cultures (4 of 5 experiments). In MHA cultures, the time point of peak cytotoxic activity varied from experiment to experiment. The cells responsible for the cytotoxic activity in cultures containing CM will be referred to as lymphokine-activated killer cells (LAK). In the presence of CM, LAK in cultures of MHA splenocytes was greater than LAK in cultures of LSH splenocytes at both 24 hr (5 of 5 experiments, $p<0.01$) and 7 days (7 of 9 experiments, $p<0.01$). No cytotoxic activity could be detected in either bone marrow or thymocytes cultured under similar conditions.

C. Titration of conditioned medium in the induction of cytotoxic cells in splenocyte cultures

Initially, concentrations of CM were used in splenocyte cultures based on the results of thymocyte proliferation assays. However, the possibility remained that reactivity of thymocytes may not adequately reflect the responsiveness of splenocytes to lymophokines in the CM. An assay was developed for determining the optimum concentrations of CM for inducing cytotoxic activity in splenocyte cultures. A fixed number of splenocytes were cultured in varying amounts of CM in microtitre plates for 6 days, at which time, ^{51}Cr-labelled target cells were added at a concentration giving an effector:target ratio of 100:1 based on input counts of effector cells. The data presented in Figure 8 show that the capacity of CM to

Figure 8

Cytotoxicity of hamster splenocytes cultured in various concentrations of rat CM. 5×10^5 normal splenocytes from MHA (O——O) and LSH (Δ——Δ) hamsters were cultured in a volume of 0.2 ml of various concentrations of rat CM. After 6 days, 0.1 ml of medium was removed and an equal volume of ^{51}Cr-labelled target cells were added at 5×10^3/well. After a further 16 hr period, cytotoxicity was assayed. Points represent the mean ^{51}Cr release ± SD of quadruplicate wells.


% SPECIFIC ^{51}Cr RELEASE
70
50
30
10
1.5
3
6
12.5
25
50
% CONCENTRATION OF CM

induce cytotoxic activity titrates out more readily in LSH splenocyte cultures than in MHA cultures, so that in the majority of experiments (3 of 4), the optimum concentration of CM was 25% for LSH, but 12.5% for MHA (3 of 5). These results do not parallel the results of the thymocyte assays, where for both strains 30% CM was optimal. In 4 of 4 experiments, MHA LAK activity was significantly greater than LSH LAK activity at concentrations of CM greater than 12% ($p<0.025$). In most experiments, CM was used at the higher concentration to best induce activity in LSH cultures.

V. Response of Lymphocytes to Purified Interleukin 2

As previously mentioned, IL2 alone augments NK activity in other species, (Henney et al 1981, Grimm et al 1983a, Miyasaka et al 1984), and is adequate to induce lymphokine activated killer cells after culture of human and murine lymphocytes (Kuribayashi et al 1981, Grimm et al 1983a, Suzuki et al 1983, Timonen et al 1983, Merluzzi et al 1984). It was of interest to determine whether hamsters were similar in this respect. As well, culture of splenocytes in purified IL2 might serve as a method of selecting for the subpopulation of lymphocytes responsible for the observed cytotoxic activity. Lastly, examination of responses to purified IL2 of both thymocytes and splenocytes might give a better indication of the relationship between the cells in the thymus and spleen

responding to lymphokines in the assays used.

A. Response of thymocytes to purified interleukin 2

Purified murine IL2 was assayed for its ability to induce proliferation of thymocytes in the presence of mitogen. The same pattern was observed as was seen with crude CM; MHA thymocytes proliferated to a significantly greater extent than LSH thymocytes in concentrations of IL2 greater than 30 U/ml plus ConA in 3 of 3 experiments ($p<0.025$) (Figure 9). In the thymocyte assay, it appeared that 33 Units/ml of murine IL2 was equivalent to approximately 15% ConA CM. One unit is the amount of IL2 in 1 ml that causes 30% of maximal proliferative response of the murine T cell line MTL2.8 obtained in the same experiment (Riendeau et al 1983).

B. Response of splenocytes to purified interleukin 2

Both murine and human IL2 were titrated for their ability to induce LAK in splenocyte cultures in the microassay. In 11 of 14 experiments, IL2 induced greater cytotoxic activity in MHA cultures than in LSH cultures, ($p<0.01$) and the activity of IL2 titrated out more quickly in LSH cultures than MHA (Figure 10). In MHA cultures, there was some lytic activity in cells cultured in as little as 3.1 half-maximal units/ml of purified human IL2, but in LSH at least 12.5 units/ml of purified IL2 were necessary to produce lytic activity above background levels. In the majority of experiments, the optimal

Figure 9

Proliferation of hamster thymocytes in response to murine IL2. Purified murine IL2 was added to. MHA (O——O) or LSH (Δ——Δ) thymocytes. Points represent the mean of quadruplicate wells ± SD minus the background proliferation determined by culture of thymocytes in medium lacking IL2.

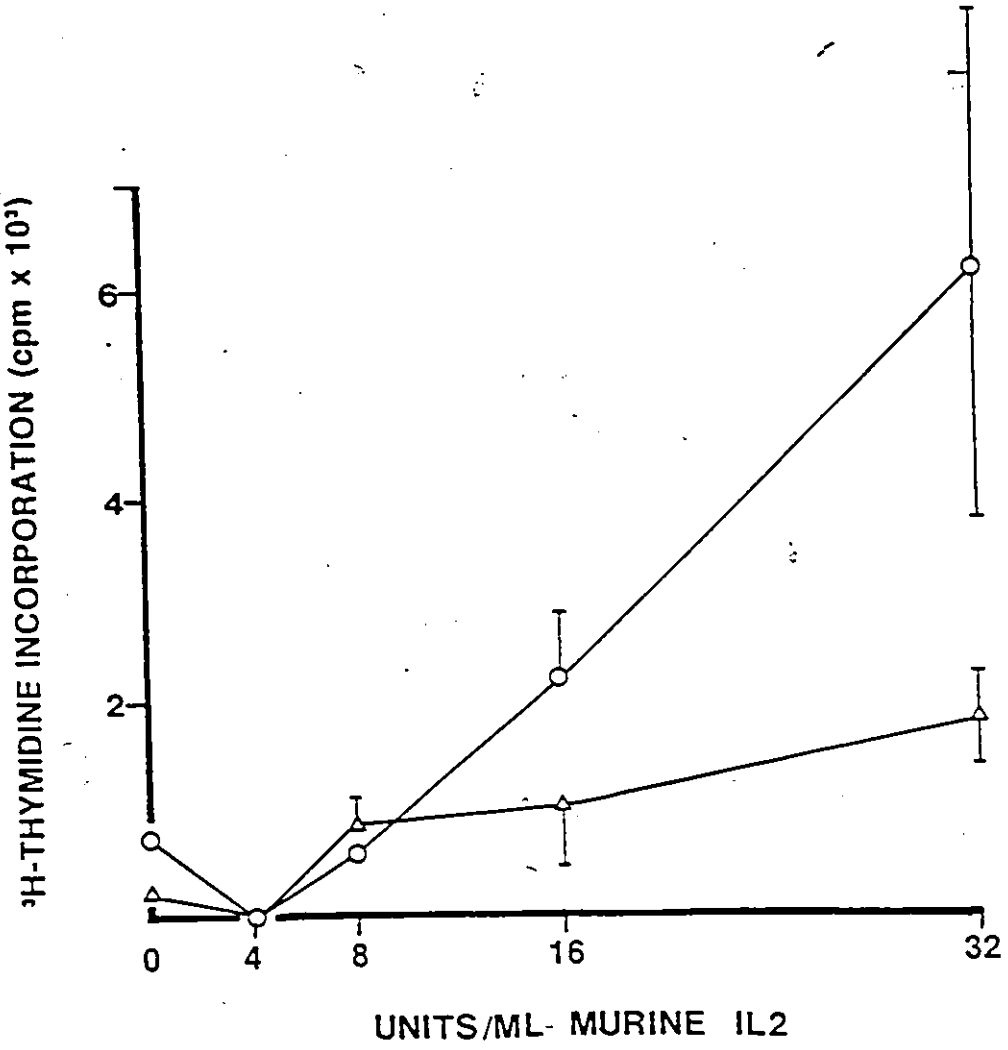
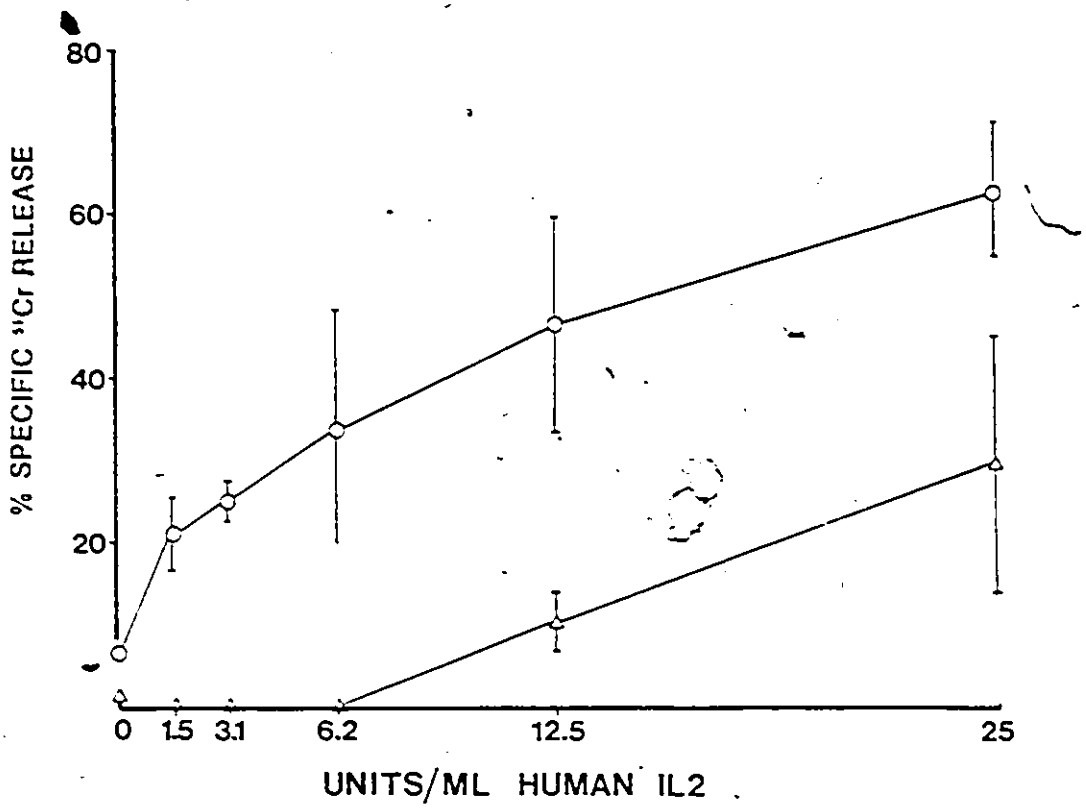

^{3}H-THYMIDINE INCORPORATION (cpm x 10^3)
6
4
2
0
4
8
16
32
UNITS/ML- MURINE IL2

Figure 10

Cytotoxicity of hamster splenocytes cultured in varying concentrations of human IL2. 5×10^5 normal splenocytes from MHA (O——O) and LSH (Δ——Δ) hamsters were cultured in a volume of 0.2 ml of various concentrations of purified human IL2. After 5 days, 0.1 ml of medium was removed and an equal volume of ^{51}Cr-labelled K562 target cells was added at 5×10^3 cells/wells. Cytotoxicity was assayed after 16 hr. Points represent the mean ^{51}Cr release ± SD of quadruplicate wells.


80
60
40
20
% SPECIFIC ^{51}Cr RELEASE
0
1.5
3.1
6.2
12.5
25
UNITS/ML HUMAN IL2

concentration of IL2 was low, 12.5-16 units/ml if the media was changed once over the course of culture, and 25-32 units/ml if the media was not changed; the total amount of IL2 required was the same.

C. Kinetics of generation of lymphokine activated killer cells in interleukin 2

The kinetics of generation of LAK in purified IL2 was examined to determine if the cytotoxic activities observed at 1 day and 5-7 days after culture in ConA CM could both be induced by IL2. At both 1 day and 5 days after culture in IL2, the cytotoxic activity was greater than that observed in cells cultured in medium alone, or cells not cultured at all (Figure 11).

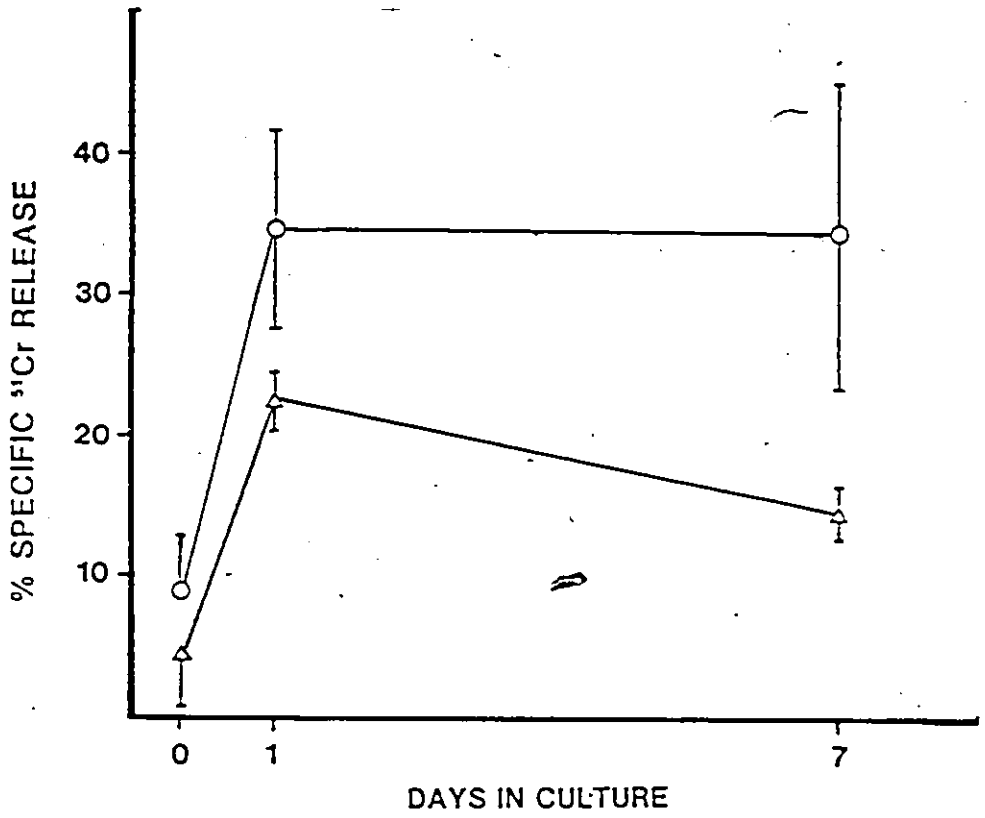
VI. Frequency of precursors of lymphokine activated killer cells in MHA and LSH spleens

A. Frequency of precursors

As in the case of the differences observed in the thymocyte assay, the differences described in the splenocyte cultures could be due to an increased number of cells in MHA spleens capable of responding to lymphokines in CM. A second possibility is that a difference exists between the hamster strains in the sensitivity of cells to CM, although their numbers may be comparable. A third possibility is that the expression of cytotoxic activity in culture is inhibited by suppressor cells, which are greater in number in LSH than in MHA spleens. To examine these

Figure 11

Kinetics of LAK induction after culture of splenocytes in IL2. Splenocytes from MHA (O——O) or LSH (Δ——Δ) hamsters were cultured in the presence of 16 U/ml of human IL2 for various times, then assayed for cytotoxicity as described in Materials and Methods. Points represent the mean ^{51}Cr release ± SD of quadruplicate wells.


% SPECIFIC ^{51}Cr RELEASE
40
30
20
10
0
1
7
DAYS IN CULTURE

possibilities, limiting dilution assays were performed to evaluate the frequency of precursors of LAK in the two strains of hamster generated after 7 days of culture in 30% CM. Initially it was established that medium alone, or medium containing 1.5 μg/ml ConA did not activate cytotoxic effectors. The data in Table III show that there were more LAK precursors in MHA than in LSH spleens, and demonstrate that a feeder layer of peritoneal exudate cells (PEC) was not a requirement for the generation of LAK. Both in the presence and absence of PEC, MHA spleens contained at least twofold more cytotoxic precursors than LSH in 6 of 9 experiments. These data suggest that MHA spleens contain more precursors of cytotoxic cells than do LSH spleens, and the differences in cytotoxic activity is not due to an intrinsic difference in the sensitivity of splenocytes to CM.

B. Effect of peritoneal exudate cells on precursor frequency

In addition to demonstrating that PEC were not necessary for the generation of cytotoxic effectors, the results suggested that PEC reduce the frequency of precursors activated in culture. Further experiments were performed to examine this concept. PEC from each strain of hamster reduced the frequency of cytotoxic precursors in both MHA and LSH spleen. In LSH, the addition of PEC often reduced the frequency beyond the sensitivity of the assay

TABLE III

Limiting Dilution Analysis of LAK Precursors in MHA and LSH Spleens

Cells/well	Wells positive	Predicted wells positive	Frequency[a]
MHA			
100,000	16/16	16/16	1/6582
20,000	15/16	15/16	
4,000	8/16	7/16	(4076-10630)
800	2/16	2/16	
160	0/16	0/16	
		χ^2=0.615	
LSH			
100,000	10/16	9/16	1/113572
20,000	2/15	2/15	
4,000	0/14	0/14	(64042-201408)
800	0/16	0/16	
160	0/16	0/16	
		χ^2=0.691	

[a] The frequency of LAK precursors in the absence of a feeder layer was calculated as described in Materials and Methods. Parentheses enclose 95% confidence limits. Using calculated frequency of precursors, the expected proportion of positive wells was calculated for each dilution of test cells.

system, so that the decrease in frequency was difficult to quantitate. For MHA, in 7 of 9 experiments, this reduction was at least 50%. In those experiments where MHA and LSH could be compared, the observed decrease in frequency appeared to be no greater in one strain than in the other, ranging from twofold to greater than hundredfold in both strains. Representative experiments are presented in Table IV.

C. Effect of depletion of splenic adherent cells on precursor frequency

Adherent cells that suppress NK activity have been demonstrated in both the spleen and peritoneal cavity of mice (Cudkowicz & Hoffman 1979, Riccardi *et al* 1981, Santoni *et al* 1982, Brunda *et al* 1983). The lower NK activity seen in LSH spleen *in vivo*, *in vitro* and in limiting dilution experiments could be the result of such suppressor cells present in LSH spleens. Relevant to this possibility was the observation that fewer wells receiving 500,000 LSH splenocytes were positive in limiting dilution assays than wells receiving 100,000 splenocytes (Figure 12). The mean percent positive wells at the highest cell concentration was 44% in 8 experiments, whereas 81% and 52% of the wells, respectively, seeded at the next two lower concentrations of cells, were positive. A similar pattern was not observed in assays of MHA splenocytes. This observation suggests that at higher concentrations of

TABLE IV

Limiting Dilution Analysis of the Effect of Peritoneal Exudate Cells on the Generation of Splenic LAK

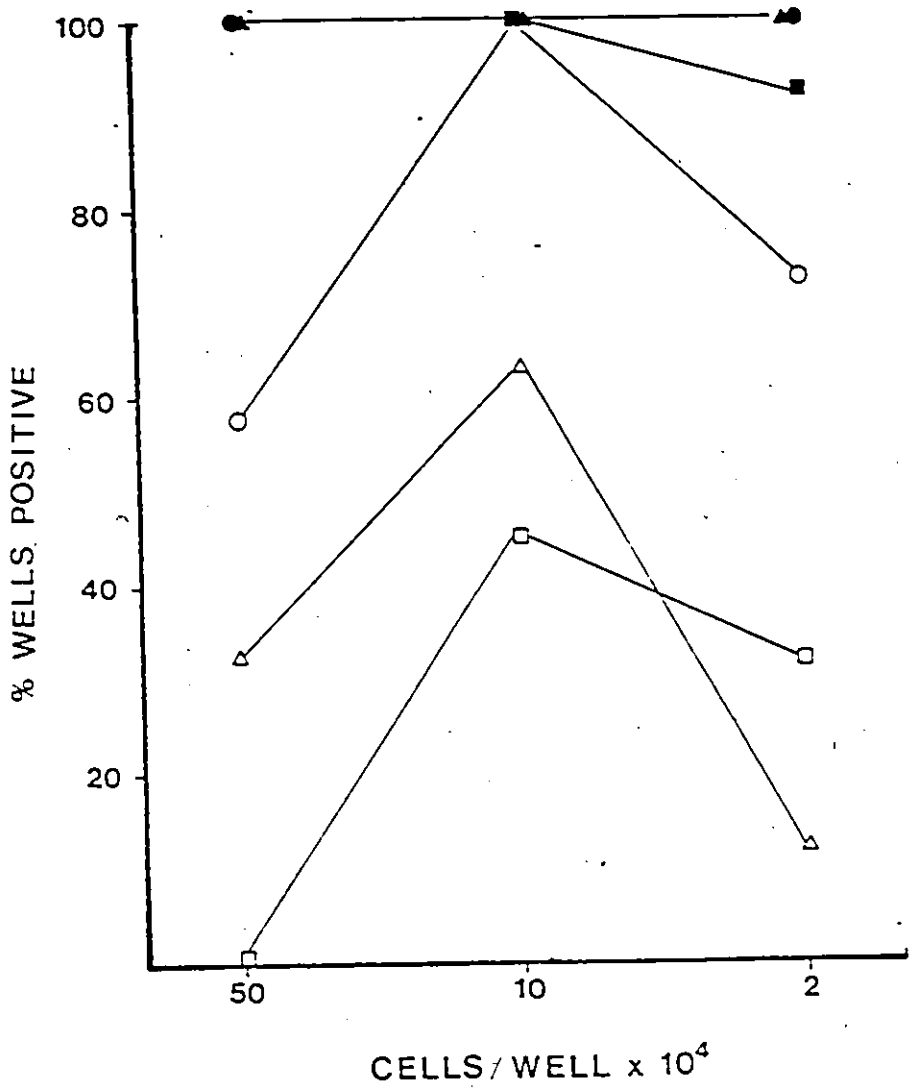
	MHA		LSH	
	with PEC	no PEC	with PEC	no PEC
Expt. 1	1/28587	1/8150	1/54970	1/7673
	(17494-46715)	(5056-13139)	(23138-130593)	(4175-12495)
Expt. 2	1/54488	1/6629	*	
	(33639-88116)	(4337-12030)		

The frequency of precursors in the presence or absence of 5×10^4 irradiated PEC (3000R) was caculated as described in Materials and Methods. Parentheses enclose 95% confidence limits.

* Results did not allow calculation of frequency of precursors.

Figure 12

Percentage of wells positive for cytotoxicity in limiting dilution assays. Normal MHA (closed symbols) and LSH (open symbols) splenocytes were cultured in 30% rat CM at varying cell concentrations as outlined in Materials and Methods. After 7 days, 0.1 ml of medium was removed and an equal volume of ^{51}Cr-labelled K562 target cells was added at 5×10^3 cells/well. Wells were considered positive for cytotoxic activity when ^{51}Cr release was greater or equal to background release plus 3 SD. Background release was the amount of ^{51}Cr released when targets were added to splenocytes cultured in the absence of rat CM. Data from 3 experiments are shown, ○ ●, □ ■, △ ▲.


100
80
60
40
20
% WELLS POSITIVE
50
10
2
CELLS / WELL x 10^4

splenocytes, the expression of LSH precursors of cytotoxic cells may have been hampered by suppressor cells; however, a suppressor effect of an adherent cell population could not be detected in the limiting dilution assay (Table V). Thus, whereas the LSH hamsters may have a greater number of functional suppressor cells than MHA hamsters, the LSH hamsters also have fewer precursor cytotoxic cells.

VII. Characterization of Hamster Cytotoxic Cells

Three populations of cytotoxic cells have been identified in normal hamster spleen; endogenous NK, 1 day LAK and 5 to 7 day LAK. Experiments were undertaken to characterize these populations of cells, particularly endogenous NK cells, and the 5 to 7 day LAK, as it is the precursors of these cells detected in the limiting dilution assays.

A. Adherence properties

It has previously been shown that endogenous splenic NK activity could be enriched by the removal of plastic adherent cells (Datta et al 1979, Gee 1979, Yang et al 1983). It has also been published that the nylon wool nonadherent population is further enriched for NK activity (Yang et al 1982). This has been confirmed in both MHA and LSH hamsters as shown in Table VI. As well, LAK harvested at both 1 day and 5 to 7 days were plastic nonadherent, as only the nonadherent cells were harvested. Both populations were further enriched after passage through

TABLE V

Limiting Dilution Analysis of LAK Precursors in Adherent and Nonadherent Spleen Cells

	Expt. 1		Expt. 2	
	MHA	LSH	MHA	LSH
Unseparated	1/5706 (3886-8377)	1/19205 (11574-31869)	1/9822 (6707-14383)	1/18905 (11900-30032)
Nonadherent[a]	1/6963 (4655-10416)	1/33465 (21139-52978)	1/17387 (9299-32508)	1/19345 (12679-29307)
Adherent	1/195719 (104705-365847)	1/166707 (73259-383925)	1/51049 (30596-85174)	1/509310 (238725-1086590)

[a]Spleen cells were diluted in microtitre plates, incubated at 37°C for 2 hr, and then nonadherent cells were transferred to a separate plate. Frequencies of precursor cells were calculated for each population after culturing for 7 days as described in Materials and Methods. Parentheses enclose 95% confidence limits.

nylon wool (Table VI). Again, MHA cultures contained greater cytotoxic activity, even after enrichment procedures, than LSH in 6 of 6 experiments ($p<0.01$).

B. Surface antigen expression

1. Thy 1.2 homologue

It is difficult to characterize hamster cytotoxic cells on the basis of surface antigen expression because of the absence of reagents detecting surface markers on hamster lymphocyte subpopulations. Based on the results of experiments titrating anti-murine Thy 1.2 sera on hamster lymphoid cells, Yang and co-workers (1982) concluded that hamster T cells express an antigen homologous to the murine theta antigen. To verify these results, two sera specific for the rat and murine theta antigens were titrated on hamster tissues for their ability to lyse cells in the presence of complement (Figure 13). There was no difference in expression of this antigen between the strains (data not shown). The distribution of cells expressing the Thy 1.2 homologue agrees with that seen by Yang and Tompkins, except for a higher expression in spleen. However, there have also been reports that both T and B cells in the hamster express this antigen when assayed by immunofluorescence (Witte and Streilein 1983). Hamster splenocytes and lymph node cells were assayed for the expression of this antigen by cytofluorometry and the results support these findings; greater than 80% of

TABLE VI

Adherence Properties of Hamster Cytotoxic Cells

Effectors[a]	Fraction[b]	Percent Cytotoxicity[c]	
		MHA	LSH
Endogenous	whole spleen	19.3±0.3	6.0±0.8
NK	plastic nonadherent	29.4±5.9	5.0±2.1
	nylon wool nonadherent	53.2±1.8	20.1±3.9
	nylon wool adherent	10.3±1.3	0
5 day LAK	plastic nonadherent	67.5±19.0	12.3±5.2
	nylon wool nonadherent	88.8±10.4	69.8±8.1
	nylon wool adherent	n.d.	n.d.

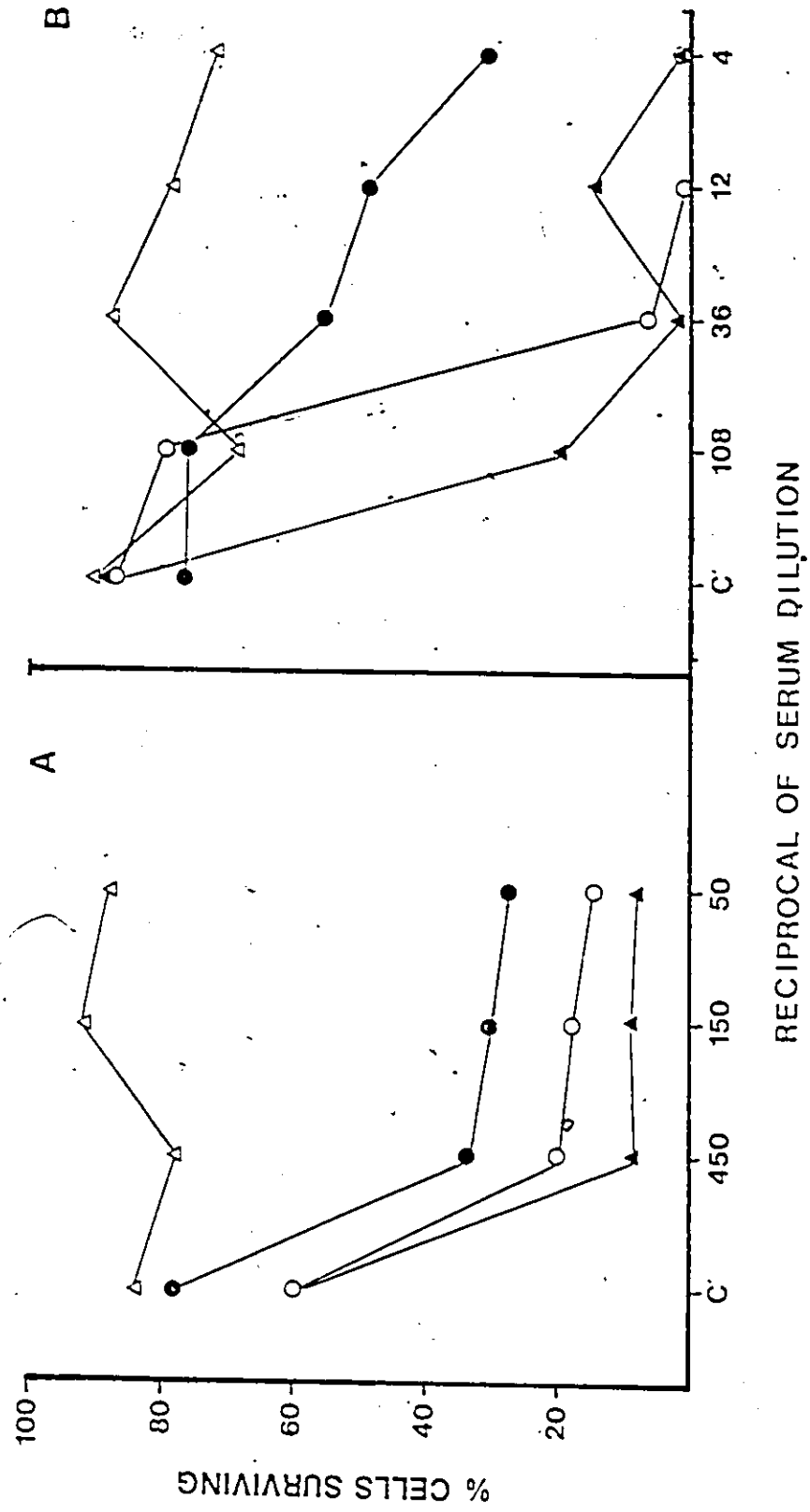
[a]MHA and LSH spleen either fresh or cultured for 5 days in 12% rat CM. Effector to target ratio is 50:1.

[b]Cells were tested for cytotoxic activity after separation of fractions as described in Materials and Methods.

[c]Percent cytotoxicity ± SD was calculated as described in Materials and Methods. n.d. signifies not done.

Figure 13

Expression of Thy 1.2 homologue on hamster lymphoid cells. Normal cells from MHA tissues were treated with varying dilutions of monoclonal anti-Thy 1.2 (A) or with rabbit anti-rat brain serum (B) plus complement. C' represents control cells treated with complement alone. Viability of cells was determined by trypan blue exclusion. Lymph node (O——O); spleen (●——●); bone marrow (Δ——Δ); thymus (▲——▲).


A
B
% CELLS SURVIVING
100
80
60
40
20
C'
450
150
50
C'
108
36
12
4
RECIPROCAL OF SERUM DILUTION

splenocytes were positive for this antigen (data not shown).

Both fresh NK cells and LAK induced after 5-7 days in culture from both strains of hamsters were tested for the expression of this homologue. After treatment with antibody and complement (C'), cytotoxicity in all groups was reduced by 70 to 90% (Table VII).

2. Asialo Gml

In the mouse, treatment of lymphocytes with low amounts of antiserum directed to the glycolipid 'asialo GM1' plus complement selectively eliminates NK activity while leaving other immune responses intact (Young et al 1980, Kasai et al 1980). Accordingly it was found that in the mouse, the antiserum diluted to 1/100 decreased NK activity by 90% (Figure 14). When titrated out on hamster spleen, dilutions of 1/30 and 1/60 reduced endogenous NK activity by 50% (Figure 15). LAK generated after 5 days in culture with CM were negative for expression of this marker (Table VIII).

C. Effect of anti-asialo GM1 serum on natural killer activity in vivo

Treatment of mice and rats with anti-asialo GM1 serum reduced splenic NK activity without altering the numbers of cells expressing Thy 1.2, the prolferative response to the T cell mitogen ConA, or the generation of effector T cells specific for allogeneic cells (Habu et al

Figure 14

Effect of anti-asialo GM1 serum plus complement on murine NK activity. Normal murine splenocytes were treated with varying dilutions of antiserum plus complement as described in Materials and Methods, then assayed for cytotoxic activity on ^{51}Cr-labelled YAC target cells. Values represent the mean release ± SD of quadruplicate wells at effector to target ratios of 100:1 (O——O) and 50:1 (●——●).

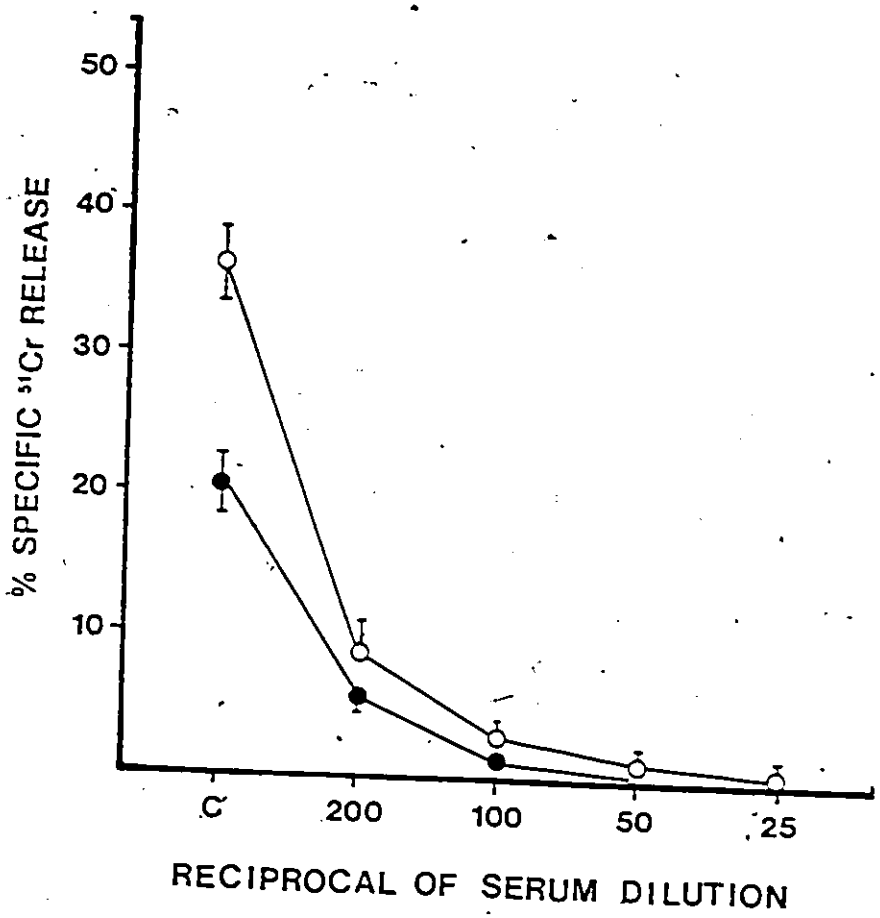

50
40
30
20
10
% SPECIFIC ^{51}Cr RELEASE
C
200
100
50
25
RECIPROCAL OF SERUM DILUTION

Figure 15

Effect of anti-asialo GM1 serum on endogenous hamster splenic NK activity. Normal hamster splenocytes were treated with anti-asialo GM1 and complement as outlined in Materials and Methods, then assayed for cytotoxic activity on ^{51}Cr-labelled K562 target cells. Values represent the mean release ± SD at an effector to target ratio of 50:1. MHA spleen (O——O), LSH spleen (Δ——Δ).

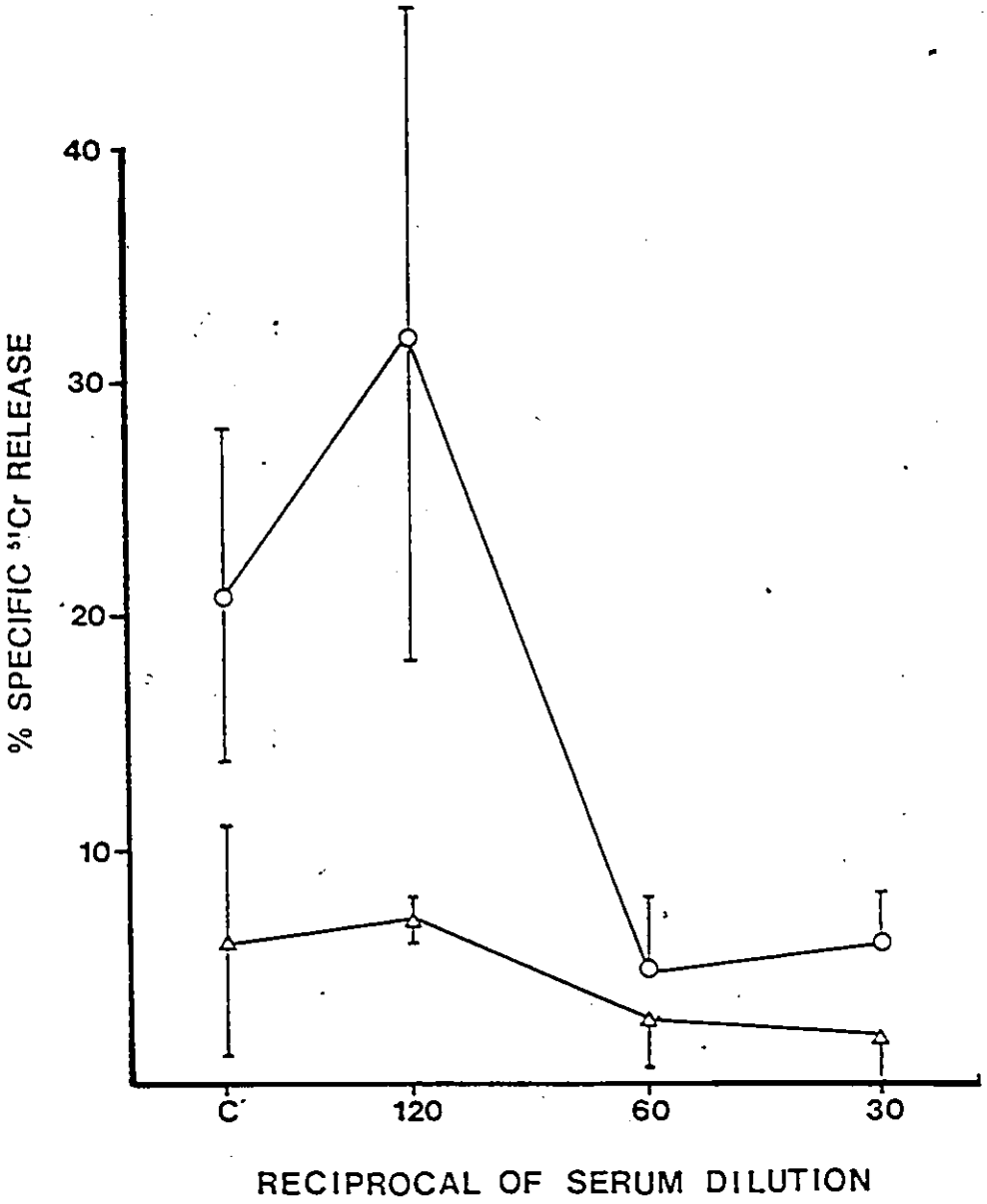

40
30
20
10
% SPECIFIC ^{51}Cr RELEASE
C
120
60
30
RECIPROCAL OF SERUM DILUTION

TABLE VII

Expression of Thy 1.2 Homologue on Hamster Cytotoxic Cells

Effectors[b]	Percent Cytotoxicity[a] Complement Control	Anti-Thy 1.2 & Complement[c]
MHA normal spleen	13.4 ± 2.9	0.5 ± 0.7
LSH normal spleen	1.6 ± 5.6	0
MHA LAK	31.9 ± 5.6	9.2 ± 1.4
LSH LAK	10.2 ± 0.2	3.2 ± 0.9

[a]Percent cytotoxicity ± SD was calculated as in Materials and Methods.

[b]MHA or LSH spleen cells either fresh or cultured for 7 d in 30% rat CM. Effector to target ratio is 50:1.

[c]Cells were tested for cytotoxicity after treatment with anti-Thy 1.2 monoclonal serum (1/100) and complement as in Materials and Methods.

TABLE VIII

Expression of Asialo GM1 Homologue on Hamster LAK

Effectors[b]	Percent Cytotoxicity[a]	
	Complement Control	Anti-Asialo GM1 & C' 1/30
5 day LAK		
MHA	19.1 ± 1.9	15.0 ± 4.6
LSH	5.5 ± 1.0	5.7 ± 0

[a]Percent cytotoxicity ± SD was calculated as in Materials and Methods.

[b]MHA and LSH spleen cells cultured for 5 days in 30% rat CM. Effector to target ratio is 50:1.

[c]Cells were tested for cytotoxicity after treatment with anti-asialo GM1 and complement as in Materials and Methods.

1981, Kawase et al 1982, Barlozzari et al 1983). In the hamster, i.p. administration of 0.4 ml of a 1/10 dilution of antiserum reduced splenic NK activity by at least 50% at 24 hr post injection, without measurably reducing cell numbers in the spleen in 4 of 4 experiments for MHA, and 3 of 4 experiments for LSH (Table IX).

VIII. Characterization of Precursors of Lymphokine Activated Killer Cells

The data from the limiting dilution experiments indicated that removal of adherent cells from the splenocyte pool did not reduce the frequency of LAK precursors, hence the precursors are found in the nonadherent fraction. This was confirmed in experiments generating LAK in CM in both bulk cultures and in the micro-assay. Unseparated, plastic nonadherent and doubly plastic and nylon wool nonadherent cells generated equivalent amounts of LAK activity after culture for 5 days in CM (Table X).

LAK precursors were also assayed for expression of asialo GM1. LAK did not express this antigen, whereas endogenous NK cells appeared to be heterogenous, and it was of interest to determine whether either of these endogenous NK populations were precursors for LAK. Treatment of normal splenocytes with anti-asialo GM1 and C' before culture in media containing CM did not result in reduced levels of LAK activity, although the NK activity in the initial population was reduced by 50% (Table XI).

TABLE IX

NK Activity in Hamster Spleen After Administration of Anti-Asialo GM1 Serum

Treatment[b]	Effector: Target	Percent Cytotoxicity[a] MHA	LSH
Normal rabbit serum	100:1	37.2 ± 2.2	17.2 ± 2.2
	50:1	22.4 ± 2.6	13.0 ± 2.1
Anti-asialo GM1	100:1	18.4 ± 4.6	4.2 ± 2.2
	50:1	9.6 ± 3.1	2.3 ± 1.7

[a]Percent cytotoxicity ± SD was determined as described in Materials and Methods.

[b]MHA and LSH hamsters were given 0.4 ml of antiserum i.p. diluted 1/10; either normal rabbit serum or anti-asialo GM1. Cytotoxicity was assayed 24 hr after administration of antiserum.

TABLE X

Adherence Properties of LAK Precursors

	Fraction[a]	Percent Cytotoxicity[b]
MHA	whole spleen	23.2 ± 8.5
	plastic nonadherent	32.2 ± 13.6
LSH	whole spleen	7.7 ± 2.3
	plastic nonadherent	6.0 ± 5.1

[a]Normal MHA or LSH splenocytes were separated into fractions as described in Materials and Methods. After separation, cells were cultured in 25% rat CM for 6 days.

[b]Percent cytotoxicity ± SD was assayed as described in Materials and Methods.

TABLE XI

Expression of Asialo GM1 Homologue on LAK Precursors

Effectors[b]		Percent Cytotoxicity[a] Complement Control	Anti-asialo GM1 1/30
MHA	NK	29.0 ± 6.6	6.4 ± 0.8
	24 hr LAK	47.2 ± 16.9	9.7 ± 7.9
	6 day LAK	28.9 ± 7.2	57.1 ± 5.2
LSH	NK	14.7 ± 1.8	11.9 ± 4.7
	24 hr LAK	29.1 ± 8.4	15.7 ± 10.0
	6 day LAK	21.4 ± 3.5	48.6 ± 8.2

[a]Percent cytotoxicity ± SD was determined as described in Materials and Methods.

[b]Normal MHA or LSH splenocytes were treated with anti-asialo GM1 serum as described in Materials and Methods, then cultured for either 24 hr or 6 days in 25 % rat CM, or assayed for cytotoxic activity without culture.

CHAPTER FOUR

THE RELATIONSHIP BETWEEN LYMPHOCYTE RESPONSIVENESS TO LYMPHOKINES AND PICHINDE VIRUS INFECTION

I. Adsorption of Pichinde Virus by Cells Enriched for Cytotoxic Activity

A. Adsorption of Pichinde virus by hamster splenocytes

Previous work had demonstrated that permissiveness of peritoneal exudate cells or nonadherent and adherent splenocytes for PV infection was not different for the two strains of hamster (Buchmeier and Rawls 1977, Gee 1979). However, this did not rule out the existence of differences in the adsorption of virus by cells enriched for NK activity. Plastic nonadherent splenocytes were incubated with virus for 1 hr, washed, then lysed. Virus associated with the cell lysate was assayed, along with virus in the first wash after infection. MHA splenocytes did not adsorb more virus than LSH splenocytes in 3 experiments, nor was there a difference in the amount of virus left in the supernates after adsorption, confirming the earlier results (Table XII).

B. Adsorption and growth of Pichinde virus in lymphokine activated killer cells

No difference could be demonstrated in the ability of nonadherent spleen cells to adsorb virus, perhaps because the relevant target cells were not adequately purified. LAK cultured in IL2 expressed high levels of NK-type cytotoxic activity and might be expected to be more homogeneous than nonadherent spleen cell preparations. Various populations of LAK were infected, and both

TABLE XII

Adsorption of Pichinde Virus by Nonadherent Splenocytes

		Virus Titre $\log_{10}$/ml	
		Cell lysate[a]	Supernate
Expt. 1 MOI=1.5	MHA	5.21	5.51
	LSH	5.03	5.50
Expt. 2 MOI=1.5	MHA	3.11	5.26
	LSH	3.65	5.32
Expt. 3 MOI=0.1	MHA	2.57	4.85
	LSH	2.89	4.78

[a]MHA and LSH splenocytes from normal animals were adsorbed with PV at the specified MOI for 60' on ice, then washed 3 times, frozen and thawed, sonicated, and the remaining lysate was assayed for pfu PV as described in Materials and Methods. The supernate from the first wash was also titrated for virus.

adsorption and replication of PV were subsequently assayed. For both 1 day LAK and 5 day, there were no differences in the amount of virus adsorbed by cells expressing high cytotoxic activity and cells expressing very little activity, nor did MHA cultured splenocytes adsorb more virus than LSH (Figures 16, 17). LAK from both strains of hamster generated after 1 day in culture in purified IL2 were equally permissive to growth of PV (Figure 16), whereas 5 day LAK from LSH hamsters generated in CM were more permissive than MHA (Figure 17). In both experiments, culture of cells in medium alone allowed greater growth of virus than growth in medium supplemented with lymphokines. Similar results were obtained in 6 experiments, where cells were cultured for up to 2 days after infection.

II. Characterization of Virus-Induced Natural Killer Activity

Because no differences could be shown in the ability of the putative target cells to adsorb virus, attempts were made to characterize the targets after infection in vivo. It had been demonstrated previously that cytotoxic cells in the spleens of hamsters infected 3 days earlier with PV expressed characteristics of endogenous NK cells, i.e. small size, plastic nonadherence, no phagocytic activity, resistance to NH_4Cl and no expression of surface Ig (Gee et al 1979). In other virus systems in hamsters, splenic NK activity did not express

Figure 16

Adsorption and growth of Pichinde virus in LAK generated after culture in IL2. Spleen cells from normal MHA or LSH hamsters were cultured in medium (□) or in medium supplemented with 16 U/ml human IL2 (▨) for 24 hr, at which time the cells were assayed for cytotoxic activity, and infected with PV as described in Materials and Methods. After further culture, cells were harvested and assayed for both IC and cytotoxicity. Log_{10} IC/10^6 cells is represented in the left panel, percent specific ^{51}Cr-release is represented in the right panel.

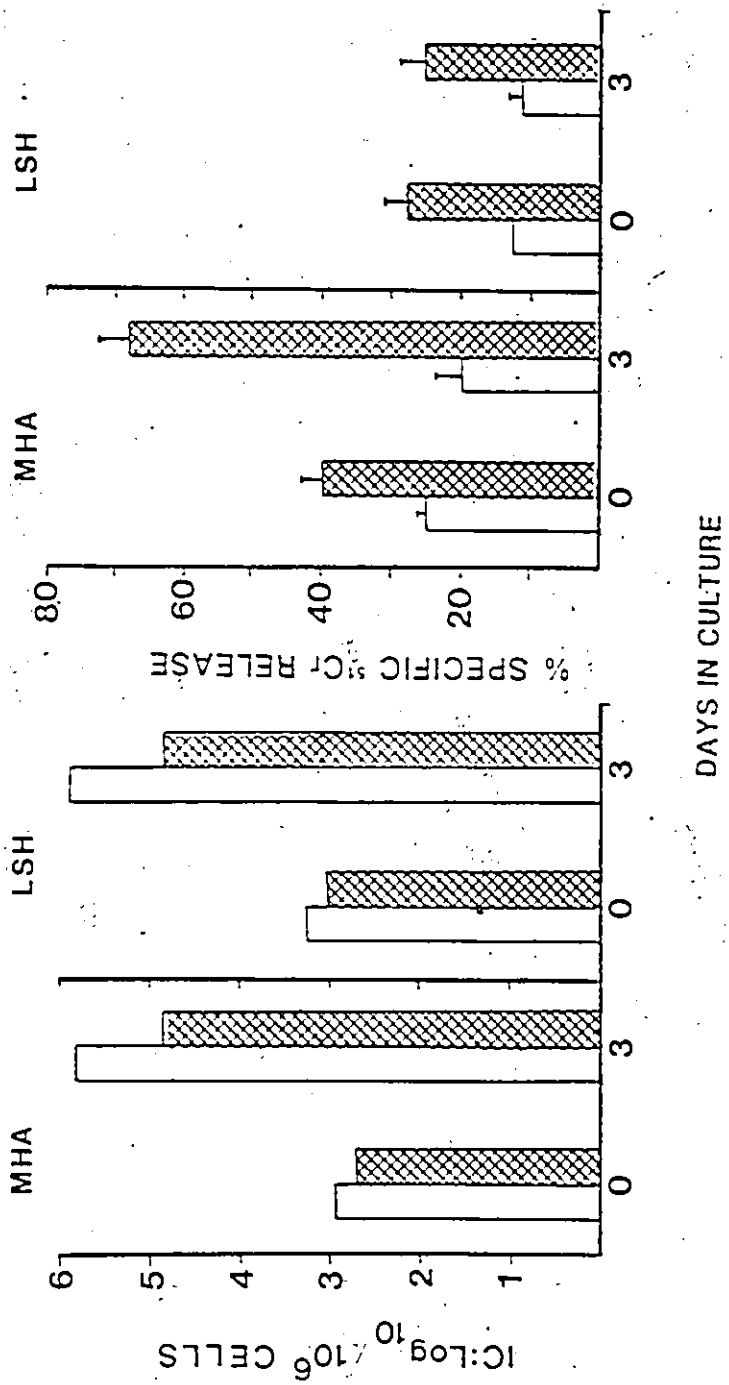
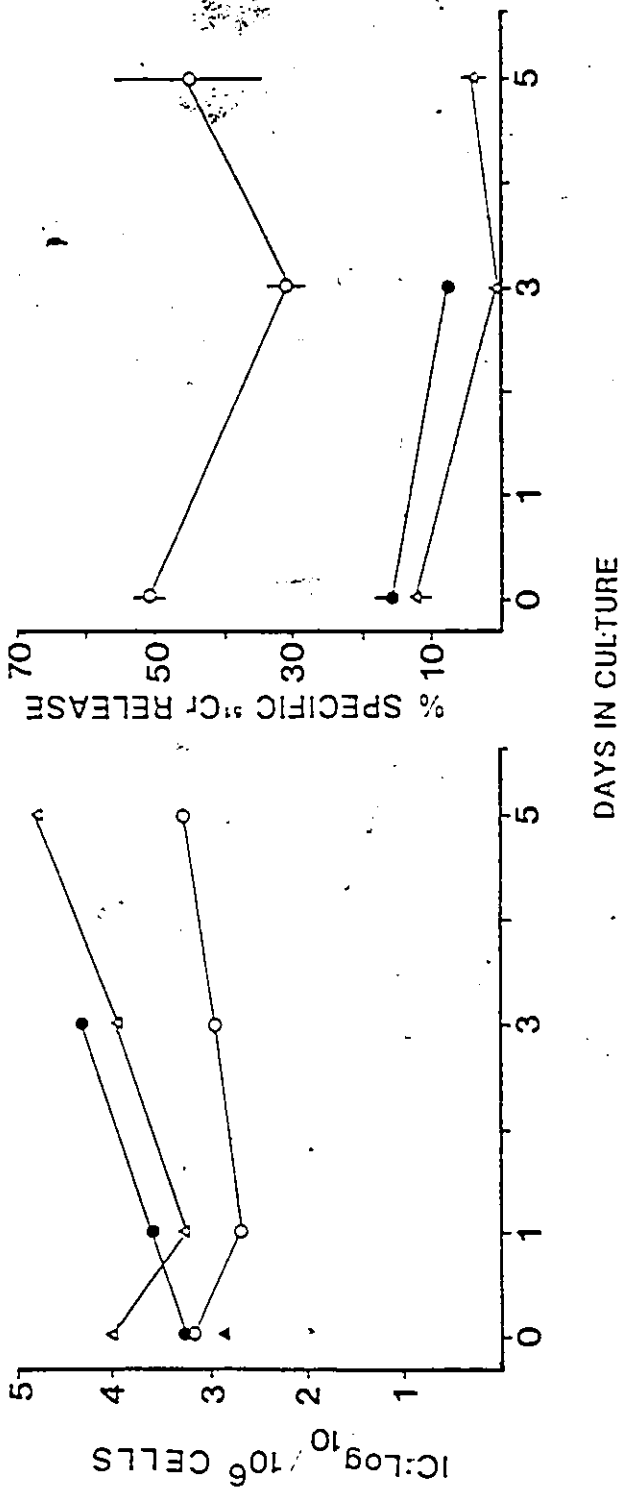

IC:Log10 /10^6 CELLS
6
5
4
3
2
1
MHA
LSH
0
3
0
3
DAYS IN CULTURE
% SPECIFIC 51Cr RELEASE
80
60
40
20
MHA
LSH
0
3
0
3

Figure 17

Adsorption and growth of Pichinde virus in LAK generated after culture in rat CM. Spleen cells from normal MHA (O—O) or LSH (Δ—Δ) hamsters were cultured in medium (closed symbols) or 25% rat CM (open symbols) for 5 days, at which time cells were harvested, assayed for cytotoxic activity, and infected with PV as described in Materials and Methods. After further culture for the times shown, cells were harvested and again assayed for IC and cytotoxicity.


% SPECIFIC ^{51}Cr RELEASE
70
50
30
10
0
1
3
5
IC:Log$_{10}$ / 10^6 CELLS
5
4
3
2
1
0
1
3
5
DAYS IN CULTURE

the theta homologue (Nelles & Streilein 1980a,b, Yang et al 1982). Cells expressing NK cytotoxic activity after infection with PV were tested for the expression of the theta homologue and asialo GM1. Treatment with anti-Thy 1.2 plus C' reduced NK activity by 50%, and with anti-asialo GM1 plus C' by approximately 60% in 4 of 5 experiments (Table XIII). Hence, PV-induced NK activity is similar to endogenous NK activity rather than LAK in expression of asialo GM1, but appear to express less of the theta homologue.

III. Characterization of Splenocytes Infected with Pichinde Virus

Splenocytes infected with PV were examined for the same traits used to characterize cytotoxic cells.

A. Adherence properties

Infectious centres (IC) associated with plastic adherent and nonadherent fractions of spleens 3 days post infection had been examined. In both fractions, MHA spleens contained more IC/10^6 cells than LSH, but the total number of IC in the nonadherent fraction in MHA spleens was 7 times that in the nonadherent fraction of LSH spleens. For the adherent pool, this ratio was 1.6:1 (Gee et al 1981a). It seemed likely from the available evidence that events early after infection were crucial in resistance, so the distribution of IC at 1 and 2 days after infection was determined. At day 1 after infection, IC could not always

TABLE XIII

Surface Antigen Expression of Pichinde Virus Induced Cytotoxic Cells

Treatment[a]	Percent Cytotoxicity[b]	
	MHA	LSH
Complement Control	43.4 ± 4.8	32.1 ± 4.2
Anti-Thy 1.2 1/100	22.1 ± 8.6	17.3 ± 3.8
Complement Control	46.5 ± 11.5	14.4 ± 2.2
Anti-Asialo GM1 1/60	20.9 ± 3.7	5.8 ± 5.4

[a]MHA or LSH spleen cells from animals infected 3 days previously with 2 x 10^3 pfu PV were treated with antisera and complement as described in Materials and Methods.

[b]Percent cytotoxicity ± SD was determined as described in Materials and Methods. Effector to target ratio is 50:1.

be detected, but when quantitation was possible, there were no differences between the strains in the numbers of IC. However, by day 2 after infection, MHA spleens contained at least twice as many IC/10^6 cells as LSH spleens in 4 of 6 experiments (Table XIV). These data confirmed earlier results (Gee et al 1981a). Examination of virus in adherent and nonadherent fractions at day 2 post infection showed that IC/10^6 cells were greater in MHA than LSH in the different fractions, as in unseparated preparations, and that neither adherent nor nonadherent fractions were consistently enriched for IC (Table XIV). When the total number of IC in each fraction was calculated, there was still no enrichment in the nonadherent fraction. Hence, the differential infection of nonadherent cells does not take place until after day 2, or the IC assay is not adequately sensitive to detect subtle differences before that time.

B. Surface antigen expression

In the LCMV system, it was possible to determine the surface antigen expression of infected splenocytes by depletion of cells with antiserum and complement (Popescu et al 1979, Lehmann-Grube et al 1983a). In this system, it was established that cells had to be viable to score as infectious centres in the plaque assay (Popescu et al 1979), and free virus could be eliminated with neutralizing antibody (Lehmann-Grube et al 1983a). Spleen cells from

TABLE XIV

Distribution of Infectious Centres in Fractions of Infected Splenocytes

Ratio of MHA to LSH[a]				
Unseparated	Nonadherent		Adherent	
$IC/10^6$	$IC/10^6$	Total IC	$IC/10^6$	Total IC
2.2 (0.8)	1.9 (0.6)	3.1 (1.9)	1.8 (0.4)	2.4(0.8)

[a]Splenocytes from animals infected 2 days previously with 2×10^3 pfu PV were separated by adherence to plastic as described in Materials and Methods. All fractions were assayed for infectious centres (IC). Values represent ratios from 6 experiments, parentheses enclose SEM.

hamsters infected 3 days earlier with Pichinde virus were treated with anti-Thy 1.2 or anti-asialo GM1 sera plus complement before plaquing to determine the phenotype of infected cells (Table XV). It can be seen that treatment with anti-Thy 1.2 plus complement reduced both infectious centres and NK activity by 40 to 50% for MHA hamsters, but reduced NK activity of LSH hamsters to a greater extent. Both asialo GM1 positive and negative cells appear to be infected with the virus, and in this case the reduction in IC is less than the reduction in cytotoxic activity for both strains of hamster. However, these data likely overestimate the number of infectious centres remaining after depletion of cells. Pichinde virus does not induce neutralizing antibody so it was impossible to control for free virus in the cell suspensions, and unlike LCMV, a considerable amount of infectious virus was associated with dead cells in two experiments (data not shown).

C. Selection of cells in interleukin 2

Cells from animals infected with PV 2 days previously were cultured for short periods of time in either medium or IL2, then assayed for cytotoxic activity and IC. Cells from both strains cultured in IL2 showed no greater infection than those in medium, indicating that IL2 responsive cells do not harbour more virus than cells surviving in medium alone, although IL2 responsive cells in

TABLE XV

Surface Antigen Expression of Splenocytes Infected with Pichinde Virus

Fraction[a]	Total Virus/ Fraction	% Reduction	Percent Cytotoxicity[b]	% Reduction
MHA Complement Control	9600		36.1 ± 7.8	
Anti-Asialo GM1 1/30	6426	33	9.9 ± 2.9	73
Anti-Thy 1.2 1/100	5617	41	19.2 ± 4.7	47
LSH Complement Control	3686		30.6 ± 4.7	
Anti-Asialo GM1 1/30	2776	25	16.8 ± 3.3	45
Anti-Thy 1.2 1/100	1942	47	9.3 ± 7.2	70

[a]Equal numbers of splenocytes from hamsters infected 3 days previously with 2×10^3 pfu PV were treated with antisera plus complement as described in Material and Methods before assay of virus and cytotoxic activity.

[b]Percent cytotoxicity was determined as described in Materials and Methods. Values represent the mean cpm ± SD of triplicate wells. Effector to target ratio is 50:1.

MHA cultures expressed significantly more cytotoxic activity than cells in medium (Figure 18).

IV. Infectious Centres in Spleens after Administration of Anti-Asialo GM1

Because of the inability to control for free virus in the system when infected cells were treated with antiserum and complement in vitro, animals were given anti-asialo GM1 to determine the effects of this antiserum in vivo. Treatment of hamsters with a single injection of anti-asialo GM1 reduced splenic NK activity by 50% (Table IX). If these NK cells serve as a target cell for PV replication, it might be expected that a reduction in NK would lead to a reduction in IC. If, however, the target cell is the asialo GM1 negative cytotoxic cell, the LAK precursor, or some other cell, NK might be reduced with no substantial effect on IC. Animals were treated with anti-asialo GM1 or normal rabbit serum, infected 24 hr later, then spleens were assayed for IC after a further 1 or 3 days. When IC were assayed 1 day post infection, virus yield was reduced over control animals (Table XVI). When IC were assayed at day 3 post infection, virus yield was equal to, or in 2 of 4 experiments, increased over the levels seen in control animals (Table XVI). Virus-induced NK activity was reduced in both sets of animals, so that at day 3, NK activity and IC were dissociated. This suggests that elimination of asialo GM1 positive cells with NK

Figure 18

Culture of infected splenocytes in IL2.

Splenocytes from MHA (O——O) and LSH (Δ——Δ) hamsters infected 2 days previously with 2×10^3 pfu PV were assayed for cytotoxic activity and IC as described in Materials and Methods, then cultured in medium (A,C) or medium supplemented with 16 U/ml human IL2 (B,D). At 1 day and 3 days of culture, cells were again assayed for cytotoxic activity or IC.

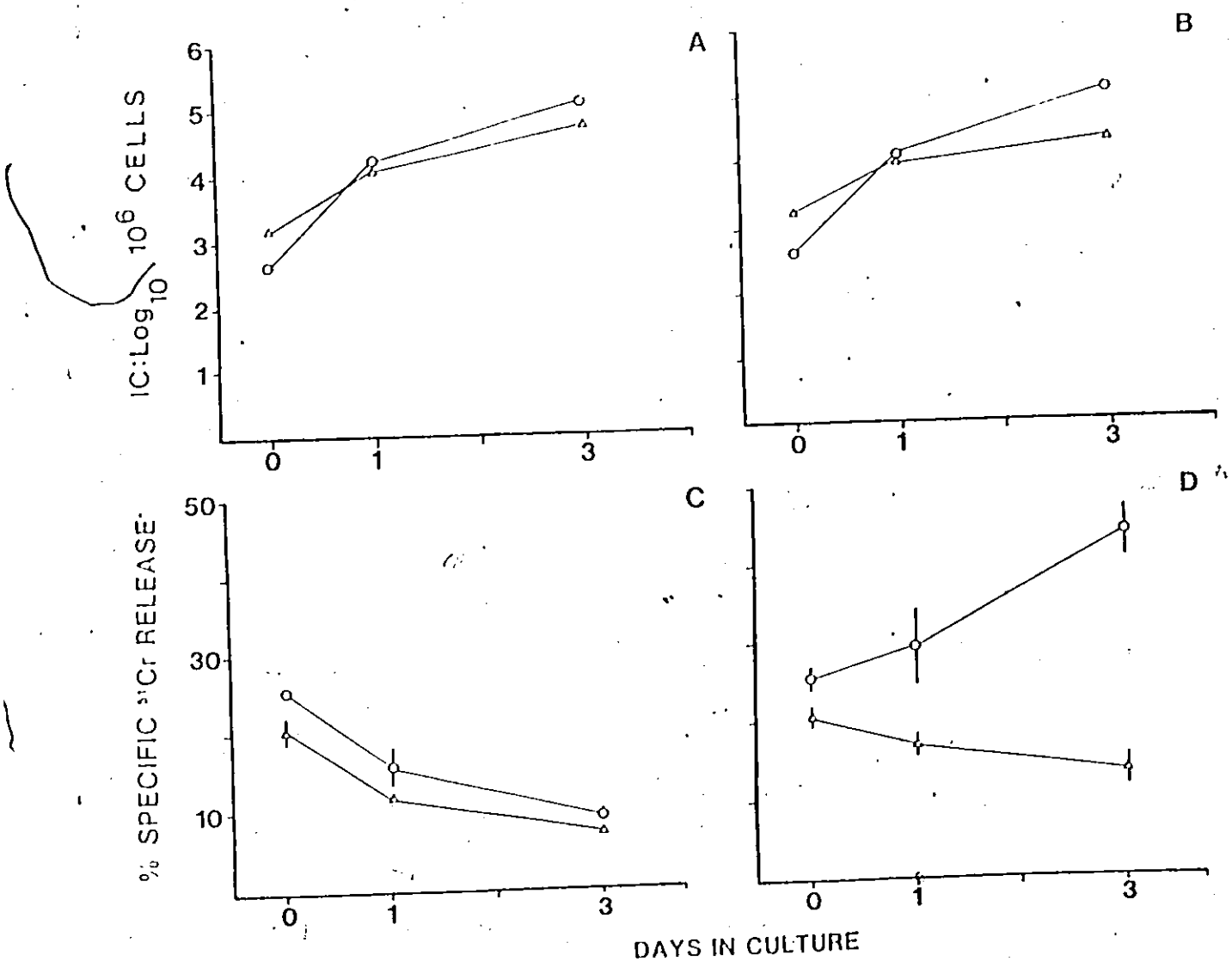

A
B
C
D
IC:Log10 10^6 CELLS
% SPECIFIC 51Cr RELEASE
DAYS IN CULTURE
6
5
4
3
2
1
50
30
10
0
1
3

TABLE XVI

Splenic Infectious Centres and Cytotoxic Activity after Administration of Anti-Asialo GM1 Serum

	IC/10^6		Percent Cytotoxicity[a]	
	NRS[b]	Asialo GM1	NRS	Asialo GM1
Day 1				
MHA	26.2	6.1	31.4 ± 5.1	24.5 ± 1.4
LSH	14.3	0.5	31.2 ± 1.5	15.2 ± 1.4
Day 3				
MHA	2008	4387	32.9 ± 4.4	9.1 ± 1.1
LSH	240	960	15.9 ± 2.0	6.6 ± 4.0

[a] Infectious centres and cytotoxic activity ± SD were determined as described in Materials and Methods. Effector to target ratio is 50:1.

[b] Animals were treated with normal rabbit serum or anti-asialo GM1, 0.4 ml of a 1/10 dilution i.p. One day later animals were infected with 2×10^3 pfu PV, then sacrificed either 1 day or 3 days post infection.

activity might reduce IC, but not adequately to shift the susceptible animals to the resistant phenotype. In these experiments there was no difference in the numbers of cells harvested from treated versus control animals, although the loss of asialo GM1 positive cells might not be detectable by simple cell counting.

V. Mortality of Hamsters After Administration of Anti-Asialo GM1 and Pichinde Virus

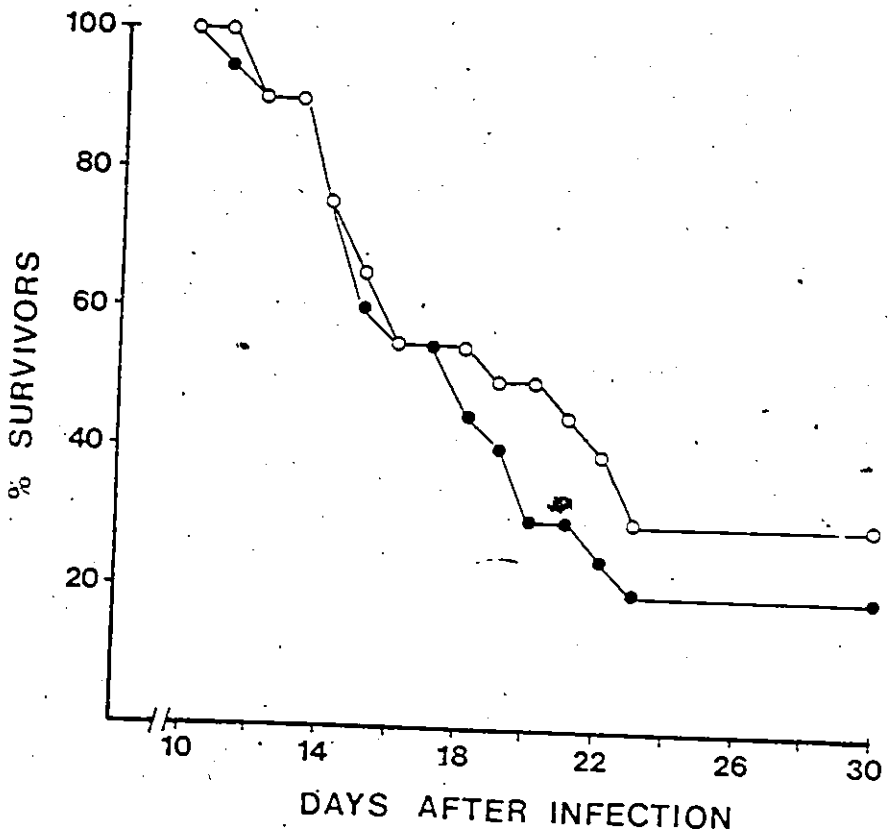
Treatment of animals with anti-asialo GM1 did not alter IC observed at 3 days after infection, but it was necessary to determine whether such treatment had any effects on mortality. Hamsters were treated with anti-asialo GM1 or normal rabbit serum as described above, then infected with PV at 24 hr. Animals were followed daily, and mortality was scored. The data in Figure 19 show that treatment with anti-asialo GM1 serum had no effect on the course of PV infection in MHA hamsters. LSH hamsters in both groups survived infection (data not shown).

VI. Mortality of MHA Hamsters after Administration of Interleukin 2 and Pichinde Virus

Anti-asialo GM1 serum did not reduce IC nor affect mortality, although NK activity was reduced. This did not rule out a role for the asialo GM1 negative LAK precursor cell as a target cell for virus replication. This precursor is responsive to IL2 in vitro as demonstrated by

Figure 19

Effect of anti-asialo GM1 serum on mortality after infection with Pichinde virus. MHA hamsters were treated with 0.4 ml of a 1/10 dilution of normal rabbit serum (● ●) or anti-asialo GM1 (O O). Twenty-four hours later all animals were infected with 2×10^3 pfu PV. For all groups, n = 20.


100
80
60
40
20
% SURVIVORS
10
14
18
22
26
30
DAYS AFTER INFECTION

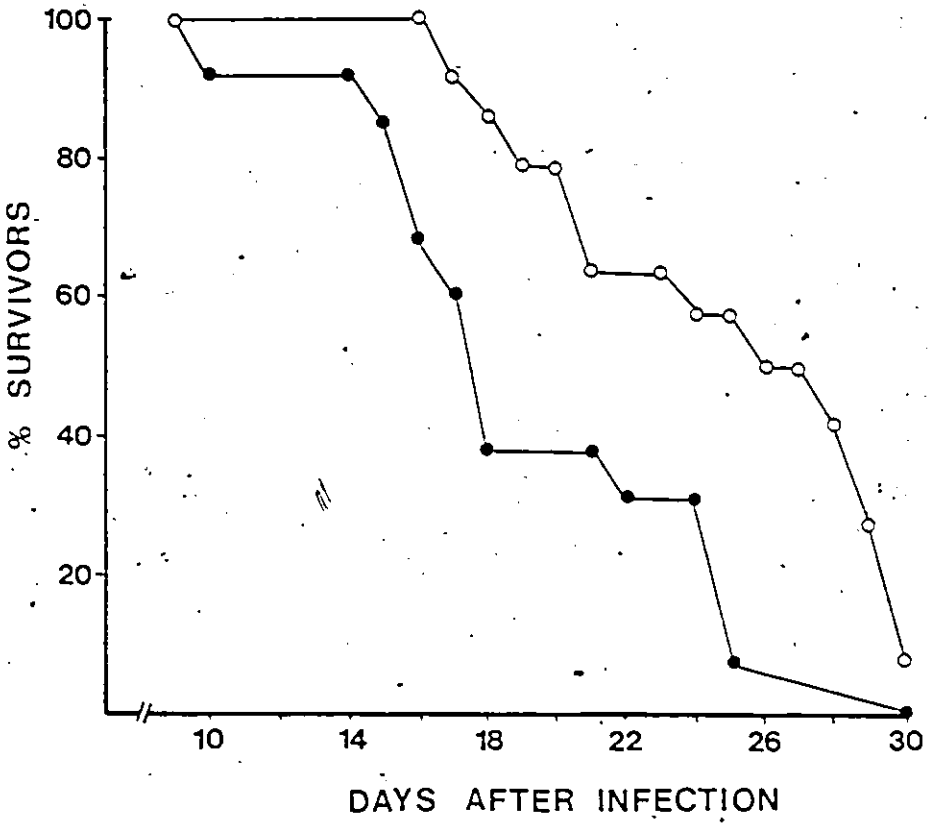
increased cytotoxic activity after culture with IL2, and the susceptible animals possess more of these cells. As well, the susceptible animals appear to produce less IL2 when lymphocytes are stimulated with mitogen. It was reasoned that if these two phenomenon were related, treatment of hamsters with IL2 might alter the size of the LAK precursor pool, and hence reduce virus replication and mortality in the susceptible strain. MHA hamsters were given 6 injections of purified human recombinant IL2 over 3 days. The total amount of IL2 equalled 900 half-maximal units; all injections were administered i.p., except the last, which was given s.c. This protocol was selected as optimal for maintaining serum IL2 levels over the course of the injections (Donohue and Rosenberg 1983, Rouse et al 1985). Control animals received an equal volume of PBS at each time point. With the last injection, all hamsters were infected with 2×10^3 pfu PV, and mortality was scored. It can be seen from the data in Figure 20 that treatment with IL2 slowed the mortality rate over that of control animals ($p<0.05$), although by day 30, the majority of hamsters in both groups had died.

VII. Infectious Centres and Cytotoxic Activity in Spleens after Administration of Interleukin 2

Because IL2 did alter mortality in the susceptible animals, the effect of the treatment on IC and NK early after infection was examined. MHA hamsters were given IL2

Figure 20

Effect of purified IL2 on mortality after infection with Pichinde virus. MHA hamsters were treated either with 900 Units interleukin 2 (IL2) (O—O) or PBS (●—●) in 6 injections over 3 days. All injections were given i.p. except the last, which was administered s.c. at the same time that 2 x 10^3 pfu PV was given i.p. For IL2 treated animals, n = 14, for the control group n = 13.


100
80
60
40
20
% SURVIVORS
10
14
18
22
26
30
DAYS AFTER INFECTION

or PBS as above, infected with PV, and IC and cytotoxic activity were assessed at day 3 post infection. The data in Table XVII indicate that IL2 treatment did not consistently reduce IC compared to control animals, nor did it measurably alter virus-induced NK activity. IL2 treatment must then slow mortality by some mechanism other than reducing virus replication, and it was concluded that IL2, as administered in these experiments, did not reduce the target cell pool.

TABLE XVII

Effect of Interleukin 2 on Infectious Centres and Virus-Induced NK in MHA Hamsters

	IC/10^6		Percent Cytotoxicity[a]	
	PBS[b]	IL2	PBS	IL2
Expt. 1 Animal a)	2360	1670	28.2 ± 2.7	28.2 ± 11.6
b)	67	5	13.2 ± 5.8	12.5 ± 3.1
Expt. 2 Animal a)	1712	3566	18.2 ± 5.3	36.5 ± 9.2
b)	0	2933	17.3 ± 9.5	38.9 ± 3.5

[a] Infectious centres per 10^6 cells was assessed as described in Materials and Methods. Percent cytotoxicity ± SD of triplicate wells was determined as described in Materials and Methods. Effector to target ratio is 50:1.

[b] MHA hamsters were administered PBS or IL2 in volumes of 0.1 ml i.p. over 3 days. A total of 900 Units of IL2 was given in 6 injections. Hamsters were infected with 2×10^3 pfu PV at the time of the last injection.

CHAPTER FIVE

DISCUSSION

The results presented here demonstrate consititutive differences in the immune systems of the inbred hamster strains, MHA and LSH, and relate these differences to relative resistance or susceptibility to infection with Pichinde virus.

I. Lymphocyte Responsiveness to Lymphokines

Both the spleen and thymi of MHA hamsters contain more cells than those of LSH hamsters, and lymphocytes within these organs appear to respond differently to ConA-induced CM.

A. Thymocyte responses: proliferation

In a thymocyte proliferation assay for lymphokines, MHA thymocytes proliferate to a greater extent than LSH in the presence of CM produced from homologous spleen (Figure 1). This increased responsiveness also occurs when assaying CM from the heterologous strain, from the rat (Figures 2A, 2B, 6) or preparations of purified IL2 (Figure 9). No amount of CM or IL2 was able to induce proliferation in LSH cultures equal to that seen in MHA cultures. Although the IL2 content of the CM was never standardized in the same units as the IL2 preparations, it appears that purified IL2 is less active than CM, suggesting that other factors in the CM participate in inducing proliferation. Both IL1 and IL3 are known to cause proliferation of thymocytes (Waksal and Colucci 1984,

Rock and Banacerraf 1984). In assays for IL1, however, higher thymocyte concentrations are required than those used in the experiments here (Oppenheim and Gery 1982, Economu and Shin 1978) and preparations of epidermal T cell activating factor (ETAF) of hamster, human and murine origin failed to induce growth of hamster thymocytes under the conditions used in these experiments (data not shown).

Because all sources of CM induced greater proliferation in MHA hamsters, it is unlikely that the observed differences are due to variations in lymphokine content of the CM preparations. Limiting dilution experiments demonstrate that the basis of the difference is a quantitiative disparity in precursor cells responding to lymphokine in the proliferative assay (Table II).

Thymocyte responses to lymphokines have been examined in attempts to delineate thymocyte maturational pathways (Waksal and Colucci 1984, Ceredig et al 1982, Phillips and Rabson 1983, Rock and Banacerraf 1984, Conlon et al 1982). Strain differences in such responses have not been examined, but differences in the response of thymocytes from murine strains in an IL1 proliferative assay have been noted (Oppenheim and Gery 1982).

B. Splenocyte responses:generation of lymphokine activated killer cells

In parallel to these findings in the thymocyte assay, differences in splenocyte responsiveness to CM are

also observed, as detected by the activation of nonspecific cytotoxic cells in vitro (LAK). Incubation of splenocytes from either strain in medium supplemented with rat CM or with purified IL2 for 24 hr or for 5 to 7 days generates such LAK activity to a greater extent in MHA cultures than in LSH cultures (Figures 7D, 11). This cytotoxic activity is independent of cell growth, as cell yields from cultures from both strains were equal (Figure 7B). Again, the low responsiveness of LSH cells is not overcome by increased amounts of lymphokine (Figures 8, 10).

The observed differences in LAK generation may simply be due to an increased fragility of LSH lymphocytes in cultures, but other manipulations do not result in descreased viability of LSH lymphocytes over MHA lymphocytes. As already discussed, LSH and MHA splenocytes survive equally well in cultures with or without CM. In addition, LAK activity was measured on a per cell basis indicating that differences are not just due to different numbers of cells surviving in the cultures. As was the case in the thymus, these differences appear to be due to a greater frequency of responsive precursor cells in MHA spleens than in LSH spleens and not to an intrinsically greater responsiveness of MHA precursors to lymphokines, as demonstrated by limiting dilution experiments (Table III). The presence of adherent peritoneal cells in the cultures reduces the frequency of LAK precursors in both strains of

hamster (Table IV), but a splenic adherent suppressor cell has not been detected (Table V). However, a suppressive effect on LAK activity was noted in LSH cultures at high cell concentrations (Figure 12). This effect was diluted out in the limiting dilution assays. Hence, the differences observed in LAK in bulk cultures may be due both to an underlying difference in the number of precursors and to the presence of a cell/factor inhibiting cytotoxic activity in LSH spleens.

The results of experiments examining LAK generation fit well with other reports. Increased NK activity has been observed after 24 hr culture of hamster spleen cells in CM (Yang et al 1983) or by culture with ConA (Singh and Tevethia 1973, Stewart et al 1985). In the latter case, it was shown that lymphokine synthesis stimulated by exposure of cells to ConA was responsible for the increased cytotoxic activity. LAK activity generated after longer incubations or after exposure to purified IL2 have not previously been described in the hamster.

Both murine and human lymphocytes display increased NK-type activity after exposure to CM (Minato et al 1981, Grimm et al 1982), interferon (Minato et al 1981, Kuribayashi et al 1981) or purified IL2 (Grimm et al 1983a, Merluzzi et al 1984). In both systems, the cells responding to interferon appear to be those cells displaying endogenous NK activity, and enhancement is

maximal after 24 to 48 hr (Minato et al 1981, Kuribayashi et al 1981, Seki et al 1985). Enhanced cytotoxic activity after culture in IL2 is generally not evident until 3 to 7 days of culture (Minato et al 1981, Teh and Yu 1983, Grimm et al 1983a, Timonen et al 1983, Burns et al 1984) although some workeres also report an early response to IL2 in human lymphocytes at 18 to 24 hr as is observed in the hamster (Burns et al 1984, Miyasaka et al 1984, Lanier et al 1985, Seki et al 1985).

Strain variation of LAK generation after culture of splenocytes in CM or IL2 has been observed in mice (Riccardi et al 1982, Riccardi et al 1983/1984, Rosenstein et al 1984). In general, mice displaying high endogenous NK activity have more precursors for LAK, with the exception of nude mice, which have high NK but few LAK precursors (Riccardi et al 1982).

C. Lymphokine production

In the initial experiments, where the source of lymphokines was hamster spleen, there existed the possiblity that the observed differences in responsiveness stemmed from disparity in the production of lymphokines by the two strains. Subsequent experiments, testing a single CM preparation on thymocytes from both strains, indicated that the differences lie at the level of the responding cells. Lymphokine production was nevertheless assayed, as differences in levels of lymphokine(s) might account for

the unequal precursor pool sizes as detected in the in vitro assays. Cells from the spleens and lymph nodes of the two strains produce equal amounts of IL1 after stimulation with lectin (Figure 5). As well, the production of ETAF, an IL1-like molecule, by keratinocytes is equal in both strains
(data not shown).

Attempts to measure the IL2 content of hamster splenic CM on a murine IL2-dependent cells line have been unsuccessful, but CM prepared from LN is active. The high responder strain, MHA synthesizes significantly less IL2 than the other strain (Figure 4). The fact that the same LN-CM contain equal activities in the thymocyte proliferation assay (Figure 3A, 3B) supports the earlier suggestion that factors in the CM other than IL2 are detected in this assay.

Only one study has examined the relative IL2 production in strains of normal mice. Differences were minor, and not related to the MHC haplotype (Hoffenbach et al 1985). Reduced IL2 production is a feature of several strains of mice that develop immunodeficiency disease (Dauphinee et al 1981, Wofsy et al 1981, Bocchieri et al 1984). The reduction of IL2 synthesis precedes disease and the magnitude of the reduction in synthesis correlates with the severity of the disease (Altman et al 1981). A similar phenomenon has been reported in humans suffering from

systemic lupus erythromatosus (SLE) (Murakawa et al 1985). It has also been reported that nude mice fail to produce IL2 (Gillis et al 1979) but more recent studies demonstrate IL2 production after long culture periods (Lees et al 1984). Interestingly, there was considerable variation in the levels of IL2 produced depending of the genetic background of the mice (Lees et al 1984). In chickens, increased, rather than decreased IL2 production was associated with autoimmune thyroid disease in birds carrying the OS gene (Schauenstein et al 1985). It is unlikely that the situation in any of these cases is analogous to that in the hamster system. In the murine examples, lower LL2 production was always associated with reduced responsiveness to mitogen and IL2 (Altman et al 1981, Wofsy et al 1981), whereas MHA animals are more responsive to IL2. MHA hamsters do not display signs of autoimmune or immunodeficiency disease.

Reduced IL2 production has also been described in certain infectious disease. For example, patients with leprotomatous leprosy versus tuberculoid leprosy produce less IL2 in response to M.leprae (Nath et al 1984). In the mouse, though, reduced IL2 production in response to M.leprae is not correlated with levels of consititutive IL2 synthesis (Hoffenbach et al 1984,1985), and both high and low producer strains are susceptible to infection.

D. Characterization of cytotoxic cells

Four populations of splenic cytotoxic cells were described in the hamster; endogenous NK, LAK induced after 1 day or 5-7 days in culture, and cytotoxic cells activated in vivo by infection with PV. The cytotoxic cells induced in limiting dilution experiments are the late LAK, so these effectors, with the cells responsible for endogenous and virus-induced NK, were characterized. These data are summarized in Table XVIII.

All populations of cytotoxic cells described here are plastic nonadherent, confirming earlier works that described endogenous and virally-induced NK activity (Gee et al 1979, Chapes and Tompkins 1979). As well, these cells are further enriched in nylon wool nonadherent populations. The precursors cells for LAK are also nonadherent and do not require the presence of adherent cells for induction, as determined in both limiting dilution and bulk assays (Tables V,X).

When examined for the expression of the theta homologue, all cytotoxic cells are positive. Effectors from the two strains of hamster do not differ in expression of this antigen. The lytic activity of endogenous NK cells was totally abrogated after treatment with anti-Thy 1.2 plus complement (Table XI). Virus-induced cytotoxic cells are more heterogeneous; activity was only reduced by 50% after the same treatment (Table XV). Expression of the

TABLE XVIII

Properties of Hamster Splenic Cytotoxic Cells

	Plastic Adherence	Theta	Asialo GM1
Endogenous NK	-	+	±
1 Day LAK	-	n.d.	-
5 Day LAK	-	n.d.	-
Virus-induced NK	-	±	±

n.d. signifies not done.

theta homologue has no implications for the lineage of cytotoxic cells, as over 80% of hamster spleen cells express the antigen (not shown) suggesting a widespread distribution on many cells. The antigen detected by monoclonal anti-Thy 1.2 has been shown to be present of both T cells and B cells in the hamster (Witte and Streilein 1983).

Other studies in the hamster, using this marker for delineation of lymphocyte subsets have shown that nonadherent endogenous NK activity, LAK induced by short culture in CM, and NK induced by vaccinia virus are all negative for expression of this antigen (Yang et al 1982, Yang et al 1983). A theta positive cytotoxic cell has been described in the peritoneal cavity of vaccinia infected hamsters, and in bone marrow cultures stimulated with CM (Yang et al 1983, Yang and Tompkins 1983). It is difficult to explain these discrepancies in results using the same antiserum at the same dilutions. However, different target cells were used to detect cytotoxicity, so perhaps different effector populations are being detected in the different laboratories.

The expression of a second marker, asialo GM1, was also examined. Endogenous NK is reduced by only 50% after treatment with the antiserum plus complement, indicating heterogeneity in this population (Figure 15). Treatment of normal animals with antiserum likewise reduces NK by

approximately 50% (Table IX). LAK generated after 5 days of culture are negative for expression of this antigen even when high amounts of reagent are used (Table VII). Virus-induced effectors resemble endogenous NK cells with respect to expression of this marker (Table XV). LAK precursors do not express asialo GM1 (Table XI). LAK might then arise from precursors negative for expression of this antigen that also mediate a fraction of endogenous NK activity. If the precursors do express lytic activity, and because these precursors differ quantitatively in the two strains, one might expect the asialo $GM1^-$ component of endogenous NK to account for a larger fraction of total activity in MHA hamsters. In other words, NK activity in MHA hamsters would be less sensitive to treatment than LSH. This does not appear to be the case, but the system of elimination and of scoring the remaining cells might not be adequately sensitive to detect these types of differences. Removal of adherent cells did not reveal any consistent differences in the proportion of endogenous NK cells sensitive to the antiserum. This suggests that although the LAK precursors are greater in number in MHA spleens, other lytic cells may also be increase. The possibility of another asialo $GM1^-$ cell not expressing endogenous NK activity, or of asialo $GM1^+$ cells changing their phenotype in culture, cannot be overlooked.

Hamster cytotoxic cells have not been evaluated for the expression of asialo GM1 antigen before. The results here suggest that hamster endogenous NK cells express this marker to a lesser extent than observed in the mouse (Figure 14) or the rat (Barlozzari et al 1983). The in vivo studies support these findings. In the mouse as little as 5 µl of undiluted serum reduced splenic NK activity by 95% (Kasai et al 1981, Habu et al 1981) and in the rat a dose equivalent to that administered to the hamsters, reduced splenic NK activity by 80% (Barlozzari et al 1983).

In the mouse and human systems, where LAK were first described (Grimm et al 1982, Minato et al 1981), there is considerable controversy about the nature of both the precursors and the effectors. It is clear that like the hamster system, heterogeneous populations of cells account for both activities. In general cells that display increased activity after 24 hours of culture are similar to cells responsible for endogenous NK activity in target cell spectrum, morphology and surface markers (Minato et al 1981, Kuribayashi et al 1981, Burns et al 1984, Lanier et al 1985). LAK generated by longer culture in CM or with IL2 are less easy to categorize. LAK have been described that arise from the endogenous NK pool and express the same features as these precursors, much like LAK generated after short-term culture (Riccardi et al 1983/1984, Suzuki et al

1983, Miyasaki et al 1984, Itoh et al 1985). Others report precursor cells devoid of NK activity generating LAK with NK-like characteristics (Miyasaki et al 1984, Shau and Golub 1985) or displaying surface features associated with T lymphocytes (Teh and Yu 1983, Grimm et al 1982). There is also evidence that some of the LAK precursors, whether they mediate endogenous NK activity or not, express antigens associated with T cells (Minato et al 1981, Grimm et al 1983b, Suzuki et al 1983, Merluzzi et al 1984, Rosenstein et al 1984, Burns et al 1984).

Virus-induced NK in the mouse resemble LAK in the acquisition of a broader target cell specificity (reviewed in Welsh 1978b) and in the increased expression of the theta antigen. In mice infected with PV, this was evident at day 4 post infection (Walker et al 1984) and in LCMV or coxsackie infected mice, but not until day 6 post infection (Kiessling et al 1980, Huber et al 1981). Target cell specificity of either virus-induced NK or LAK in the hamster was not determined, nor was the expression of the T cell antigens. However, with respect to asialo GM1, virus-induced NK are more similar to endogenous NK than LAK. This suggests that cells positive and negative for asialo GM1 expression respond to viral stimulus to show enhanced cytotoxic activity. At a time later than 3 days after infection virus-induced NK activity may share traits with LAK.

E. Summary and hypothesis

How are the phenomena of increased thymocyte and splenocyte responsiveness related to one another? One possibility is that the responding lymphocytes in the two organs are the same. The data suggest that the lymphocytes from the spleen and thymus responding to CM and IL2 are functionally different. Recently several reports have appeared that described NK type activity in the murine thymus. Thymocytes pre-incubated with the thymic hormone, thymic serum factor (Kaislerian et al 1983a) or thymocytes from animals bearing the Lewis lung carcinoma (Kaislerian et al 1983b) displayed enhanced killing of murine NK targets, and a subpopulation of thymocytes expressing high NK activity was isolated from normal thymus by density gradient centrifugation (Zoller et al 1982). Culture of murine thymocytes in medium supplemented with CM resulted in the growth of granular cells that also expressed NK activity (Born et al 1983, Toribio et al 1983). Of interest was the discovery that responsiveness of thymocytes to the thymic hormone was strain dependent (Kaislerian et al 1983a). Short term culture of hamster thymocytes under similar conditions to those used for spleen cultures did not induce any detectable lytic activity.

A second possibility is that both the thymic and splenic responding cells represent stages of maturation in

the same cell lineage, or share the same precursor cells. Several researchers have conducted experiments designed to determine the subpopulations within the thymus responding to IL2 in the proliferation assay. Most believe that only mature immunocompetent cells are capable of the proliferative response (Kisielow et al 1982, Boedeker et al 1980, Hayward et al 1981, Herron et al 1983), but others claim that a small population of immature thymocytes is capable of a transient response (Wei-Feng et al 1982, Conlon et al 1982). More recent studies have examined the proliferative response of fetal and neonatal thymocytes to IL2 compared to the response of adult thymocytes and have found that thymocytes from neonates displayed a response five times that seen with adult cells (Bocchieri et al 1983, Hardt et al 1985). Examination of IL2 receptor expression in neonatal thymocytes support these results. Habu and co-workers (1985) found that 50% of thymocytes were positive for the receptor at day 15 of gestation, but by birth this had diminished to 2 to 3% and in adults less than 1% of thymocytes carry the IL2 receptor. Lugo and colleagues (1985) also reported increased IL2 receptor-bearing thymocytes in neonatal mice relative to adults.

Although the cells responding to CM in the spleen are functionally different from those in the thymus, they may belong to the T cell lineage. In other systems, there

exists convincing evidence that cells expressing T cells markers account for a fraction of the endogenous NK activity (Mattes et al 1979, Koo et al 1980). In the spleens of mice undergoing regeneration after treatment with cyclophosphamide , NK cells recently recruited from the marrow expressed more theta antigen than observed in normal adults (Hurme and Sihvola 1983, 1984), and the human peripheral T cell marker, T3, was characteristic of cells expressing NK activity in neonates compared to adults (Abo et al 1983). These workers note that the presence of the T cell markers does not imply thymic processing; Thy 1.2 positive cells are frequent in the marrow of both rat and mouse (Schrader et al 1982, Basch and Berman 1982), and these cells may migrate directly to the spleen. Merluzzi and colleagues (1985a,1985b) examined the cytotoxic potential in the spleens of mice recovering from bone marrow transplantation and sublethal irradiation, both situations of active lymphopoiesis . These mice displayed a diminished capacity to mount T cell responses and reduced IL2 production but splenocytes from these mice had a capacity for LAK generation equal to that of normal mice. Here, then , is an example of reduced IL2 production coupled to a responsiveness to IL2 in vitro that is characteristic of an actively regenerating immune system. This situation may be analogous to the conditions described

above in the spleens of mice recovering from cyclophosphamide treatment and in neonates.

The following series of events are proposed to explain the differences between MHA and LSH hamsters. Both hamsters produce a stem cell in the marrow that is committed to the T cell lineage. This cell migrates to the thymus, where it matures into an immunocompetent T cell. Both immature and immunocompetent cells are detected in the thymocyte assay, and both immature cells, that require some further maturational event in the periphery, and immunocompetent thymocytes are exported (Stutman 1985). In the spleen, these 'post-thymic progenitors' may participate in endogenous NK activity and also serve as LAK precursors. With further maturation these cells also become T effector cells, as proposed by Merluzzi (1985b). Equally possible is a scheme whereby cells in the spleen are derived directly from the bone marrow from the same stem cell as the thymic cells, but does not undergo any thymic processing.

The difference between the hamsters may arise at the stem cell stage, so that MHA hamsters simply produce larger numbers of these cells that migrate to the thymus and then to the spleen, increasing the size of these organs, and the frequency of precursors responding to the lymphokines. In other words, MHA hamsters are in a more active state of lymphopoiesis characteristic of a more

immature but functional immune system. Alternatively, the strains may produce equal numbers of the precursor cell, but MHA animals possess a partial blockage somewhere in the maturational pathway, related to the decreased production of IL2, so that relatively immature cells accumulate in the lymphoid organs. However, as already noted, this blockage must not be total, as MHA animals do not display obvious immune defects. We cannot rule out the possibility that the difference lies in the lymphopoietic process in the marrow in such a way that most lymphoid precursors are produced in excess in MHA hamsters. In this case, the splenic and thymic responses to CM may indeed be due to cells of separate lineages.

III. Relationship between Lymphokine Responsiveness and Susceptibility to Pichinde Virus

A. Adsorption and growth of Pichinde virus in cells enriched for cytotoxic activity

The initial purpose of these experiments was to determine the underlying cause of susceptibility to PV in MHA hamsters. Preliminary experiments had indicated that genetic susceptibility was not due to qualitative differences in target cell permissiveness for infection, nor to an inability to mount an immune response to the virus (Buchmeier and Rawls 1977, Gee et al 1979, Gee et al 1980), but to a quantitative difference in a target cell

that also mediates cytotoxic activity (Gee et al 1979, 1981a).

However, these previous experiments examining permissiveness of target cells for infection may have been unsuccessful because the relevant cells were not adequately enriched. However, normal nonadherent splenocytes and LAK generated in either CM or IL2 from the two strains show no difference in the adsorption or growth of PV in vitro, although both populations display increased cytotoxic activity (Table XII, Figure 16). As well, splenocytes cultured in medium share a permissiveness for PV equal to or greater than the permissiveness of cells grown in CM. The reduced growth of PV in cells cultured in CM or IL2 might be due to the presence of interferon in the CM, or by the induction of interferon synthesis by IL2 (Handa et al 1983, Kawase et al 1983, Weigent et al 1983). Whether this is the case or not, virus adsorption and replication were independent of cytotoxic activity expressed by the various cell populations (Figures 16, 17).

B. Characterization of cells expressing cytotoxicity and harbouring Pichinde virus after infection

The preceding experiments examining lymphokine responsiveness in the two strains of hamster suggested that this putative target cell in the spleen might be the IL2 responsive LAK precursor cell that is present in larger numbers in MHA animals, or the resultant activated

cytotoxic cell. Preferential infection of immature lymphocytes by arenaviruses is not an unprecedented concept. LCMV infects macrophages, B cells and T cells (Popescu et al 1979, Lohler and Lehmann-Grube 1981, Lehmann-Grube et al 1983b), but T cells are only susceptible in neonates, suggesting that only at a particular stage of development do they serve as targets (Lehmann-Grube et al 1983a, Lehmann-Grube 1984b).

Attempts were made to characterize the cells infected in vivo, and to compare these to cells mediating cytotoxic activity. Virus-induced cytotoxic cells at day 3 post infection are nonadherent cells heterogeneous for the expression of asialo GM1 and the theta homologue. It was known that at day 3 post infection, the largest differences in IC between the strains were also in the nonadherent fraction (Gee et al 1981a). Differences in IC in unseparated splenic populations were detectable at day 2 and sometimes at day 1 post infection, indicating that the inability to limit viral replication is evident very early after infection. Attempts to determine whether more virus was sequestered in nonadherent splenocytes at these early times were negative (Table XIV). These data imply that the differential infection of nonadherent cells is not critical in the susceptibility of MHA hamsters, and may simply be the result of early decisive events. Alternatively, infection of these nonadherent cells is important, and

either the assay is not sensitive enough to detect subtle differences early in infection, or the critical event in susceptibility only occurs at day 3, when the nonadherent cells become infected.

The experiments using antisera plus complement to characterize IC are difficult to interpret because of the inability to eliminate virus free in the suspensions or associated with cell debris. Hence, the estimates of infectious centres remaining after depletion of cells are probably high, and a certain proportion of infectious virus is due to free virus in the suspension or released from dead cells. However, with these reservations in mind; it does appear that cells infected with Pichinde virus are heterogeneous with respect to both asialo GM1 and the theta homologue, but that the reductions in IC after treatment with antiserum and complement are of smaller magnitude than the reductions in cytotoxic activity (Table XV).

Splenocytes from infected hamsters were assayed for their responsiveness to IL2 by short term culture in either medium or IL2. MHA cells cultured in IL2 express high levels of cytotoxic activity. However, these cells are not infected to a greater extent than cells cultured in medium that fail to express any cytotoxic activity. LSH splenocytes cultured in medium or IL2 mediate very little cytotoxicity, but harbour equal amounts of virus. Culture in IL2, at least for up to 3 days, does not select out a

cell preferentially infected with PV in either strain of hamster, although these culture conditions induce high lytic activity in MHA cells. In both medium and IL2, MHA cells do contain more virus than LSH, analogous to the in vivo situation (Figure 18).

C. Modulation of Pichinde virus pathogenesis

The experiments in vitro indicated that cells expressing asialo GM1 could serve as target cells for PV replication, but they were not the exclusive targets. The later experiments show that a single injection of antiserum eliminates these asialo $GM1^+$ targets, as assessed by the reduced NK activity in the spleens of treated animals (Table IX). Animals infected with PV when splenic NK activity is reduced still display depressed NK activity 2 and 4 days after injection of the antiserum (Table ~~XVI~~). At the same time, IC are reduced at day 1, but by day 3 are equal to or increased over control levels (Table XVI), and the initial reduction of IC is not adequate to shift the susceptible animals to the resistant phenotype (Figure 19). These findings suggest that, although cells expressing asialo GM1 can serve as targets for Pichinde virus replication, they are not the critical target cells resulting in increased virus replication in MHA hamsters. This lends support to the contention that MHA hamsters are susceptible because of increased numbers of the remaining asialo $GM1^-$ LAK precursors that also become infected.

The data do not clearly demonstrate that the initial reductions in NK activity and IC are due to an actual loss of cells. Equal numbers of splenocytes were recovered from treated and control animals, but small changes due to the loss of asialo GM1$^+$ cells might not be demonstrable in haemocytometer cell counts. In the mouse and rat, a single injection of anti-asialo GM1 reduces NK activity by over 80% (Kasai et al 1981, Suzuki et al 1985, Barlozzari et al 1983, Habu et al 1981), and apparently does so by reducing the number of splenic cells expressing asialo GM1 (Habu et al 1981, Suzuki et al 1985) or cells with the morphology associated with NK activity (Barlozzari et al 1983). The reduced NK activity does not recover until day 7 to 11 after injection in these systems, and presumably this is the time necessary for the regeneration of cells expressing this antigen. If the same is true in the hamster, then reduced NK and possibly reduced IC are both due to a decrease in the number of asialo GM1$^+$ effector cells. Alternatively, antiserum treatment might interfere with both NK activity and early viral replication by a mechanism other than elimination of cells, so that the target cells are still present in the spleens or in the circulation, and become infected later than day 1.

The possibility that NK activity and IC are reduced because of the loss of other asialo GM1$^+$ cells that act indirectly on these parameters cannot be ruled out. A small

proportion of resident peritoneal macrophages in the mouse also express asialo GM1 (Wiltrout et al 1985) although cytolytic activity of peritoneal macrophages is not susceptible to treatment with anti-asialo GM1 (Kawase et al 1983, Keller et al 1983). Whether splenic macrophages similarly express some asialo GM1 or not is unknown. Studies in the mouse claiming specificity of the antiserum for NK effector cells have demonstrated that T effector cells are negative for asialo GM1 (Young et al 1980, Kasai et al 1980, Kawase et al 1982) but that progenitor cells may express this antigen (Beck et al 1982, Suzuki et al 1985). The number of cells expressing the theta antigen in the murine spleen is not affected by treatment with anti-asialo GM1 (Habu et al 1981, Suzuki et al 1985).

The effect of treatment with anti-asialo GM1 serum on viral pathogenesis has been examined in mice. In some, but not all, viral systems examined, treatment with the antiserum resulted in increased viral titres at the same time that NK activity was reduced (Bukowski et al 1984, 1985, Habu et al 1984). These data are used to support the hypothesis that NK activity is a relevant anti-viral defense. The increased viral titres observed in the spleens of hamsters treated with the antiserum are unlikely to result from reduced NK activity, as susceptibility in control animals correlates with high, not low, NK activity. NK activity did not appear to play a significant role in

limiting viral titres in mice infected with LCMV (Bukowski et al 1984). Experiments scoring mortality rather than IC show that the control levels of virus at day 3 after infection in treated MHA hamsters correlate with mortality, and treatment of LSH hamsters did not alter their resistance to infection (Figure 19).

The last attempt to alter PV pathogenesis met with more success. It was reasoned that deficient IL2 production by MHA hamsters results in a larger pool of immature cells. Administration of purified IL2 might alter this pool size, and hence protect the animals. Although the mortality rate of IL2 treated animals was slowed compared to controls (Figure 21), this was not due to a decrease in virus load early after infection (Table XVII). A possible explanation for these results is that IL2 administration elicits an earlier immune response than normally observed after infection. Death could be due to continued viral replication that could no longer be limited after IL2 levels declined in the hamsters, or to IL2-induced immune mediated pathology. The latter course of events is suggested by the rapid death of the animals at day 10. If this is the case, these results cannot explain the susceptibility of normal MHA hamsters, as these animals show no evidence of immunologically mediated disease at the time of death (Murphy et al 1977).

The dose of IL2 administered might not have been adequate to see the desired effects on the spleen cell pool size. In cyclophosphamide treated mice, or mice recovering from bone marrow transplants, larger amounts of IL2 were administered over longer periods of time, before CTL precursors were demonstrable (Merluzzi et al 1983, 1985a), although a single dose of IL2 was reported to augment NK and alloreactive CTL in another study (Hefeneider et al 1983). Ettinghouse and colleagues (1985) have demonstrated proliferation of lymphoid cells in lungs, liver, kidney, spleens and lymph nodes of normal mice after administration of IL2, but the doses used were over 100-fold more than those used in this study. These doses also increased rather than decreased LAK after 4 days of treatment. IL2 has been reported to elicit T helper cells for antibody reponses in nude mice (Stotter et al 1980). The effect of IL2 on the course of viral infections has not been examined except in one set of experiments where immune cells plus IL2 transferred to infected mice limited viral growth more efficiently than immune cells alone, presumably by inducing growth of the cells (Rouse et al 1985).

C. Summary

To restate the hypothesis, it was proposed that nonadherent splenic lymphocytes mediating NK activity and serving as precursors for LAK, are targets for PV replication and that quantitative differences in these

cells account for the observed differences in susceptibility to infection in the two strains of hamster. In light of experiments presented in the first section of this thesis, it was suggested that increased numbers of this target cell are present in MHA hamsters because of diminished IL2 production that results in the accumulation of relatively immature cells in the lymphoid organs.

The data presented in the second section neither support nor refute the hypothesis with certainty. It is clear that differences in genetic resistance to the virus are not due to differences in the permissiveness of target cells for infection, and hence it may be quantitative rather than qualitative differences in a target cells that are crucial. The data presented here supporting this hypothesis include the increased number of cells in MHA lymphoid organs, and in particular, in LAK precursors, and the shared expression of the theta homologue and asialo GM1 on NK effector cells and on cells infected with virus as demonstrated <u>in vitro</u> and <u>in vivo</u>. Other data accumulated in the course of these experiments, refute the hypothesis. Elimination of cells expressing asialo GM1 reduced both NK and IC initially, but had no effect on later replication of the virus, nor on survival of MHA hamsters. This may suggest that the remaining LAK precursor cells are the relevant target cells, but there was no evidence that the asialo $GM1^+$ or asialo $GM1^-$ component of endogenous NK

activity was selectively increased in MHA hamsters. Although LAK precursors were increased, other leukocyte populations might be equally increased. At day 2 after infection, when the spleens of MHA hamsters already contained twice as much virus as LSH spleens, there was no preferential infection of nonadherent cells, nor were more infected cells selected from MHA spleens relative to LSH spleens by culture in IL2. In culture, virus titres and cytotoxic activity were dissociated. The fact that reduced virus titres at day 1 after infection were not able to overcome the genetic susceptibility of these animals also suggests that later events control the outcome of infection, and this may be independent of actual virus titre.

The arenaviruses infect lymphoid organs early in the course of infection, including macrophages (Laguens et al 1983a, Lohler and Lehmann-Grube 1981, Murphy et al 1977) but there is only one instance where infection of macrophages is responsible for disease and mortality. A virulent strain of Junin virus infects both macrophages and dendritic cells, whereas the attenuated strain of the virus only infects dendritic cells (Laguens et al 1983a). There is no evidence that quantitative or qualitative differences in the infection of macrophages are responsible for the susceptibility of MHA hamsters to PV. However, macrophages may play a more active role in the pathogenesis of PV

infection. The only aspect of macrophage function examined in this thesis was IL1 production after exposure to the mitogen LPS, and this was the same in the two strains, but other macrophage functions might be deficient. In the LCMV system, macrophages are activated at the time that virus titres decline (Lohler and Lehmann-Grube 1981). Infection of murine peritoneal macrophages with PV interferes with their responsiveness to macrophage growth factor, so that proliferation is inhibited (Friedlander et al 1984). This growth failure is evident 4 days after exposure to the virus. These kinetics are similar to those observed for the limitation of viral replication in LSH hamsters after infection. Perhaps the macrophages of LSH hamsters are more resistant to this effect of PV infection. Unfortunately, the available evidence indicates that activated macrophages do not express direct anti-Pichinde effects (Friedlander and Jahrling 1982), but activated macrophages might contribute to resistance through other mechanisms, such as elicitation of early cell-mediated immune responses. If MHA macrophages fail to become activated after infection with PV, but not after infection with other viruses, IL1 production and consequentially IL2 production might be diminished also and hence the administration of exogenous IL2 would be beneficial as was noted. This would explain the restriction of MHA

susceptibility to PV, and the situation would be analogous to that described for responses to M. leprae.

Genetic differences in the resistance of animals to the other arenaviruses are limited to a few studies of LCMV infection. Mortality after infection with LCMV is immune-mediated in most instances, so genetic resistance is often due to decreased specific immune responses directed to virus-infected tissues (Tosolini and Mims 1971, Lehmann-Grube and Lohler 1981, Moskophides and Lehmann-Grube 1983, Oldstone and Dixon 1969). One set of experiments examined the pathology of LCMV disease in suckling mice which was found to resemble the disease induced by interferon administration to young mice (Gresser et al 1975, Riviere et al 1977, Ronco et al 1980). Injection of anti-interferon serum reduced mortality in LCMV infected suckling mice (Riviere et al 1977), but more interesting was the discovery that the genetic susceptibility of several strains of mice correlated not with viral titres but with increased levels of interferon induced by the virus (Riviere et al 1980). In these studies, interferon was thought to interfere with synthesis of basement membranes in the affected organs, primarily the liver and the kidney. Increased levels of interferon have also been implicated in the pathogenesis of LCMV disease in adult mice, but in this instance interferon appears to cause disease by potentiating the specific immune response

(Jacobson et al 1981). More severe disease after infection of monkeys with Machupo virus (Stephen et al 1977) or humans with Junin virus (Laguens et al 1984) is also associated with higher interferon titres. The genetic susceptibility might be due to increased interferon production after infection with PV. Higher interferon titres would also account for the higher NK activity observed after infection. The induction of virus-induced NK activity in mice infected with LCMV parallels the production of interferon (Welsh 1978b). Genetic studies of interferon production in the mouse have demonstrated that the genes responsible for interferon induction differ with the stimulus used (deMaeyer et al 1970, Pugliese et al 1980). This would explain the specificity of the susceptibility of MHA hamsters to PV. However, this hypothesis does not account for the initial high virus titres, which may simply be due to the increased number of cells in the spleen, nor is it easy to understand why virus titres would continue to increase in the presence of interferon.

Other aspects of the nonspecific inflammatory response might regulate susceptibility. Complement profiles are altered in guinea pigs (Rimoldi & deBracco 1980) and humans infected with Junin virus (deBracco et al 1978). Laguens (1984) proposed that interferon associated with Junin virus infection activates complement that then

damages the tissues. Another intriguing possiblity is that of a difference in a lymphokine other than interferon that is synthesized by T lymphocytes and directly interferes with viral replication in surrounding cells. Such a lymphokine is thought to be responsible for the clearance of LCMV from the spleens of mice early after infection before specific T cell reponses can be detected (Lehmann-Grube et al 1985). It could be that MHA hamsters fail to synthesize this molecule, because of a specific unresponsiveness of T cells to PV antigen, and this underlying failure to respond may be linked to macrophage defects, or to failure to produce IL2 after infection.

A review of the literature of viral studies in hamsters revealed only one other study of genetic resistance to a virus. Both MHA and LSH hamsters are susceptible to an intraperitoneal injection of VSV while a third strain of hamsters is resistant (Fultz et al 1981a). The eptible animals survive if the virus is introduced by any route other that i.p., and in these surviving animals initial virus growth is the same as that observed in i.p. infected animals. However, after 42 hr, virus yields decline in surviving animals (Fultz et al 1981a, 1981b). No mechanism for resistance was postulated, although interferon levels induced by the virus were not correlated with survival (Fultz et al 1982). Initial events in the peritoneal cavity appear to be crucial, as

infection by this route was more rapidly fatal. In MHA hamsters, both i.p. and s.c. infection are fatal, indicating that the pathogenesis VSV infection is different from that of PV. Other studies in the hamster lend little to this discussion; various parameters of the immune system have been examined after infection with vaccinia (Nelles & Streilein 1980b, et al 1981), measles (Byington et al 1975, Cremer et al 1982) parainfluenza (Henderson 1979) and influenza viruses (Stein-Streilein et al 1981, Kimmel et al 1982), but none of these studies compared responses in strains of hamsters, nor were these models analogous to the type of infection observed with PV.

III. Conclusions

In sum, the data show that cells responding to IL2 are heterogeneous, as are cytotoxic effectors and these two populations appear to overlap, but in the absence of reagents specific for hamster lymphocyte populations, no particular cell infected with PV can be isolated, characterized and quantitated. This is particularly true if the cell of interest is only one of many cells that become infected, as is the case here. The fact that in several instances IC and cytotoxic activity were dissociated suggests that the hypothesis is incorrect, and that some cell other than those mediating cytotoxic activity is the crucial target, or that some factor other

than the presence or absence of a target cell accounts for the susceptibility of MHA hamsters.

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