ANALYSIS OF VIRUS-INDUCED CELL-MEDIATED IMMUNE RESPONSES IN SUSCEPTIBLE AND RESISTANT STRAINS OF INBRED HAMSTERS

By

© MARCIA ARLENE CHAN, B.Sc., M.Sc.

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AUTHOR: Marcia Arlene Chan, M.Sc. (University of Toronto)
B.Sc. (University of Toronto)

SUPERVISOR: Dr. William E. Rawls

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ABSTRACT

The arenaviridae are a group of enveloped, negative-stranded RNA viruses. These viruses characteristically produce persistent infections in their natural host, which is how they are maintained in nature. Experimentally-induced arenavirus disease has been found to possess similar pathological features of human disease. In the inbred MHA strain of Syrian hamster, Pichinde virus causes a fatal infection when inoculated intraperitoneally. Investigation of the factors leading to fatal Pichinde virus disease in Syrian hamsters revealed that susceptibility was genetically determined. Susceptibility of MHA hamsters to a lethal infection of the virus appeared to be related to the presence of a splenic target cell for Pichinde virus replication that was minimally expressed in resistant hamster strains. In addition, during studies on cell-mediated immune responses to Pichinde virus antigens, it was discovered that MHA hamsters survived infection when the virus was given in the footpad. However, unlike the resistant LSH and LVG strain hamsters, the MHA hamsters did not manifest a footpad swelling response.

The present studies were initiated to determine some characteristics and possible mechanism(s) underlying the lack of footpad swelling response to a primary inoculation of Pichinde virus in the MHA strain hamster. MHA unresponsiveness was not due to a lack of immune recognition of Pichinde virus antigens, since this strain was shown to produce complement-fixing antibodies to Pichinde virus. Furthermore, footpad-inoculated MHA hamsters were protected against a subsequent intraperi-

toneal challenge with Pichinde virus.

Experiments designed to elucidate the genetic basis for presence or absence of footpad swelling revealed that expression of footpad swelling to Pichinde virus was a dominant trait controlled by a single gene or closely linked genes. Furthermore, the lack of responsiveness in MHA hamsters appeared to be specific for Pichinde virus, since footpad swelling could be elicited in this strain by footpad injection of either vaccinia virus or vesicular stomatitis virus. Histological analysis of the virus-injected footpad showed that footpad swelling was associated with an influx of mononuclear cells at the site of injection. These observations suggested that MHA unresponsiveness could not have been caused by a general defect in effector mechanisms that mediate the footpad swelling response.

A search for Pichinde virus-induced suppressor activity was then undertaken. Treatment of MHA hamsters with cyclophosphamide, a drug known to augment delayed hypersensitivity responses by inhibiting suppressor cell activity resulted in a significant footpad swelling response following Pichinde virus injection. Furthermore, transfer of lymphoid cells but not serum from 5 day footpad-inoculated MHA hamsters could specifically suppress footpad swelling to Pichinde virus in the LSH recipient.

Another parameter of cell-mediated immunity studied was the generation of cytotoxic cells. Pichinde virus primed MHA and LSH hamsters were capable of generating cytotoxic cells, after restimulation in vitro with virus but the cytotoxicity lacked specificity. When spleen cells from 7 day footpad-inoculated MHA hamsters were added to spleen cells from LSH hamsters, the cytotoxic activity of the latter

cells was significantly suppressed.

These results taken together suggested that the absence of footpad swelling in the MHA strain hamster was caused by the generation of a cell-associated suppressor mechanism that appeared to be induced specifically by Pichinde virus.

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LIST OF ABBREVIATIONS

alpha-MEM	alpha formulation of Eagle's Minimal essential medium
B lymphocyte	bone marrow-derived lymphocyte
BHK	baby hamster kidney cells
CF	complement fixation, complement-fixing
cpm	counts per minute
DTH	delayed-type hypersensitivity
FBS	fetal bovine serum
FP	footpad
HaSA	hamster serum albumin
HBSS	Hanks' balanced salt solution
HEPES	hydroxyethylpiperazine-N'-2-ethanol sulfonic acid
IP	intraperitoneal(ly)
IV	intravenous
LEF	LSH embryo fibroblast
LSH	an inbred strain of Syrian hamster originally bred at the London School of Hygiene
LVG	an outbred, closed colony of Syrian hamster maintained in Lakeview, N.J. (Lakeview Golden)
2-ME	2-mercaptoethanol
MEM F15	F-15 formulation of Eagle's Minimal essential medium
MHA	an inbred albino strain of Syrian hamster originally bred at Mill Hill (Mill Hill Albino)
MHC	major histocompatibility complex
MLN	mesenteric lymph node
NK lymphocyte	natural killer lymphocyte

LIST OF ABBREVIATIONS (cont'd)

PBS	phosphate-buffered saline
PE, PEC	peritoneal exudate cell(s)
pfu	plaque-forming unit(s)
PLN	popliteal lymph node
RNA	ribonucleic acid
SD	standard deviation
SEM	standard error of the mean
SPL	spleen
T lymphocyte	thymus-derived lymphocyte
T-EDTA	trypsin-ethylenediaminotetraacetic acid
VBD	veronal buffered diluent

CHAPTER 1

INTRODUCTION

1.1 The immune response

The immune response is envisioned as consisting of highly regulated, multiple, interlocking cell circuits in which the intensity, duration and type of immune reaction is dependent on the messages that are passed among specialized cells of the immune system, following antigenic stimulation (Cantor and Gershon, 1979). The specialized cells of the immune system are small lymphocytes capable of initiating immune responses after recognition of antigen. In mammals as exemplified by the mouse, the small lymphocytes are divided into two distinct populations, both of which appear to arise from hemopoietic stem cells located in the bone marrow. A proportion of these stem cells migrate to the thymus where differentiation and maturation takes place. In the mouse, during this process, the cell surface antigens, theta (Reif and Allen, 1964) and TL (Boyse, Old and Stockert, 1968) are acquired. Subsequently, a second differentiation step occurs in which a segment of thymus lymphocytes lose the TL antigen and migrate to peripheral lymphoid tissues, where they are designated 'thymus-derived' or 'T' lymphocytes (Weissmann, 1967; Davies, 1969). The other lymphoid population, referred to as 'bone marrow-derived' or 'B' lymphocytes (Roitt et al., 1969) is not processed by the thymus but appears to remain in the bone marrow, where induction of differentiation occurs (Owen, 1972) and a coat of membrane-bound immunoglobulin is acquired (Ritter, 1975). Other cells which participate in immune responses include macrophages (Rosenstreich, Farrar and Dougherty, 1976) and other accessory cells (Talmage and Hemmington, 1973). Upon antigenic stimulation, the T lymphocytes develop into "sensitized" T

cells and the B lymphocytes become antibody-secreting cells. The T cells are responsible for the cell-mediated aspects of immunity, such as delayed hypersensitivity and graft rejection, whereas the B cells are responsible for the humoral aspects of the immune response.

The T lymphocytes are the major components of the immunoregulatory cell circuits that have been described. These cell circuits, in general, consist of inducer cells, precursor or amplifier cells and effector cells, communicating with each other using antigen-specific products and major histocompatibility complex (MHC) gene products. Some of these cells appear to produce soluble factors which function as signals that appear to be integrated and transmitted by macrophages to the appropriate target cell (Gershon, Naidorf and Ptak, 1980). Through the discovery of two antigens, Ly (Kisielow et al., 1975) and Qa-1 (Stanton and Boyse, 1976) present on the cell surface of T lymphocytes, these cells have been separated into subpopulations with different immunological functions (Cantor and Boyse, 1975; Jandinski et al., 1976). By the use of Lyt antisera, the T lymphocytes can be divided into three subsets - Lyt-1$^+$, Lyt-23$^+$ and Lyt-123$^+$. Cells of the Lyt-1$^+$ phenotype appear to be programmed to act as inducers or initiators. The Lyt-1$^+$ helper cell induces B lymphocytes to make antibody (Cantor and Boyse, 1975; Cantor and Boyse, 1977; Eardley et al., 1977); macrophages and other nonspecific inflammatory cells to participate in delayed-type hypersensitivity reactions (Huber et al., 1976); precursor killer cells to differentiate into killer-effector cells (Cantor and Simpson, 1975; Plate, 1976) and precursor suppressor cells to differentiate into suppressor cells that can exert a potent feedback inhibitory action on the Lyt-1$^+$ inducer cell. Whether all inducing functions of the Lyt-1$^+$ cell set are the

property of a single group of cells or whether the Lyt-1$^+$ cell set is a heterogeneous collection of cells with each inducer function being mediated by individual Lyt-1$^+$ subsets is not known. However, there is evidence suggesting that the Lyt-1$^+$ T cell subset that is specialized in inducing suppressive activity bears I-J encoded antigens on its surface (Ptak et al., 1979) whereas the Lyt-1$^+$ cell subset that is involved in B cell activity is I-J$^-$ (Gershon et al., 1981). Furthermore, it has recently been discovered that the subset of cells mediating delayed hypersensitivity responses possess the Lyt-1$^+$ phenotype (Leung and Ada, 1980a).

The Lyt phenotype of cytotoxic and suppressor cells remains somewhat controversial. Originally described as bearing the surface antigens, Lyt-23 (Cantor and Boyse, 1977; Eardley et al., 1978), recent evidence suggest that both cytotoxic and suppressor cells can also possess the Lyt-1 surface antigen (Maier, Levy and Kilburn, 1980; Nakayama et al., 1980; Vidovic et al., 1981). It appears that different sets of cytotoxic cells can be generated dependent upon the conditions in which they were induced. For example, cytotoxic cells raised against allogeneic cells have been reported to express the Lyt-123$^+$ phenotype whereas killer cells generated against syngeneic tumour cells have been reported to be of the Lyt-23$^+$ phenotype (Maier, Levy and Kilburn, 1980). Similarly, two populations of suppressor cells have been found to exist, those that suppress a humoral response have been found to be Lyt-23$^+$ (Green, Gershon and Eardley, 1981) and those that suppress the delayed-type hypersensitivity reaction have been found to be Lyt-1$^+$ (Liew and Russell, 1980).

Cells that possess the Lyt-123$^+$ phenotype are generally regarded as precursor or amplifier cells. This cell subset has been shown to be required for the maturation of the Lyt-23$^+$ suppressor/cytotoxic effector cell (Cantor and Boyse, 1977; Burakoff et al., 1978; Eardley et al., 1978; Wagner et al., 1979; Wagner et al., 1980) and to serve as a precursor for the Lyt-1$^+$ inducer cells (Gershon et al., 1977; Shen et al., 1980) and for the effectors of the delayed hypersensitivity response (Kaufmann, Simon and Hahn, 1979; Leung, Mak and Ada, 1981).

In the initiation of the immune response by antigen, a series of T cell - T cell interactions appear to take place and the Lyt-1$^+$ inducer cells seem to play a central role in determining the type, intensity and duration of the immune response that ensues. When antigen enters the body, its first confrontation with the host immune system is usually with a macrophage. The mechanism by which the macrophage interacts with the antigen is not clear. Nevertheless, it appears that the macrophage binds antigen through an active metabolic, nonimmune mechanism and preserves the antigen in a form that remains immunogenic for extended periods of time (Unanue and Askonas, 1968; Waldron, Horn and Rosenthal, 1974). Several investigators found that macrophages from either normal or tolerant donors could present antigen as well as macrophages from immunized animals (Levis and Robbins, 1970; Seeger and Oppenheim, 1970). Thus, it seems that the macrophage does not possess immune specificity in its binding to antigen. Following the initial encounter, a series of events occur. With some antigens, this results in the presentation of the antigen in association with gene products of the I subregion of the MHC on the surface of the macrophage and release by the macrophage of a

soluble Ia-restricted factor. This factor, in turn, induces both an amplifier cell and precursor T helper cell to differentiate into a Lyt-1$^+$ T helper cell (Erb et al., 1977). The Lyt-1$^+$ T helper cell can then induce a number of different immunologic effector cells, as mentioned above. In other models, the macrophage has been shown to release an antigen-nonspecific factor, termed interleukin 1 that appears to also induce helper cell activity (Mizel, 1982).

The quality and quantity of the immune response, i.e. active immune state or suppressed state, that develops appears to depend on such factors as the dose of antigen and site of antigen encounter as well as the genetic constitution of the host. Several investigators have demonstrated that suppression in vitro (Eardley and Gerson, 1976; Kontiainen and Feldmann, 1976; Weitzman, Shen and Cantor, 1976; Watson and Collins, 1980) and suppression in vivo (Collins and Watson, 1980) of the immune response can occur when high antigenic doses are used. Furthermore, the route by which antigen is presented to the immune system seems to be an important determinant of the nature of the immune response. Experimental, intravenous inoculation of antigen has been shown by a number of researchers to lead to enhanced skin graft survival in recipient mice when allogeneic cells are used (Greene and Bach, 1979) or to the emergence of an increased number of regulatory or suppressor cells (Bach et al., 1978; Greene and Weiner, 1980; Leung and Ada, 1980b). In contrast, a subcutaneous inoculation of the same antigen preferentially has been shown to lead to an active immune state.

Delayed hypersensitivity reactions in the mouse can be inhibited by intravenous inoculation of antigen. Generation of suppressor cells have been described as the cause of suppression of delayed hypersensiti-

vity responses to the hapten, azobenzenearsonate (Bach et al., 1978) and picryl chloride (Thomas, Natkins and Asherson, 1979). At least two and possibly three separate suppressor cells have been characterized in the pathway leading to suppression. In the azobenzenearsonate model, intravenous inoculation of mice with hapten-derivatized syngeneic cells culminates in the development of three distinct suppressor cells - a cyclophosphamide-sensitive afferent suppressor cell (Ts_1), a cyclophosphamide-resistant efferent cells (Ts_2) and possibly a third cell (Ts_3) that may impose the suppressive effect. Collaboration between the cells is mediated by the soluble factors, TsF_1 and TsF_2 that are produced by the respective suppressor cells (Germain and Benacerraf, 1981). Similarly, in the picryl chloride system, both afferent and efferent suppressor cells have been described (Asherson and Zembala, 1980). However, the suppressor factor that is produced by the efferent suppressor cell in this system also collaborates with a macrophage, so that the latter cell has the capacity to suppress. Therefore, it appears that cell-mediated suppression derives from a complex pathway consisting of a variety of suppressor cell interactions and their soluble factors.

Although most of the studies defining the cells participating in the immune response have been done in the mouse, some studies have also been done in the hamster. Hamsters appear to possess T and B lymphocytes and macrophages. Four classes of immunoglobulins have been described - IgM, IgG, IgA and IgE (Robles et al., 1981) and all the components of the classical complement system have been identified (Barta et al., 1981). In addition, hamsters appear capable of developing cellular immunity. They can reject skin allografts and manifest graft-versus-host reactivity (Duncan and Streilein, 1981) and can develop contact hypersensitivity to simple haptens (Maguire, 1980; Streilein, Sullivan and

Thompson, 1980). However, hamsters do seem to have some interesting immunological properties. The hamster cytotoxic T effector cell lacks MHC restriction when killing virus-modified targets (Kimmel, Wyde and Glezen, 1982), unlike the equivalent murine cytotoxic cell. This could be due to the fact that limited polymorphism appears to exist at the class I locus of the hamster MHC (Hm-1), which is analogous to the murine H-2 K/D locus. Nevertheless, it seems that natural killer cells or reactivity mediated by antibody-dependant mechanisms are predominantly responsible for cytotoxic activity generated in hamsters. Natural killer cells appear to dominate responses to tumours (Trentin and Datta, 1981) and to many virus infected tissues (Lausch et al., 1981). Moreover, Nelles, Duncan and Streilein (1981) have reported that cytotoxic activity generated in vaccinia virus-infected hamsters results from antibody-dependent and natural killer cell mechanisms rather than from cytotoxic T cell activity. Our understanding of the hamster immune system is hampered by the lack of specific reagents capable of identifying the cell-surface markers of T and B lymphocyte subsets, macrophage subsets and other cells participating in the immune response. At the present time, hamster lymphocytes are being characterized using murine monoclonal antisera. Yang and Tompkins (1982) have shown that murine monoclonal anti-mouse Thy 1.2 antiserum can detect a Thy 1.2 homologue on hamster T cells and can be used to distinguish these cells from splenic NK cells.

Despite the current limited knowledge of the hamster immune system, these animals appear to be useful in the investigation of a variety of infectious and oncologic diseases. Pathogenesis of experimentally induced diseases in hamsters appears to mimic, in many systems, the

human disease counterpart. For example, respiratory infections in hamsters with Mycoplasma pneumoniae have been reported to be similar to the human disease (Clyde and Fernald, 1981).

1.2 Effect of viral infections on the function of the immune system

Invasion of a host by virus usually results in the activation of the host's immune system. Antiviral antibodies are formed and the development of antiviral effector cells occurs, leading to the recovery of the host from infection. Thus, manifestations of viral disease are greatly affected by the immune system. Conversely, the immune system can be significantly altered by viral infections.

The first documentation that viral infections can effect the function of the immune system was made in 1908 by Von Pirquet who reported that a loss of tuberculin skin reactivity occurred in children suffering from measles virus infection. Since that time, other investigators have substantiated Von Pirquet's observation (Mascia, Chick and Levy, 1953; Starr and Berkovich, 1964) and have further demonstrated that immune reactivity can be altered by natural infection or vaccine-induced infection with other viruses such as smallpox virus (Huges, Smith and Kim, 1968), rubella virus (Kauffman et al., 1974; Chantler and Tingle, 1980), influenza virus (Kantzler et al., 1974), varicella virus (Belsey, 1967) and mumps virus (Hall and Kantor, 1972).

In most cases, viral infection depress ensuing immunologic functions and it has been suggested that the T lymphocytes are the primary targets of viral-induced immunosuppression, such that subsequent alterations in T lymphocyte reactivity produce impaired effects in both humoral and cell-mediated immune responses (Doyle and Oldstone, 1979).

However, Katsura, Minato and Nishikawa (1981) have shown that vesicular stomatitis virus infection of mice at the time of immunization with antigen leads to a persistent and augmented delayed hypersensitivity response specific to the immunizing antigen.

Woodruff and Woodruff (1975) have proposed several mechanisms by which viruses might mediate immune dysfunction. One mechanism might be the impairment of macrophage function. Macrophages play a major role in the inductive phase of the immune response by virtue of their role in antigen processing and presentation, so that abnormalities in macrophage function as a result of viral replication in the cell might lead to immunologic disorder. For example, poliovirus appears to exert an immunosuppressive effect on lymphocytes by replicating in macrophages (Soontiëns and Van Der Veen, 1973). A second mechanism might be the destruction of lymphocytes. Chaturvedi et al. (1981) have shown that dengue type 2 virus exerts a direct cytotoxic effect on lymphocytes through the production of a cytotoxic factor in the spleen of infected mice. Mice treated intravenously with the factor were reported to have a reduced number of T lymphocytes in their spleen and a reduced hemolysin titre following intraperitoneal immunization with sheep red blood cells.

Viral infections might also affect the distribution of lymphocytes and their route of circulation through lymphoid tissue. A continuous circulation of lymphocytes through lymphoid tissue could be important in facilitating the induction and expression of immunity by promoting the interaction between immunologically competent cells and antigens. Thus, any alterations in lymphocyte traffic might lead to a suppressed response. Evidence of viral-induced disturbances in

lymphocyte traffic has been obtained in studies determining the fate of transfused radiolabelled lymphocytes. Woodruff and Woodruff (1974) reported a difference in distribution of radioactivity in lymphoid tissue between recipients that had been infused with Newcastle disease virus-treated ^{51}chromium labelled lymphocytes and recipients that had received untreated radiolabelled cells. The virus appeared to direct the homing of many donor cells from lymph nodes and spleen to the liver.

Viruses might also exert their immunosuppressive effect by inhibiting lymphocyte activation and proliferation. The mechanism by which viruses could mediate such inhibition might be either directly by the arrest of lymphocyte macromolecular synthesis or indirectly via viral-induced factors or suppressor cells. Viruses depend solely on the host cells to provide the cellular machinery for the replication of their nucleic acid and synthesis of their proteins. Thus, when the virion penetrates the host cell, a complete shutdown of host cell macromolecular synthesis may occur and further normal cell growth and function is prevented. Lymphocyte populations, stimulated either nonspecifically by mitogens or specifically by antigen have been shown to support the replication of a large number of viruses *in vitro*. For example, measles virus has been found to infect lymphocytes *in vitro*, resulting in an altered response of the lymphocytes to various antigens and mitogens (Huddlestone, Lampert and Oldstone, 1980). Furthermore, these authors isolated infectious measles virus from lymphoid cells taken from patients with measles virus infection and were able to infect T lymphocytes *in vitro* from healthy immune subjects with the isolated virus. Similarly, Chantler and Tingle (1980) reported that human peripheral blood lymphocytes previously stimulated with phytohaemagglutinin or pokeweed mitogen would support

rubella virus replication, resulting also in a depressed response to mitogen.

One of the early antiviral mechanisms that is produced by the host in response to viral invasion is interferon. Interferon is produced by virus-infected cells and its role is to limit virus spread by inhibiting viral replication in neighbouring cells. However, it can also have a detrimental effect on the immune system. Interferon induced by viral infection can inhibit both humoral and cell-mediated immune responses. Baron and coworkers (1979) have suggested that interferon is capable of indirectly suppressing precursor T and B lymphocytes by activating a macrophage with suppressor function. Moreover, Koltai, Mécs and Kása (1981) have shown that mice, previously injected with Sendai virus did not respond to a footpad inoculation of two potent inflammatory agents, carrageenan and serotonin creatinine sulphate. These authors concluded that Sendai virus induced the production of interferon which in turn, inhibited the inflammatory response. De Maeyer, De Maeyer-Guignard and Vandeputte (1975) have also found that interferon induced by injection of Newcastle disease virus into mice inhibited delayed-type hypersensitivity responses in these animals to picryl chloride and sheep erythrocytes.

Viruses might mediate their effects on immune function through the stimulation of suppressor cells. Evidence for a role of suppressors has been suggested by the results of experiments with reovirus and influenza virus. Mice injected intravenously with UV-inactivated reovirus did not exhibit a delayed hypersensitivity response when challenged 6 days later with live virus in the footpad. This depressed state was demonstrated to be mediated by suppressor cells since the infusion of spleen cells from tolerant animals prevented the induction of delayed hypersensitivity

in recipients that were previously immunized with live virus. Immunization with live virus always resulted in a competent delayed hypersensitivity response (Greene and Weiner, 1980). A similar observation has been reported in studies with mice injected with UV-inactivated influenza virus (Leung et al., 1980; Liew and Russell, 1980). Furthermore, injection of UV-inactivated virus appeared also to induce the formation of suppressor cells that selectively inhibited cytotoxic T cell responses to influenza virus (Leung et al., 1980).

Viral infections have been shown to have a profound effect on the functioning of the immune system. Their influence is primarily suppressive. The ability of many viruses to replicate in lymphocytes may either result in a depletion of lymphoid cells through a cytopathic effect or render the cells unresponsive to antigenic or mitogenic stimulation. Alternatively, viruses may exert their influence indirectly through either viral-induced factors, such as interferon or induction of suppressor cells.

However, there are some viruses that affect the immune system in such a manner, that the immune system becomes detrimental to the host. Lymphocytic choriomeningitis virus is the causative agent of lymphocytic choriomeningitis, a naturally occurring viral disease of mice. The clinical disease and pathological changes following intracranial inoculation of the virus into adult mice appears to be a direct result of the immune response. An intense lymphocytic-monocytic infiltration, induced by viral growth in the brain results in damage to the host cells involved in maintaining the physiologic blood-brain ion concentration barrier (Cole and Nathanson, 1974). Evidence of the detrimental effect of the specific host immune response was elucidated in experiments in which

immunosuppressed mice were selectively reconstituted with specific subpopulations of sensitized lymphoid cells. Both Cole and Nathanson (1974) and Volkert and colleagues (1975) found that only the adoptive transfer of T lymphocytes could reproduce the inflammatory lesions and lethal disease in recipient mice.

1.3 Pathogenesis of Pichinde virus in inbred strains of Syrian hamsters

Pichinde virus is a member of the arenavirus group. The arenaviridae are enveloped and appear as round, oval or pleomorphic particles with a mean diameter of 110-130 nm (reviewed by Pedersen, 1979; Rawls and Leung, 1979; Howard and Simpson, 1980). Surface projections cover the virion and ribosome-like particles are present within the virion core. The genome of Pichinde virus consists of negative-stranded ribonucleic acid (RNA). Of five distinct RNA species that have been found, three (28S, 18S and 4 to 6S) are of host ribosomal origin (Carter, Biswal and Rawls, 1973). The two remaining species, 31S (L) and 22S (S) with molecular weights of $2.63-2.83 \times 10^6$ daltons and $1.26-1.31 \times 10^6$ daltons, respectively, appear to be virus specific (Ramsingh et al., 1980). Through cross-hybridization experiments, the L and S RNA species were found to contain unique sequences (Leung et al., 1981) but no messenger function appeared to be associated with either fragment (Leung, Ghosh and Rawls, 1977).

Three primary gene products appear to be encoded by the virion genome. These include a large (L) protein, having a molecular weight of 2×10^5 daltons; a nucleoprotein (NP), having a molecular weight of 6.4×10^4 daltons and a glycoprotein (GPC), having a molecular weight of 7.9×10^4 daltons (Harnish, Leung and Rawls, 1981). The glycoprotein,

GPC, which appears to be derived from a 4.2×10^4 dalton nonglycosylated polypeptide has been shown to cleave to yield two virion glycoproteins, GP-1 (5.5×10^4 daltons) and GP-2 (3.6×10^4 daltons) (Harnish et al., 1981). The nucleoprotein, NP, has been found to contain antigenic determinants that cross-react serologically with proteins from other members of the arenavirus group (Buchmeier and Oldstone, 1978) and also may be responsible for the induction of complement-fixing antibodies (Buchmeier, Gee and Rawls, 1977). Recent evidence, reported by Leung and colleagues (1981) has suggested that NP and GPC are products of messenger RNA transcribed from the viral S RNA species. However, at the present time, it is not known if L protein is encoded in the L RNA species.

Pichinde virus was isolated from a cricetine rodent, Oryzomys albigularis in the Pichinde valley of Colombia, South America (Trapido and Sanmartin, 1971). Similar to the other members of the arenavirus group, Pichinde virus can cause persistent infection in its natural host. Age at infection appears to be important in the development of persistence. Animals that have been infected early in life excrete virus in the urine and saliva throughout life, possess little or no detectable neutralizing antibody and may not show any clinical symptoms of infection (Johnson, Webb and Justines, 1973; Webb, Justines and Johnson, 1975). Moreover, some of the arenaviruses can cause severe disease in humans. These include the agents responsible for Argentine hemorrhagic fever (Junin virus), Bolivean hemorrhagic fever (Machupo virus), Lassa fever (Lassa virus) and lymphocytic choriomeningitis (Lymphocytic choriomeningitis virus). The arenaviruses appear to gain entry into the host either by the alimentary or upper respiratory tract. Replication begins in the local lymphoid tissue or lymph nodes and then invasion of the reticulo-

endothelial system occurs. Post mortem studies following arenavirus infection have illustrated generalized hemorrhages in tissues, focal liver and renal necrosis, and hyperplasia of reticulum cells (Child et al., 1967; Elsner et al., 1973; Winn and Walker, 1975).

Serological evidence suggests that Pichinde virus may infect humans but no significant human disease has been reported to date (Buchmeier, Adam and Rawls, 1974). Nevertheless, Pichinde virus can cause a lethal infection in newborns of all strains of hamsters tested and in one adult strain (MHA) of Syrian hamsters (Mesocricetus auratus) (Buchmeier and Rawls, 1977). The inbred MHA strain was shown to be markedly susceptible to Pichinde virus infection both as a newborn and as an adult. After 8 days of infection virus titres in the blood of infected MHA reached 10^8 plaque-forming units per ml of serum. In contrast, the random bred LVG strain acquired resistance to lethal Pichinde virus infection at about 5-7 days of age and virus titres of approximately 10^3 plaque-forming units per ml of serum were obtained from the LVG hamster strain 8 days after infection. Moreover, histologic analysis of tissues from Pichinde virus infected hamsters revealed marked similarities to the pathological changes induced in humans by arenaviruses (Child et al., 1967; Elsner et al., 1973; Winn and Walker, 1975).

Murphy, Buchmeier and Rawls (1977) have extensively studied the pathogenesis of Pichinde virus infection in these two hamster strains using immunofluorescence, light and electron microscopic techniques. Following intraperitoneal injection, the principal target organs for Pichinde virus replication appeared to be spleen, liver and kidney. The initial signs of infection in the spleen of MHA hamsters were seen

at day 4. Both marginal zones and the periarteriolar lymphatic sheath were infected. By day 7, extensive necrosis was observed in both white and red pulp of the spleen and complete destruction of these areas was noted 10 days after infection. The major target for viral growth appeared to be a macrophage. Large numbers of granulocytes invaded all necrotic sites in the spleen, but there was no evidence of mononuclear cell infiltration. In contrast, a limited amount of viral antigen was seen in the spleen of LVG hamsters at day 4. However, the only remaining evidence of infection at 10 days was a granulocytic infiltration.

Infection of the liver of the MHA hamster proceeded in a similar manner as the spleen. Viral antigen was detected at 4 days of infection. By day 10, there was extensive focal necrosis of Kupffer cells and hepatocytes. A great deal of cellular debris was present in the lesions and in nearby sinusoids. However, at this time, no inflammatory cell infiltration in these lesions was evident. In the liver of the LVG hamster, viral antigen was detected in the capsule and in a few hepatocytes at day 4, but at no time afterwards.

In the lymph nodes of both MHA and LVG hamsters, viral antigen was demonstrable in a few foci in reticular cells. However, macrophage involvement was evident only in MHA lymph nodes. Furthermore, in MHA bone marrow, viral antigen was detected in approximately 1 in 10,000 marrow cells and in 10% of marrow megakaryocytes. No viral antigen was present in LVG marrow and antigen was never found in the thymus of either strain. However, a granulocytosis was observed in the blood of all infected hamsters but it was more extensive in the MHA strain than in the LVG strain. In addition, a reduction in the number of large lymphocytes was apparent in both strains. Thus, the major destructive

effects of the infection were in the spleen and liver. Macrophages were the primary targets for viral growth in the spleen and Kupffer cells in the liver.

Brain and kidney of MHA hamsters were also affected. Foci of viral antigen were found in meninges and in the epithelium of choroid plexuses of MHA brain. Moreover, widespread infection of interstitial tissue and focal infection of tubular epithelium of MHA kidney was evident. In contrast, neither viral antigen nor histologic changes were seen in either brain or kidney of LVG hamsters.

Like other arenaviruses, Pichinde virus has been shown to express a reticuloendothelial tropism. In the MHA strain of Syrian hamsters, Pichinde virus induced extensive and severe reticuloendothelial necrosis, involving primarily destruction of the spleen and liver. However, unlike other rodent arenavirus infections, a mononuclear infiltration into lesion sites was absent, suggesting that direct viral cytopathic effects were responsible for lesion development and death.

1.4 Strain differences in susceptibility of Syrian hamsters to lethal Pichinde virus infection

Pichinde virus produces a lethal infection accompanied by high levels of viremia, when inoculated intraperitoneally, in the adult MHA strain of Syrian hamster, but not in the adult LVG or LSH strains (Buchmeier and Rawls, 1977). Susceptibility has been demonstrated to be under genetic control (Gee, Clark and Rawls, 1979; Gee et al., 1980). Both survival and the ability to limit viral replication are dominant phenotypes and appear each to be controlled by a single gene or linked genes.

A general target cell defect or specific macrophage defect did not appear to be responsible for the differences in susceptibility between the hamster strains. Pichinde virus grew in vitro equally well in primary kidney and peritoneal exudate cells derived from both resistant LVG and susceptible MHA hamsters (Buchmeier and Rawls, 1977). In addition, no major differences in the humoral immune responses to viral antigen by LVG and MHA hamsters were apparent. The kinetics and production of complement-fixing antibodies and antibodies directed against antigen on the surface of virus-infected cells were similar in both strains and the peak titres of antibody were comparable (Buchmeier and Rawls, 1977). Thus, it was postulated that susceptibility of the MHA hamster to Pichinde virus may be the result of an inadequate cell-mediated immune response to the virus. Cell-mediated immunity has been suggested to play a significant role in limiting Pichinde virus replication in the LVG hamster. Treatment of Pichinde virus-infected LVG hamsters with cyclophosphamide was shown to effectively eliminate the age-acquired resistance of this strain (Buchmeier and Rawls, 1977).

In assessing the cell-mediated immune response of the susceptible MHA hamster to Pichinde virus, it was discovered that the MHA hamster survived a footpad inoculation of the virus and was able to limit viral replication to low levels, independent of the dose of virus inoculated (Gee et al., 1981a). Furthermore, footpad-immunized MHA hamsters were protected against a normally lethal intraperitoneal challenge with Pichinde virus, which indicated that this hamster strain had the capacity to develop a protective immune response against the virus.

Since Pichinde virus appeared to exhibit a tropism for cells of the reticuloendothelial system, Gee and colleagues (1981a) examined

possible target cell differences within the spleens of susceptible and resistant hamsters. As early as 2 days after an intraperitoneal injection of virus, spleens of MHA hamsters were found to contain approximately 10-fold more virus-producing cells than spleens of resistant LSH hamsters. Moreover, unlike the MHA strain, LSH hamster spleens subsequently showed a decline in the number of infected cells and virtually no virus-producing cells were detected in LSH spleens 8 days after infection. When spleen cells from infected MHA and LSH hamsters were separated into plastic-adherent and non-adherent populations, the majority of the virus-producing cells in the MHA spleen were associated with the non-adherent fraction. In fact, there were six to seven times more infectious centres in this fraction of spleen cells of MHA hamsters compared to that of LSH hamsters. Further separation of the non-adherent cell populations according to size by velocity sedimentation revealed that the infectious centres contained in the MHA spleen were concentrated primarily in cells that sedimented at a rate typical of a small to medium-sized lymphocyte. In contrast, few infectious centres were detected in the corresponding fraction of spleen cells of LSH hamsters. Instead, virus was predominantly located in a population of cells that sedimented at a rate typical of macrophages.

In addition, Gee, Clark and Rawls (1979) evaluated the role of cytotoxic cells in Pichinde virus infection in the susceptible MHA and resistant LSH strain. Spleen cells from MHA hamsters were found to exhibit appreciable levels of cytotoxic activity against syngeneic and allogeneic tumor cells. Moreover, this cytotoxic activity increased after Pichinde virus infection. In contrast, LSH hamsters showed lower endogeneous cytotoxic activity and less enhancement after virus infection. Characterization of the cytotoxic effector cell revealed a similar-

ity to murine natural killer (NK) cells, i.e., small, non-adherent, non-phagocytic lymphocyte lacking detectable surface immunoglobulin (Welsh, 1978). Furthermore, in elucidating the properties of the MHA hamster splenic NK cell, it was discovered that the cytotoxic cell co-purified with the non-adherent, small to medium-sized population that contained the majority of infectious centres found in the MHA spleen. Thus, it was suggested that the susceptibility of the MHA hamster to Pichinde virus was a consequence of the presence of an additional splenic target cell that had NK activity. Possession of this extra target cell was postulated to result in an early, overwhelming proliferation of virus in the susceptible strain when the virus was inoculated intraperitoneally.

Route of inoculation appeared to be important in the pathogenesis of Pichinde virus infection in Syrian hamsters. As previously mentioned, MHA hamsters that died after an intraperitoneal inoculation of virus, survived an infection initiated by a footpad inoculation of the virus (Gee et al., 1981a). Investigations of splenic NK activity and development of infectious centres in the spleen of footpad-inoculated MHA hamsters revealed a 2-day delay in these two parameters compared to those of intraperitoneal-inoculated MHA animals (Gee et al., 1981b). Therefore, it was suggested that the delay in the rise of splenic NK activity and developing infectious centres was responsible for the survival of footpad-inoculated MHA hamsters. The footpad route of injection appeared to have retarded Pichinde virus spread sufficiently to permit the MHA animal to generate a protective immune response.

Furthermore, when Pichinde virus was inoculated into the footpads of the resistant LSH and LVG hamsters, a swelling response which was

maximal 8 days after inoculation was observed in these two strains (Gee et al., 1981b). In contrast, no footpad swelling was noted in the MHA hamster strain. Thus, the MHA hamster strain differed from other strains of hamsters not only in the fatal outcome of an intraperitoneal inoculation of Pichinde virus but also in the absence of footpad swelling following injection by this route. This thesis focuses on the difference between the MHA and LSH strain of hamsters in the footpad swelling response to Pichinde virus.

1.5 Hypotheses

The MHA strain hamster, unlike the other strains of hamsters tested, did not manifest a footpad swelling response after Pichinde virus injection. The purpose of the investigation, described in the thesis was to determine the mechanism(s) responsible for the lack of responsiveness.

Footpad swelling in response to a local injection of antigen is generally regarded as an indicator of cell-mediated immunity. In order for such an immune response to occur, immune recognition of antigen, generation of effector mechanisms and factors that regulate the expression of the effector mechanisms are required. A defect at any of the three levels could result in the lack of a response.

In the MHA strain hamster, it is unlikely that unresponsiveness was caused by a defect in immune recognition. This strain produced complement-fixing antibodies to Pichinde virus after footpad injection and was protected against a subsequent lethal intraperitoneal challenge with the virus. Therefore, the defect in the MHA hamster could be the result of a problem in effector mechanisms or in the regulation of the response. A regulation problem, such as enhanced suppressor activity,

could account for the lack of a footpad swelling response in this strain to Pichinde virus.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

Inbred Syrian hamster strains LSH and MHA and a random-bred LVG strain of hamster were purchased from Charles River/Lakeview, New Jersey. Both male and female hamsters aged 5-10 weeks were used to monitor footpad swelling.

F_1(LSHxMHA) and back-cross progeny were bred in the animal quarters at McMaster University. Animal rooms received 14 hours of light daily between 2:00 a.m. and 4:00 p.m., to facilitate breeding. The animals were housed in wire-topped polycarbonate cages and were fed Purina chow and water ad libitum.

To measure response to virus challenge, $2x10^3$ pfu of virus in 0.05 ml was injected into the right rear footpad. In some cases, a similar volume of control antigen, which was a sonicated preparation of BHK cells, was injected into the left hind pad.

2.2 Cell lines and cell cultures

Baby hamster kidney (BHK) cells and Vero cells were grown in Eagle's minimal essential medium (MEM F-15, Flow Laboratories, Mississauga, Ontario) containing 10% v/v heat-inactivated fetal bovine serum (FBS) (Grand Island Biological Co. (Gibco), Grand Island, New York), 1% v/v 0.75 g/l sodium bicarbonate, 1% v/v 1M hydroxyethylpiperazine-N'-2-ethanol sulfonic acid pH 7.24 (HEPES) buffer (Sigma, St. Louis, Missouri), 2.0 mM L-glutamine (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco).

LSH embryo fibroblast cells were derived from 9-day old embryos. The embryos were asceptically excised from LSH hamsters and washed three

times with phosphate buffered saline (PBS). The embryos were then minced into small fragments with scissors and the fragments were made into single cell suspensions by incubation in 20 ml of 1.0% trypsin-ethylenediaminotetraacetic acid (T-EDTA) (Gibco) in PBS at room temperature. Following trypsinization, the cells were washed once with Hanks' balanced salt solution (HBSS) (Gibco) supplemented with 10% v/v heat-inactivated FBS and resuspended in alpha-MEM (Gibco) supplemented with 10% v/v heat-inactivated FBS, 1% v/v 0.75 g/l sodium bicarbonate, 1% v/v 1M HEPES buffer, 2.0 mM L-glutamine, 3% v/v distilled water and antibiotics. This medium is subsequently referred to as culture medium. The suspension was then passed through sterile cheese cloth to eliminate blood clots and coagulated material. The final suspension of cells was poured into 150 cm^3 plastic tissue culture dishes (Corning Glass Works, Corning, New York) containing culture medium. The resulting cell line was designated LEF (LSH embryo fibroblasts).

2.3 Virus and virus assays

Pichinde virus

Pichinde virus strain AN3739 was originally isolated by Trapido and Sanmartin (1971) from the blood of a rodent, Oryzomys albigularis. A stock of virus was grown by infecting monolayers of BHK cells with 10^3 plaque-forming units (pfu) of virus per cell. The virus was allowed to adsorb for 1.0 hr at 37°C. The cells were then refed with culture medium. After 48 hr of incubation at 37°C, virus was harvested and aliquots of virus were stored at -90°C.

The virus was assayed by plaque formation on monolayers of Vero cells. Ten-fold dilutions of infected cell suspensions were made and

0.2 ml of the appropriate dilution was added to drained monolayers of Vero cells that had been grown in 60 mm tissue culture dishes (Corning Glass Works, Corning, New York). Following incubation for 1.0 hr at 37°C, the monolayers were overlaid with 1.75% w/v Bactoagar (Difco Laboratories, Detroit, Michigan) diluted in HBSS (2x) supplemented with 2% v/v 100x BME amino acids (Gibco, Grand Island, New York) 2% v/v 100x BME vitamins (Gibco), 4.5 g/l sodium bicarbonate, 0.02M HEPES buffer, 200 U/ml penicillin, 200 µg/ml streptomycin, 20% v/v heat-inactivated FBS and 50 U/ml mycostatin (E.R. Squibb and Sons, Inc., Princeton, New Jersey). Four days later, a second overlay containing 0.1 g/l neutral red (Gibco) was added and plaques were counted after an additional day of incubation at 37°C.

Vaccinia virus

A stock of vaccinia virus, strain WR, was prepared and titred as described by Niederkorn-Buchmeier (1977).

Vesicular stomatitis virus

Vesicular stomatitis virus, strain HR-LT of the Indiana serotype was a gift from Dr. L. Prevec, McMaster University. The method of preparation and titration of this virus has been described by SenGupta and Rawls (1979).

Herpes simplex virus

Herpes simplex virus type 1, strain KOS was used throughout the study. The virus isolation, propagation and purification has been described by Rawls et al. (1968). A stock of virus was grown by infecting monolayers of Vero cells with 1-3 pfu of virus per cell. After adsorption for 1.0 hr at 37°C, the cells were refed with culture medium. The

infected cells were then incubated at 37°C until a complete cytopathic effect was visible. The supernate, containing virus, was centrifuged at 29,000 rpm for 1.0 hr in a Damon/IEC B60 ultracentrifuge. The pellet of virus was then added to a cell lysate, which was obtained by freeze-thawing infected cells and centrifuging at a low speed to remove debris. The virus was assayed by plaque formation on monolayers of Vero cells, as described by Rawls (1979). Aliquots of virus were stored -90°C.

UV-inactivation of virus

UV-inactivation of virus was carried out by exposing virus 10 cm away from a germicidal light source (General Electric G30T8) for 5 min. In the case of herpes simplex virus, the virus was gently sonicated in a Bransonic 220 sonicator (Branson Inst. Co., Shelton, Conn.) for 30 sec before being exposed to the germicidal lamp. This time exposure has been shown to eliminate 99% infectivity of Pichinde virus (Carter et al., 1973).

2.4 Iodination of hamster serum albumin

Sodium ^{125}iodide (NEN Canada Ltd., Lachine, Quebec) (1.0 mCi) in a volume of 0.05 ml 0.05 M phosphate buffer was added to 25 µg hamster serum albumin in 0.05 M phosphate buffer, pH 7.6, followed by the rapid addition with immediate mixing of 50 µl chloramine T (5 mg/ml) in 0.05 M phosphate buffer, pH 7.6. After 1.0 minute, 10 µg sodium thiosulfate (10 mg/ml) and 100 µl KI (10 mg/ml) in 0.05 M phosphate buffer, pH 7.6 was added. The solution was then placed on a Sephadex G-25 column (1.75 x 30 cm) that had been previously equilibrated with 0.05 M phosphate buffer, pH 7.6 and treated with 2% v/v FBS. After the solution was placed on the column an additional 200 g of KI was then added to rinse free iodine from the column. Forty drop fractions were collected in test tubes containing 0.10 ml of 0.05 M phosphate buffer, pH 7.6 and 2% v/v FBS. A 0.01 ml sample was removed

from each test tube and counted in a gamma counter (Gamma 300, Beckman Instruments Inc., Fullerton, California). Fractions 10 through 15 off the column, which usually contained the major peak of radioactivity, were pooled and used for further studies.

2.5 Kinetics of disappearance of ^{125}iodine-labelled hamster serum albumin from blood

Hamsters of the LVG strain were inoculated intraperitoneally (IP) with $1x10^7$ counts per minute (cpm) ^{125}I-labelled hamster serum albumin (^{125}I-HaSA) in a volume of 0.04 ml. At various time intervals after injection, 0.10 ml of blood was withdrawn by cardiac puncture using a 23-gauge needle. The blood samples were counted directly in the gamma counter.

The apparent half-life ($t_{\frac{1}{2}}$) of the labelled albumin from the animals' bloodstream was calculated from simple exponential kinetics, using the equation

$$cpm_t = cpm_o e^{-kt} .$$

2.6 Footpad swelling

Two methods were used to quantify footpad swelling. A spring-loaded micrometer was used to measure the hind footpads of animals before and after they received an inoculation of virus into the right hind pad and BHK supernate into the left. Results were expressed as the mean difference in measurements between the test (right) and control (left) hind footpad.

The second method of assessment utilized a radioisotopic assay. In this assay $1x10^6$ cpm of ^{125}I-HaSA was injected intraperitoneally (IP) into hamsters and 24 hr later the hind feet were amputated and counted

in the gamma counter. Results were expressed as:

$$\frac{\text{cpm in test foot}}{\text{cpm in control foot}}$$

2.7 Histological studies

Hamsters were euthanized using ether and the hind feet were amputated and fixed in 10% buffered formalin. The fixed samples were decalcified and embedded in paraffin wax, sectioned and stained with hematoxylin-eosin.

Evaluation of the cellular infiltrate was carried out by counting the number of mononuclear cells that were present in a 1 cm^2 grid that was subdivided into 25 segments (Wild Leitz, Germany). Three different high-powered (400x) fields were chosen per sample for evaluation.

2.8 Cyclophosphamide treatment

Cyclophosphamide (PROCYTOX^R, Horner Ltd., Montreal, Quebec) was prepared at a concentration of 10 mg/ml in sterile double distilled water immediately prior to use. At intervals before and after primary footpad inoculation of virus, hamsters were given an IP injection of either 25 mg/kg, 50 mg/kg or 100 mg/kg body weight of cyclophosphamide as indicated in the text. Non-infected MHA and LSH hamsters that received cyclophosphamide in one control experiment revealed no footpad response due to cyclophosphamide treatment alone.

2.9 Adoptive transfers

2.9.1 Lymphoid cell transfers

The effect of adoptiye transfer of hamster lymphoid cells on primary footpad swelling response was studied by harvesting aseptically

lymph nodes and spleens from MHA and LSH donors. Some donors previously received an inoculation of $2x10^3$ pfu of Pichinde virus into both hind footpads at various days before sacrifice. Spleens collected from groups of 3 animals and lymph nodes were pooled in tissue culture plates containing 5 ml of culture medium. The tissues were then minced with scissors into small fragments and gently pressed through 60-gauge sterile wire mesh screens using the barrel end of a 5 cc syringe. The screens were washed once with culture medium and the cells were collected and suspended in culture medium. To remove large aggregates of cellular debris, the cell suspension was underlaid with 2-3 ml of FBS and allowed to stand for 10 min at room temperature. The supernatant was recovered and centrifuged at 200 g for 10 min. The cells were then resuspended in 0.172 M NH_4Cl for 15 min at 4^oC to lyse red blood cells. The cell suspensions were again layered over FBS and immediately centrifuged at 1000 rpm for 10 min. Viable cell counts were determined on the pellet by trypan blue exclusion in a hemocytometer and the cell concentration adjusted according to need.

Prescribed numbers of viable cells were inoculated into the saphena of the left hind limb of ether anesthetized LSH recipients. Immediately after the inoculation of cells, $5x10^6$ pfu of Pichinde virus was injected into the right hind footpad of each recipient. Footpad swelling was measured 7 or 8 days later.

2.9.2 Serum transfers

To study the effect of "immune serum" on the footpad swelling response, blood was collected by intracardiac puncture of animals which had received a footpad inoculation of $2x10^3$ pfu of Pichinde virus 8 days

or 8 weeks previously. Sera were separated from the blood samples and inoculated in a volume of 0.4 ml into the saphena of the left hind limb of ether anesthetized LSH recipients. Immediately after the injection of serum, recipients were inoculated with $5x10^6$ pfu of Pichinde virus into the right hind footpad. Footpad swelling in these recipients was measured 8 days later.

2.10 Generation of cytotoxic cells in vitro

Peritoneal exudate (PE) cells were obtained from hamsters stimulated 3-5 days earlier by an IP injection of 5.0 ml of sterile paraffin oil. PE cells were harvested aseptically by rinsing the peritoneal cavity three times with a 30 ml volume of HBSS (Gibco, Grand Island, New York) supplemented with antibiotics and 0.5% v/v 0.75 g/l sodium bicarbonate. The PE cells were centrifuged at 200 g for 10 min and suspended in culture medium which was supplemented with 10^{-4} M 2-mercaptoethanol (2-ME) (BDH Chemicals, Toronto, Ontario) and 10% v/v conditioned medium (see below). If red blood cells were present, then the PE cell suspension was treated with 0.172 M NH_4Cl for 15 min at 4^oC prior to suspension in culture medium plus 2-ME. Viable cell counts were determined by trypan blue exclusion in a hemocytometer and the cells were adjusted to a concentration of $2.5x10^6$ cells/ml. The PE cells were subsequently exposed in suspension to 1.0 pfu/cell of either UV-inactivated Pichinde virus or UV-inactivated herpes simplex virus. The treated PE cells were then mixed 5-6 hr later in a 1:10 ratio with various lymphoid cell suspensions obtained from either uninfected animals or hamsters which had received a FP inoculation of virus. Culture flasks containing the cell mixtures were

incubated in an upright position at 37°C in a CO_2 incubator (5%). Five days later, these cells were recounted and adjusted to a concentration of $1x10^7$ cells/ml in culture medium and tested against uninfected, Pichinde virus-infected and herpes simplex virus-infected LEF cell targets in a chromium release assay (see below).

2.10.1 Preparation of conditioned medium

Spleens were removed aseptically from normal LSH hamsters and fragmented, as described, into single cell suspensions. The cell suspensions were allowed to stand over FBS to remove large aggregates of cellular debris and then the supernatant cells were centrifuged at 1000 rpm for 10 min. Viable cells were counted by trypan blue exclusion in a hemacytometer and adjusted to a concentration of $1x10^6$ cells/ml. The cells were incubated for 5 days in 50 ml of culture medium supplemented with $5x10^{-5}$ M 2ME and 3.3 µg/ml concanavalin A (Sigma, St. Louis, Missouri) at 37°C in a CO_2 incubator. The cells were then harvested and centrifuged at 200 g for 10 min. The cell pellet was discarded and the supernatant collected and filtered. Aliquots of supernatant were then stored at -20°C.

2.11 ^{51}Chromium release assay

Cell suspensions to be tested for cytotoxic activity were diluted 2-fold with culture medium in 96-well flat-bottom tissue culture plates (Linbro, Flow Laboratories, Mississauga, Ontario). A cell line derived from 9-day old LSH embryos was used as target cells (LEF). Trypsinized target cells were labelled with 1 mCi sodium ^{51}chromate (NEN Canada Ltd., Lachine, Quebec) for 90 min at 37°C in HBSS without Ca^{++} or Mg^{++} (Gibco, Grand Island, New York), supplemented with 10%

v/v heat-inactivated FBS, 0.75 g/l sodium bicarbonate and antibiotics. The labelled cells were washed three times, once in HBSS and twice in culture medium and resuspended in culture medium at a final concentration of $1x10^5$ viable cells/ml. In some cases, the target cells were infected with virus. The target cells, in a total volume of 0.1 ml were then added to individual wells of the 96-well flat-bottom tissue culture plates containing doubling dilutions of effector cells in a total volume of 0.1 ml. The final effector to target ratios ranged from 100:1 to 6.25:1. After incubation at 37^oC in a CO_2 incubator for 16 hr, the plates were centrifuged at 200 g for 10 min and 0.1 ml of supernatant was removed and counted in a gamma counter. Values for maximal release were obtained by mixing 0.1 ml of the labelled target cells with 0.1 ml water containing 1% Nonidet P-40 (NP-40) (BRL Laboratories, Rockville, Maryland). Spontaneous release was determined by incubating the labelled target cells in 0.1 ml of culture medium for the duration of the assay.

Percent target lysis was estimated using the standard formula,

$$P(\text{percent specific } {}^{51}Cr\text{-release}) = 100 \times \frac{(\text{cpm with effector cells} - \text{spontaneous cpm})}{(\text{total releasable cpm with NP-40} - \text{spontaneous cpm})}.$$

It has been shown that where P is >20% in cytotoxic cell assays, the percent specific release value provides a misleading estimate of the frequency of cytotoxic effector cells in the test cell suspension (Miller and Dunkley, 1974). To obtain a quantitative estimate, the frequency of effector cells was determined from the formula,

$$P = 100\ (1-e^{-N\alpha t}).$$

as described by Miller and Dunkley (1974), where N is the number of cells tested, t is the interaction time and α is a constant proportional to the frequency of effector cells. The average value of αt was determined from several values (usually 4-5) of N and the data expressed as $N\alpha t \times 10^3/10^6$ test cells. An $N\alpha t \times 10^3/10^6$ value of 1000 corresponds to 63% lysis by 10^6 cells and a value of 100 corresponds to 10%. 223 $N\alpha t \times 10^3$ units represents that amount of activity required to lyse 20% of the cells (1 Lytic $Unit_{20}$).

2.12 Complement fixation test

Antibodies against Pichinde virus were quantitated using the method of Kagan and Norman (1970) but adapted to microtiter plates. Doubling dilutions of sera to be tested were made in round-bottom 96-well Microtiter plates (Cooke Engineering Co., Alexandria, Virginia) in a total volume of 0.025 ml of veronal buffered diluent (VBD), using constant delivery (0.025 ml) Microtiter dilution loops (Cooke Engineering Co., Alexandria, Virginia). Five units of antigen were then added in a total volume of 0.025 ml to each of triplicate wells. One unit of antigen was defined as that amount of antigen which, in the presence of excess anti-Pichinde virus antibody, fixed 50% of the available complement. The positive antigen was prepared by sonicating and freeze-thawing Pichinde virus-infected BHK cells. This preparation had a high titre when reacted with normal hamster sera. The negative control antigen consisted of a sonicated preparation of BHK cells, diluted 1:10 in VBD. To each well containing antibody and antigen, 5 complement CH50 units of guinea pig complement (Gibco, Grand Island, New York) were added in a total volume of 0.05 ml. The guinea pig complement has previously been titred against sensitized sheep red blood cells and one complement CH50 unit was defined as that dilution of complement which

lysed 50% of a 1.4% v/v suspension of sensitized sheep red blood cells. Each dilution of sera and both positive and negative control antigen preparations were screened for anticomplementary activity by adding 5, 2.5 and 1.25 complement CH50 units and no complement, in the absence of antigen and antibody, respectively. In all tests, known positive and known negative antisera were included as controls. Antibody and antigen were allowed to react with complement overnight at $4^{o}C$ and then warmed for 15 min at room temperature. Meanwhile, sheep red blood cells were washed three times with VBD and a 2.8% v/v cell suspension was made in VBD. Sheep red blood cells were sensitized by incubating an equal volume of the washed 2.8% v/v cell suspension with an equal volume of 1:2000 dilution of haemolysin (Gibco) for 10 min at room temperature. The dilution of haemolysin had previously been determined as optimal. Then, 0.025 ml of the 1.4% v/v sensitized sheep red blood cell suspension was added to each well. The plates were incubated for 1.0 hr at $37^{o}C$, at which time the complement controls exhibited the appropriate amount of lysis. The wells were scored for the amount of lysis on a 0-4+ scale, where 0 represented 100% lysis and 4+ indicated a clear supernate with an intact red cell button. A reaction of 3-4+ was considered positive.

2.13 Bone marrow transfers

Bone marrow cells were obtained by asceptically removing right and left femora from MHA and LSH hamsters and flushing the femora with culture medium, using a 20-gauge needle. The cells were centrifuged at 1000 rpm for 10 min and resuspended in culture medium. Viable cell counts were determined by trypan blue exclusion in a hemocytometer and the cells were adjusted to a concentration of 7.5×10^{7} cells/ml.

LSH and F_1(LSHxMHA) hamsters that were used as recipients, were irradiated in Lucite containers with a dose of 950 rads of X-rays (250 kv, 30mA, HVL 1.33mm Cu^{2+}, dose rate 95 R mm^{-1}) (Maxitron-250 X-ray, General Electric). Immediately after irradiation, the hamsters were injected IV with $3x10^7$ bone marrow cells in a total volume of 0.4 ml from either MHA or LSH donors. The irradiated recipients were then kept in cages covered with filter paper and treated with tetracycline for 14 days. Eight weeks later, the survivors were inoculated with $2x10^3$ pfu of Pichinde virus in the right hind footpad and footpad swelling was assessed 7 days later.

CHAPTER 3

RESULTS

3.1 Assays of delayed type hypersensitivity in hamsters

Classically, footpad swelling which is recognized as a measure of delayed type hypersensitivity has been quantified using a micrometer. However, in order to measure more precisely changes in footpad thickness, two methods which included micrometer determination and the radioisotopic method of Paranjpe and Boone (1972) were used. According to the method of Paranjpe and Boone, radiolabelled serum protein was injected IP into the animal and the amount of radioactivity (cpm) that was present in the swollen foot was compared to that which was present in the control foot and expressed as a foot count ratio.

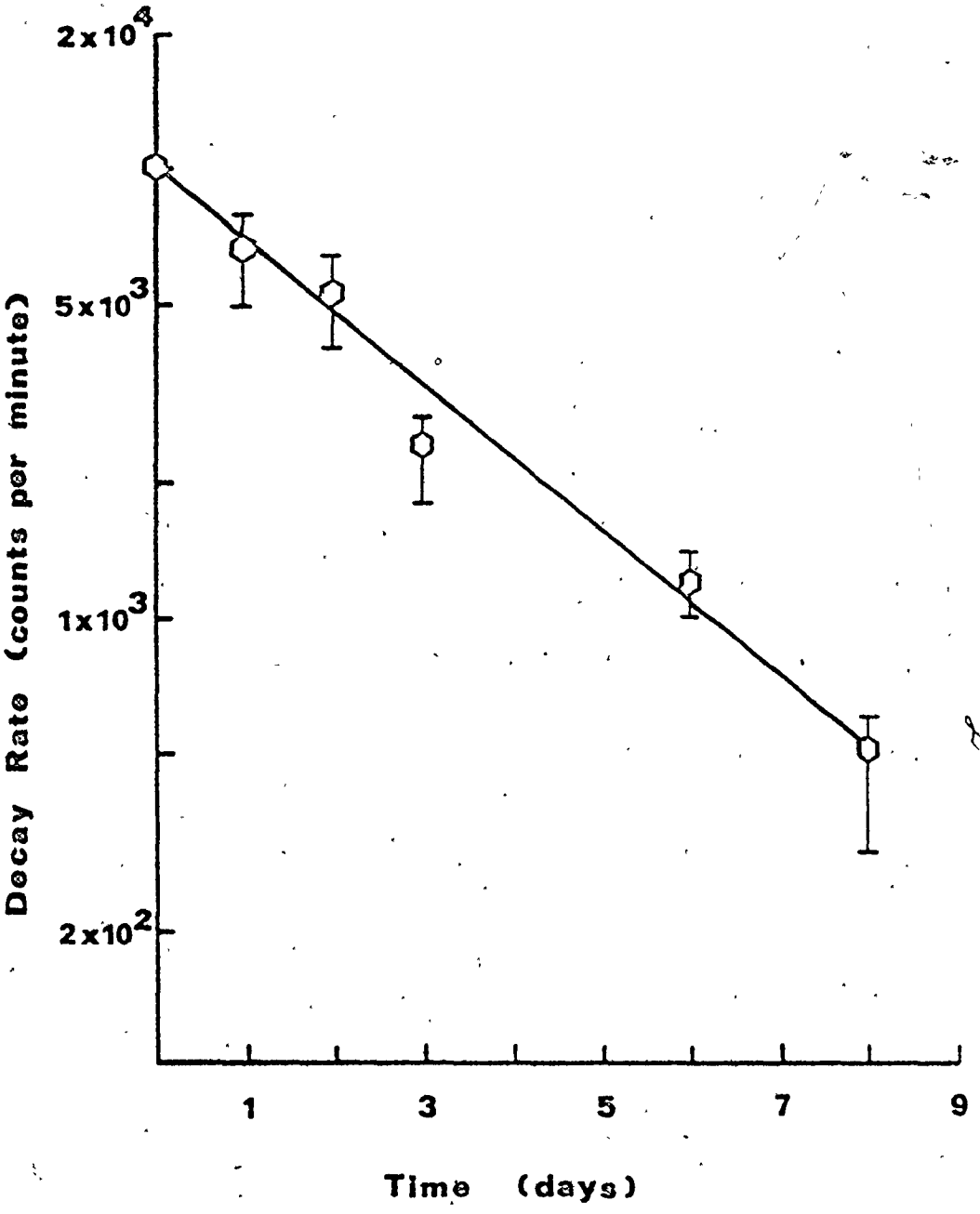
3.1.1 Kinetics of disappearance of ^{125}iodine-labelled hamster albumin from blood

To determine how quickly ^{125}I-HaSA was eliminated from the hamsters' bloodstream, blood samples were taken at various days, by cardiac puncture from LVG hamsters which had received an IP inoculation of $1x10^7$ cpm ^{125}I-HaSA and counted in the gamma counter. The maximum amount of ^{125}I-HaSA that appeared in the bloodstream of the hamsters represented approximately 40% of the initial radioactivity that was injected into the animals. Furthermore, ^{125}I-HaSA decayed quickly in the hamsters with a 30% reduction in activity over the first 24 hr (Figure 1). The half-life of ^{125}I-HaSA was calculated to be 44.4 hr.

Since ^{125}I-HaSA seemed to decay rapidly in the hamsters, injection of the isotope 24 hr before feet were excised was chosen for subsequent experiments.

Figure 1

"Kinetics of disappearance of ^{125}I-HaSA". LVG hamsters were inoculated with approximately $1x10^7$ cpm ^{125}I-HaSA. At various time intervals after injection, 0.1 ml of blood was taken by cardiac puncture and counted in a gamma counter. The half-life of ^{125}I-HaSA was calculated to be 44.4 h . Each point represents the mean of 3 hamsters $\pm$ standard error of the mean (SEM).


2x10^4
5x10^3
1x10^3
2x10^2
Decay Rate (counts per minute)
1
3
5
7
9
Time (days)

3.1.2 Relationship between foot count ratio and micrometer measurements

To assess whether the radioisotopic method and the micrometer measurements were recording similar changes in footpad response after viral injection, 68 foot count ratios and their corresponding micrometer measurements were subjected to a correlation analysis. A correlation coefficient (r) value of 0.76 was obtained which was found to be significant ($p<.005$). Thus, a positive association appears to exist between foot count ratios and micrometer measurements. A scatterplot and least squares line of the data on which the statistical analysis was performed are illustrated in Figure 2. Therefore, both methods appeared to correlate similarly to footpad swelling. However, when measurements of 48 LSH hamsters tested in 13 experiments were examined, a larger standard deviation was found in the micrometer measurements than in the foot count ratios.

This observation suggested that either the micrometer measurements were not quite as precise as the foot count ratios, or that the two methods were measuring different aspects of the footpad swelling response. Nevertheless, both methods were used interchangeably.

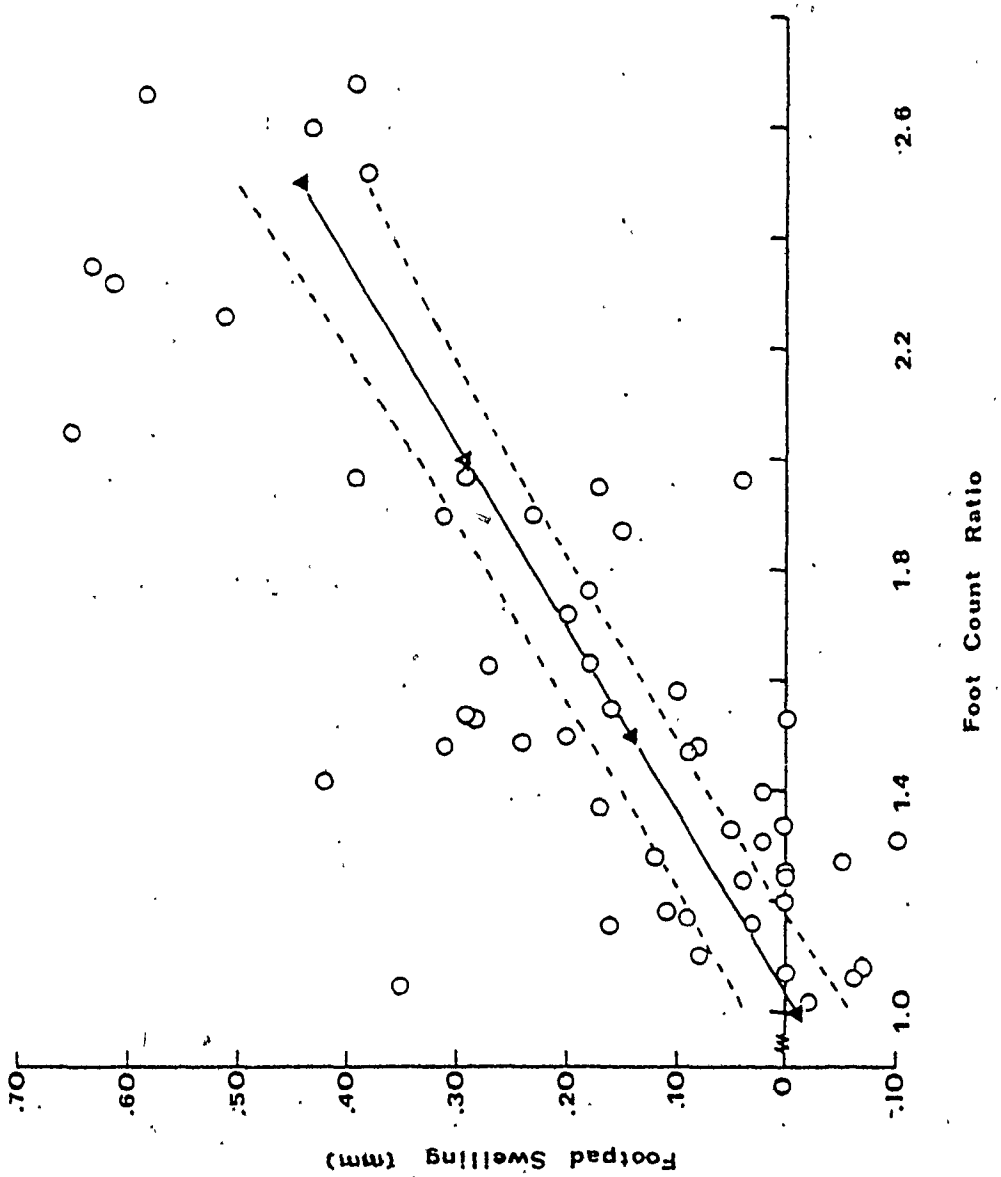
3.2 Differences between hamster strains in footpad swelling response to viruses

3.2.1 Response to Pichinde virus

LVG, LSH and MHA hamsters were inoculated subcutaneously in the right hind footpad with $2x10^3$ pfu of Pichinde virus. An appreciable swelling response was elicited in both LVG and LSH hamsters, as measured by both micrometer and radioisotopic methods and a peak response occurred 7-8 days after injection. In contrast, footpad swelling could not be

Figure 2

"Scatterplot of foot count ratios and corresponding micrometer measurements". Foot count ratios and their corresponding micrometer measurements were subjected to a correlation analysis. A correlation coefficient value of 0.76 was obtained, suggesting that a positive association exists between foot count ratios and micrometer measurements ($p<.005$). Least squares line (▲——▲); 95% confidence limits of the least squares line (---).


Footpad Swelling (mm)
.70
.60
.50
.40
.30
.20
.10
0
-.10
1.0
1.4
1.8
2.2
2.6
Foot Count Ratio

detected by either method in the MHA hamster, at this time (Figure 3A and 3B).

This unresponsive nature of the MHA strain was reproducible and consistent in 10 experiments in which footpad swelling was monitored in LSH and MHA hamsters.

3.2.2 Pichinde virus replication in footpads

To ensure that the lack of footpad swelling response observed in the MHA hamster was not attributable to a failure of Pichinde virus to infect the MHA animals, homogenates of footpads of LSH and MHA hamsters that had been footpad (FP)-injected 3, 5 and 8 days previously, with $2x10^3$ pfu of Pichinde virus were assayed for infectious virus on monolayers of Vero cells. As demonstrated in Figure 4, high titres of Pichinde virus were detectable in the footpads of both hamster strains and peak virus titres occurred at 5 days of infection.

This result confirmed that FP-inoculated MHA hamsters could be infected with Pichinde virus.

3.2.3 Complement-fixing antibodies to Pichinde virus

An additional study was done to determine if the absence of footpad swelling in the MHA hamster was due to a lack of immune recognition of Pichinde virus antigens by the MHA strain hamsters. LSH and MHA hamsters that received a FP-inoculation of $2x10^3$ pfu of Pichinde virus were bled by cardiac puncture 8 days later. The sera were collected and assayed for antibodies to Pichinde virus by the complement fixation test. Table 1 shows that both MHA and LSH hamsters produced complement-fixing antibodies to Pichinde virus. Moreover, MHA hamsters seemed to generate higher antibody titres than LSH animals.

3.2.4 Response to vesicular stomatitis virus and vaccinia virus

To determine whether MHA hamsters had a general defect in footpad swelling for viruses, MHA and LSH hamsters were FP-injected with either $2x10^3$ pfu of vesicular stomatitis virus or $2x10^3$ pfu of vaccinia virus. As illustrated in Figure 5, MHA and LSH animals elicited pronounced footpad swelling 5 days after an injection of vesicular stomatitis virus. Similarly, both hamster strains displayed footpad swelling to vaccinia virus 6 days after injection but the degree of swelling was considerably less than the response observed to vesicular stomatitis virus (Figure 6).

Of the viruses tested, these results suggested that the failure of the MHA hamster strain to respond to a FP-inoculation of Pichinde virus was restricted to Pichinde virus.

3.2.5 Response to mixtures of vaccinia virus and Pichinde virus

Since MHA hamsters could mount a footpad swelling response to a FP-inoculation of vaccinia virus but not to Pichinde virus, it was of interest to test whether Pichinde virus could nonspecifically suppress the response to vaccinia virus if both viruses were injected simultaneously into the same footpad of MHA hamsters. Groups of 3-4 MHA hamsters were FP-inoculated with either $2x10^4$ pfu of vaccinia virus, $2x10^4$ of vaccinia virus and $2x10^3$ pfu of Pichinde virus, $2x10^3$ of Pichinde virus, $2x10^4$ pfu of vaccinia virus followed one day later with $2x10^3$ pfu of Pichinde virus or $2x10^3$ pfu of Pichinde virus followed one day later with $2x10^4$ pfu of vaccinia virus. Footpad swelling was measured 5 days later. A footpad swelling response occurred in all groups of MHA hamsters except those hamsters that received a FP-inoculation of Pichinde

virus alone (Figure 7). These observations implied that Pichinde virus did not nonspecifically suppress footpad swelling in the MHA hamsters. The result of the experiment did not, however, exclude the possibility that Pichinde virus might induce a specific suppressor mechanism that blocked the footpad swelling response to its own antigens.

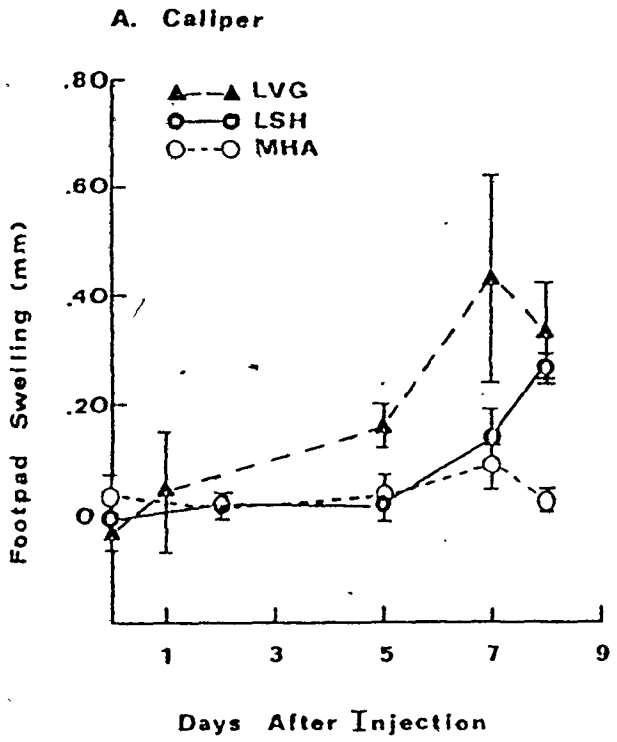
3.2.6 Histological studies

In some experiments histological sections were prepared from footpads of MHA and LSH hamsters that had been FP-inoculated with either Pichinde virus, vesicular stomatitis virus, vaccinia virus or in some cases, vaccinia virus and Pichinde virus together. It was anticipated that the histological sections would reveal the nature of the cellular response and an approximate number of inflammatory cells present in the footpad at the time when peak swelling occurred, as determined by the micrometer measurements.

LSH hamsters that received a FP-injection of either Pichinde virus, vesicular stomatitis virus or vaccinia virus showed a dense mononuclear cell infiltrate in the virus-injected foot as compared to the corresponding uninjected control foot (Figure 8, Figure 9 and Figure 10, respectively). Enumeration per high-powered field (400x) of the cells present in the virus-injected foot revealed an approximate 10 to 40-fold increase over that cell number present in the uninjected foot (Table 2). Similarly, a dense mononuclear cell infiltrate was present in the virus-injected feet of MHA hamsters that had been FP-inoculated with either vesicular stomatitis virus or vaccinia virus (Figure 11 and Figure 12, respectively) and the cell number in the virus-injected feet was increased compared to the uninjected control feet (Table 3). In contrast, a cell infiltrate was not detected in Pichinde virus-

FIGURES 3A AND 3B

"Footpad swelling response to Pichinde virus". LVG, LSH and MHA hamsters were inoculated with $2x10^3$ pfu of Pichinde virus in the right rear footpad and with control antigen from uninfected cells in the left rear footpad. At various times after injection, the hind feet were measured using both the micrometer method (A-caliper) and radioisotopic method (B-^{125}I-HaSA). Each point represents the mean of 3 animals $\pm$ standard error of the mean (SEM). Footpad swelling was monitored up to 12 days after injection, at which time swelling had subsided in both LSH and LVG hamsters. During this time interval, MHA hamsters did not manifest a footpad swelling response.


A. Caliper
LVG
LSH
MHA
Footpad Swelling (mm)
.80
.60
.40
.20
0
1
3
5
7
9
Days After Injection

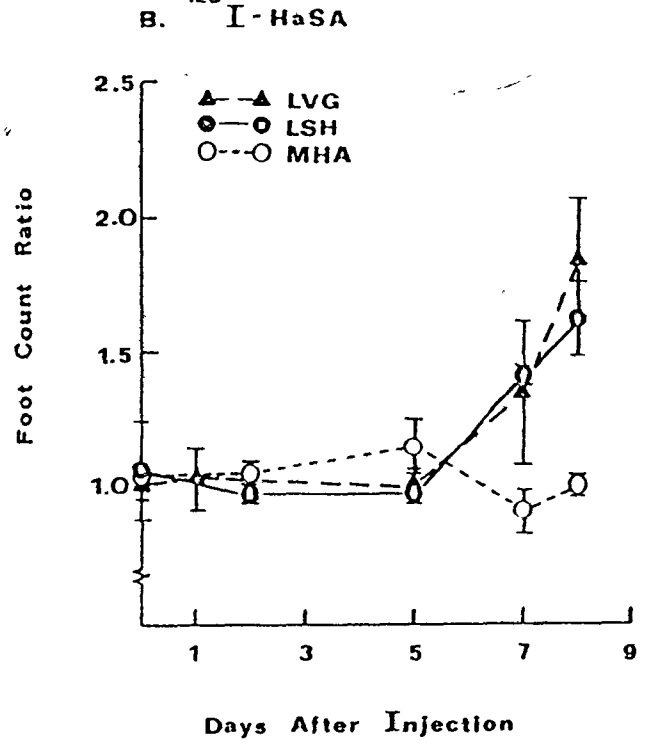

B. 125I-HaSA
LVG
LSH
MHA
Foot Count Ratio
2.5
2.0
1.5
1.0
1
3
5
7
9
Days After Injection

FIGURE 4

"Kinetics of Pichinde virus replication in footpads". Groups of 3 MHA and LSH hamsters were inoculated with $2x10^3$ pfu of Pichinde virus into the right rear footpad. At various times after injection, the feet were excised, homogenized and assayed on monolayers of Vero cells for infectious Pichinde virus ($\log_{10}$ PFU/footpad). Each point represents the mean of 3 animals $\pm$ SEM.

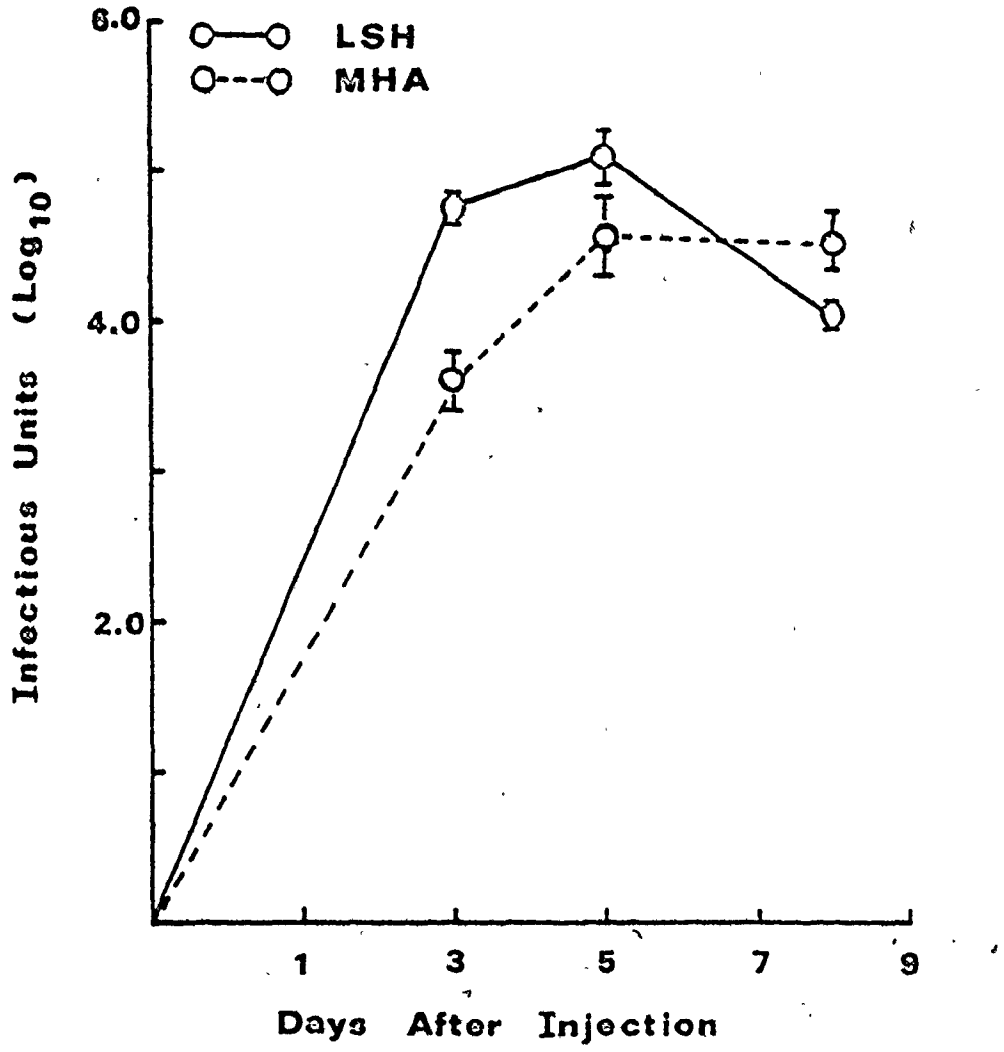

6.0
4.0
2.0
LSH
MHA
Infectious Units (Log10)
1
3
5
7
9
Days After Injection

FIGURE 5

"Footpad swelling response to vesicular stomatitis virus". LSH and MHA hamsters were inoculated with $2x10^3$ pfu of vesicular stomatitis virus in the right rear footpad. At various times after injection, the hind feet were measured with a micrometer and the difference in thickness was plotted. Each point represents the mean of 5 animals $\pm$ SEM.

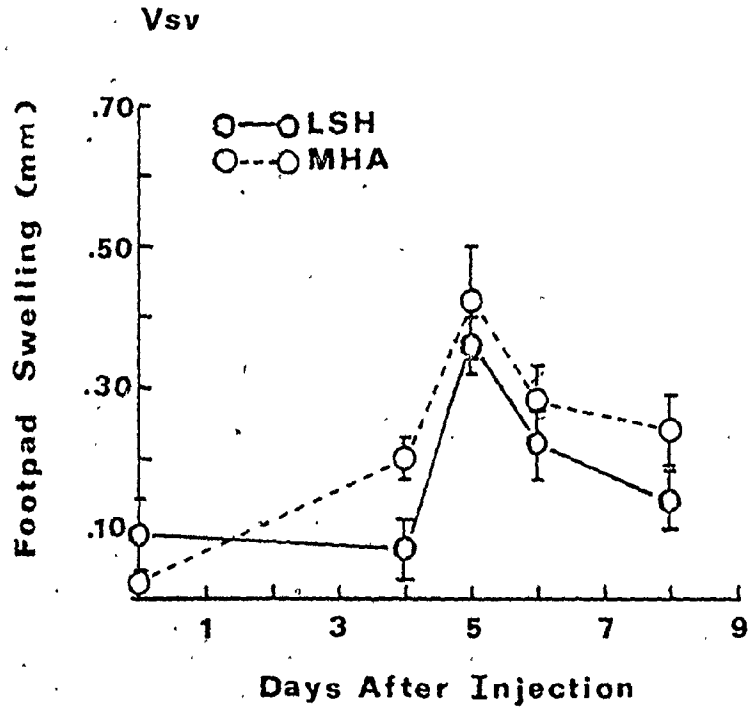

Vsv
Footpad Swelling (mm)
.70
.50
.30
.10
LSH
MHA
1
3
5
7
9
Days After Injection

FIGURE 6

"Footpad swelling response to vaccinia virus". LSH and MHA hamsters were inoculated with $2x10^3$ pfu of vaccinia virus in the right rear footpad. At various times after injection, the hind feet were measured with a micrometer and the difference in thickness was plotted. Each point represents the mean of 5 animals $\pm$ SEM.

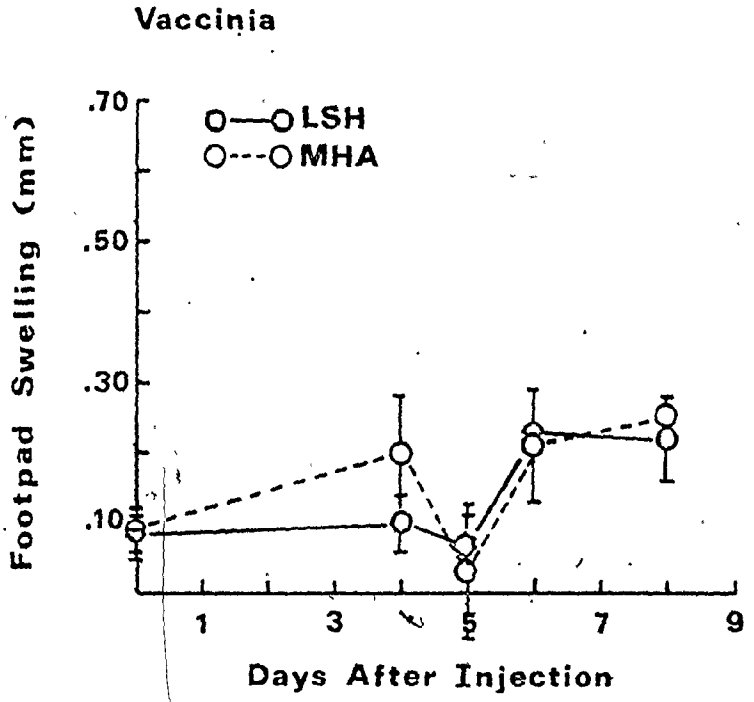

Vaccinia
LSH
MHA
Footpad Swelling (mm)
.70
.50
.30
.10
1
3
5
7
9
Days After Injection

FIGURE 7

"Footpad swelling response to vaccinia virus and Pichinde virus". Groups of 4 MHA hamsters were FP-inoculated with either $2x10^4$ pfu of vaccinia virus, $2x10^3$ pfu of Pichinde virus, $2x10^4$ pfu of vaccinia virus and $2x10^3$ pfu of Pichinde virus, $2x10^4$ pfu of vaccinia virus followed one day later with $2x10^3$ pfu of Pichinde virus or $2x10^3$ pfu of Pichinde virus followed one day later with $2x10^4$ vaccinia virus in the right rear footpad. 6 days from the start, the hind feet were measured and the difference in thickness was plotted. Each bar represents the mean of 4 hamsters $\pm$ SEM.

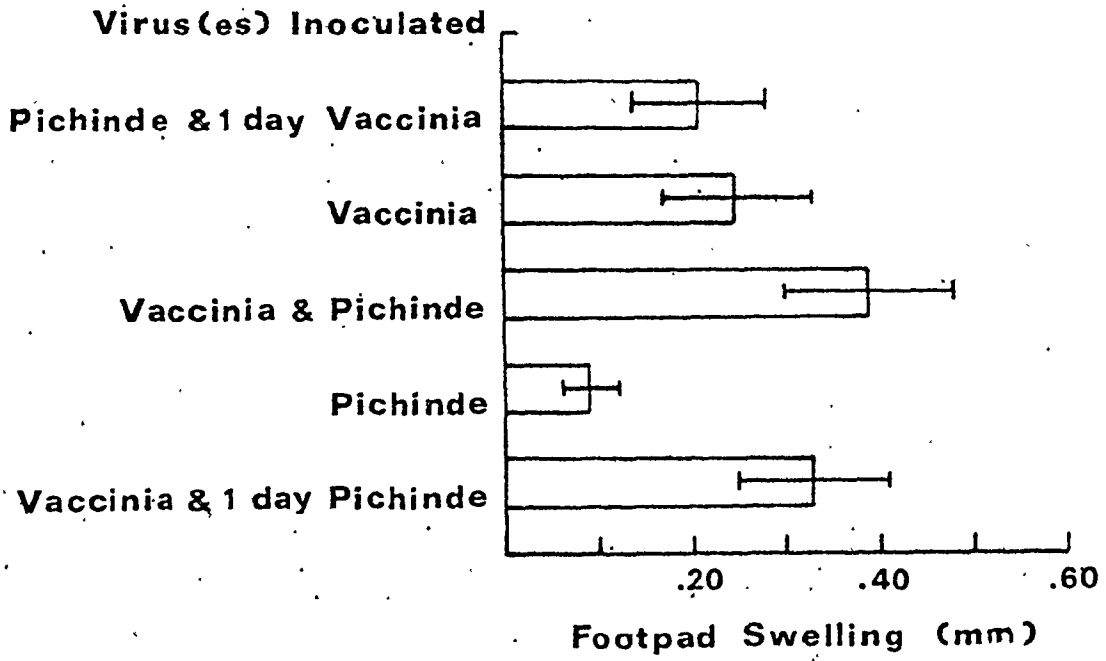

Virus(es) Inoculated
Pichinde &1 day Vaccinia
Vaccinia
Vaccinia & Pichinde
Pichinde
Vaccinia & 1 day Pichinde
.20
.40
.60
Footpad Swelling (mm)

Table 1

Complement-fixing Antibody Titres to Pichinde Virus

Hamster strain[a]	CF Antibody Titre[b]
MHA	10.7±2.7
LSH	3±1

[a] MHA and LSH hamsters were injected with $2x10^3$ pfu of Pichinde virus in the right rear footpad. 8 days later, blood was obtained from each hamster by cardiac puncture. Sera were collected and assayed for antibodies to Pichinde virus by the complement fixation test.

[b] Reciprocal titres are expressed as the mean of 3 hamsters ± SEM.

injected feet of MHA hamsters (Figure 13) and the average cell number present in the Pichinde virus-injected feet of MHA hamsters was not significantly different from the cell number present in the corresponding uninjected control feet (Table 3).

In cases where MHA hamsters had been FP-inoculated with either vaccinia virus and Pichinde virus simultaneously or in sequence, the histological sections of the virus-injected feet revealed an extensive mononuclear cell infiltrate (Figure 14, Figure 15 and Figure 16). Moreover estimation of the mononuclear cell number present in these virus-injected feet revealed an approximate 5-fold increase over the cell number present in feet that were injected with Pichinde virus alone (Table 3).

The results of the histological studies were in agreement with the footpad response as shown by the micrometer measurements. The MHA strain hamster was capable of manifesting an inflammatory reaction in response to viruses other than Pichinde virus, such as vesicular stomatitis virus and vaccinia virus and to combinations of vaccinia virus and Pichinde virus.

3.3 Genetic control of footpad swelling response to Pichinde virus

Genetic studies were performed on progeny of MHA and LSH hamsters in an effort to determine whether the lack of footpad swelling to a footpad inoculation of Pichinde virus in MHA hamsters was genetically acquired. Individual F_1 (LSH X MHA) and backcross progeny were inoculated with Pichinde virus in the right hind footpads and the radioisotopic method was used to assess swelling 8 days later. Progeny were classified as having the LSH phenotype if their foot count ratio was within 1 standard deviation of the LSH mean. Progeny with lesser

Table 2

Number of Mononuclear Cells Present in Virus-Inoculated Footpads of LSH and MHA Hamsters

Strain	Virus	Footpad	Mononuclear Cell Number (Mean ± SEM)
LSH	Pichinde	Right	385 ± 44
	-	Left	6 ± 2
LSH	Vaccinia	Right	311 ± 76
	-	Left	69 ± 23
LSH	VSV	Right	423 ± 5
	-	Left	10 ± 1
MHA	Pichinde	Right	81 ± 7
	-	Left	57 ± 14
MHA	Vaccinia	Right	317 ± 47
	-	Left	23 ± 2
MHA	VSV	Right	533 ± 47
	-	Left	6 ± 1

VSV: vesicular stomatitis virus

Histological sections were prepared from feet of LSH and MHA hamsters that had been FP-inoculated with either $2x10^3$ pfu of Pichinde virus, $2x10^3$ pfu of vaccinia virus, or $2x10^3$ pfu of vesicular stomatitis virus. When peak swelling occurred, as determined by micrometer measurements, feet were excised and fixed for sectioning. The mononuclear cell number recorded for each footpad represents the mean cell count of 3 randomly selected fields of view per footpad ± SEM.

Table 3

Number of Mononuclear Cells Present in Virus-Inoculated Footpads of MHA Hamsters

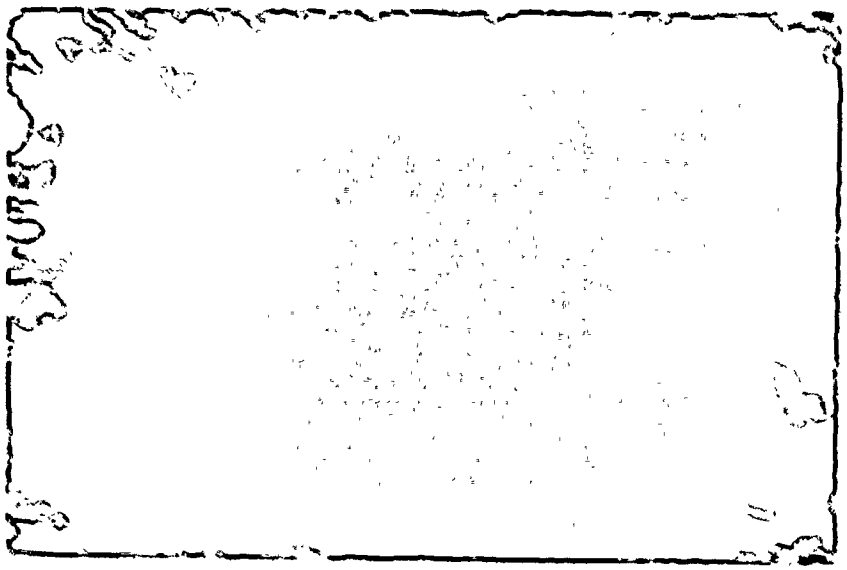
Virus	Animal No.	Mononuclear Cell Number (Mean ± SEM) Exp. 1	Animal No.	Exp. 2
Vaccinia	1.	225±53	1.	308±84
	2.	167±22	2.	292±8
Vaccinia + Pichinde	1.	225±75	1.	475±218
	2.	283±44	2.	485±94
Pichinde	1.	67±17	1.	100±14
	2.	32±11	2.	150±52
Vaccinia + 1 day Pichinde	1.	417±79	1.	692±84
	2.	433±87	2.	692±60
Pichinde + 1 day Vaccinia	1.	504±121		ND

Histological sections were prepared from the right feet of MHA hamsters that had been FP-inoculated 5 days earlier, with either $2x10^4$ pfu of vaccinia virus, $2x10^4$ pfu of vaccinia virus together with $2x10^3$ pfu of Pichinde virus, $2x10^4$ pfu of vaccinia virus followed one day later with $2x10^3$ pfu of Pichinde virus or $2x10^3$ pfu of Pichinde virus followed one day later with $2x10^4$ pfu of vaccinia virus. The mononuclear cell number recorded for each footpad represents the mean cell count of 3 randomly selected fields of view per footpad ± SEM.

FIGURE 8

"Histological section of feet of a Pichinde virus-FP-inoculated LSH hamster". Haematoxylin-eosin stained section of right and left footpads of a LSH hamster 8 days after injection of the right footpad with $2x10^3$ pfu of Pichinde virus. A. Right foot showing the presence of a dense cellular infiltrate (312x). B. Uninfected left foot (312x).

A



B

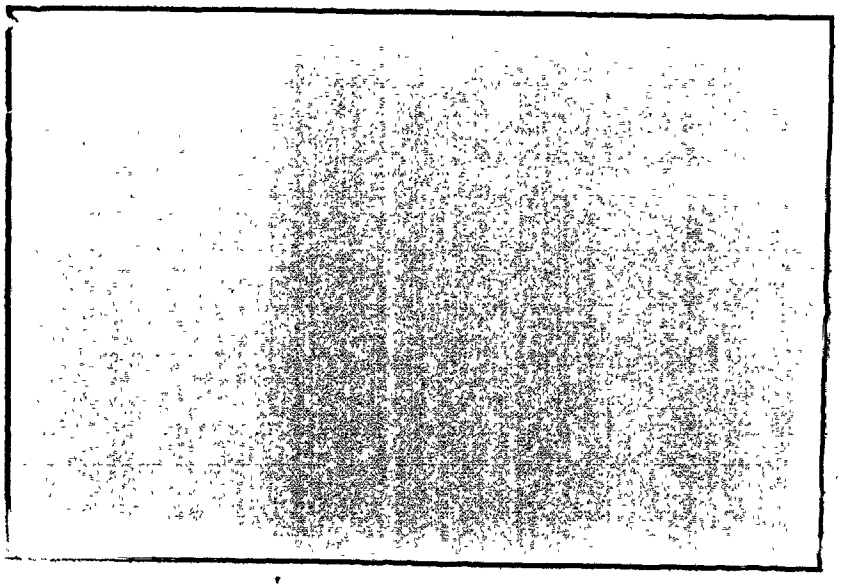
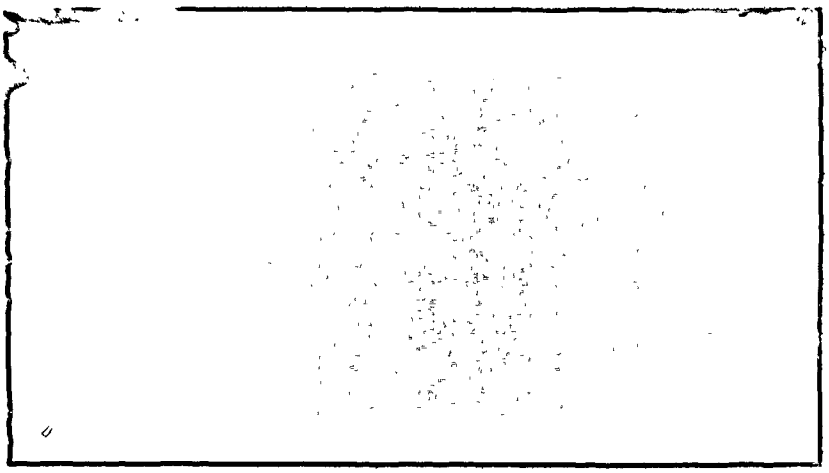


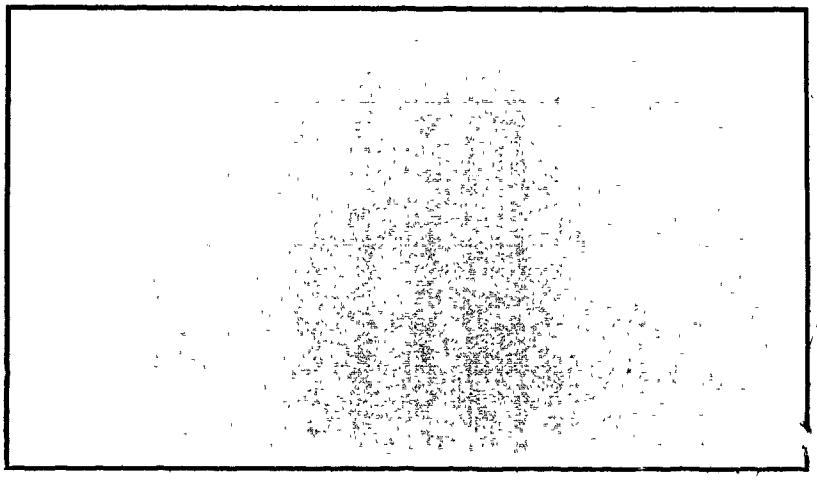
FIGURE 9

"Histological section of feet of a vesicular stomatitis virus-FP inoculated LSH hamster". Haematoxylin-eosin stained section of right and left footpads of a LSH hamster 6 days after injection of the right footpad with $2x10^3$ pfu of vesicular stomatitis virus. A. Right foot showing the presence of a dense cellular infiltrate (312x). B. Uninfected left foot (312x). C. Magnification of right foot showing that the infiltrate consisted mainly of mononuclear cells (1250x).

A



B



C

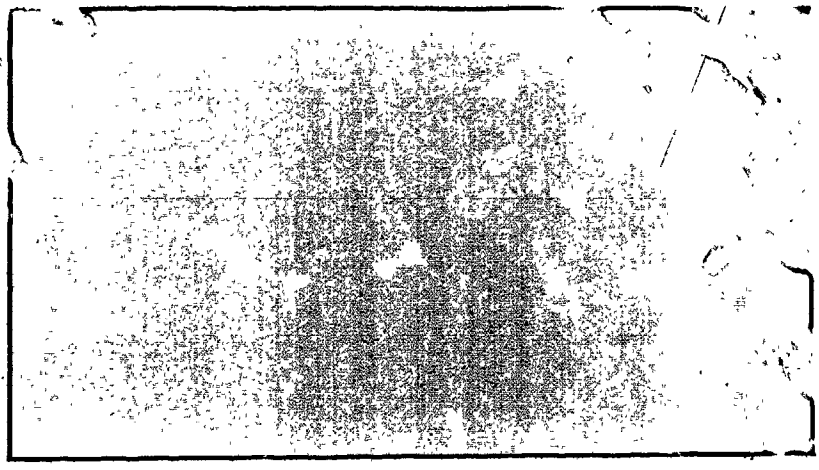
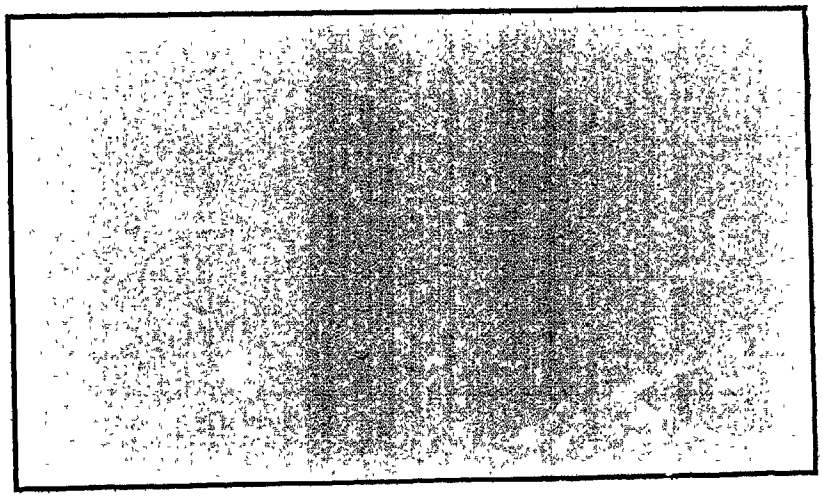


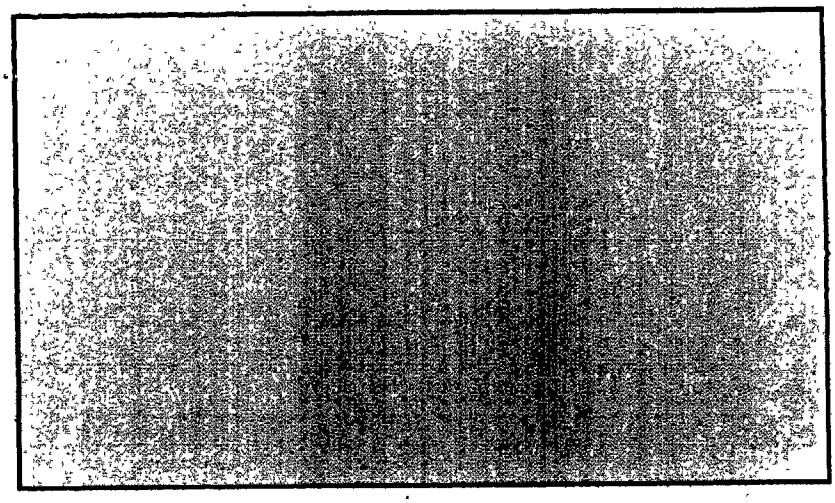
FIGURE 10

"Histological section of feet of a vaccinia virus-FP-inoculated LSH hamster". Haematoxylin-eosin stained section of right and left footpads of a LSH hamster 6 days after injection of the right footpad with $2x10^3$ pfu of vaccinia virus. A. Right foot showing the presence of a dense cellular infiltrate (312x). B. Uninfected left foot (312x). C. Magnification of right foot showing that the infiltrate consisted mainly of mononuclear cells (1250x).

A



B



C

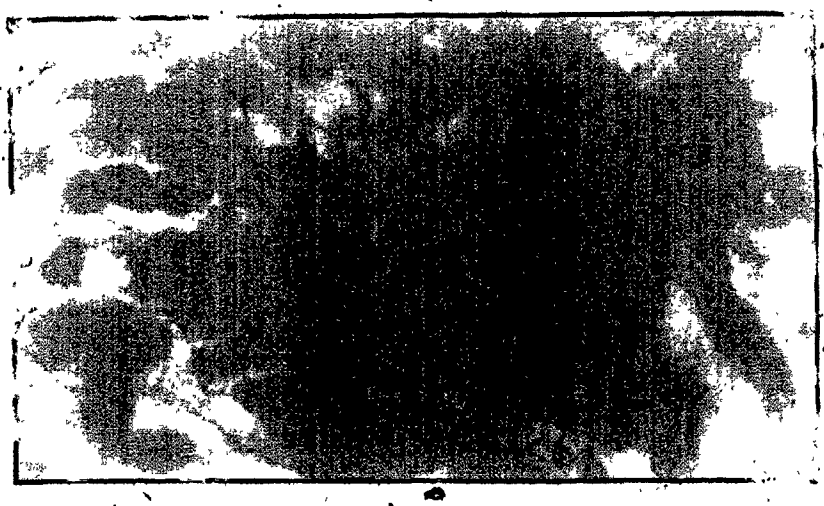
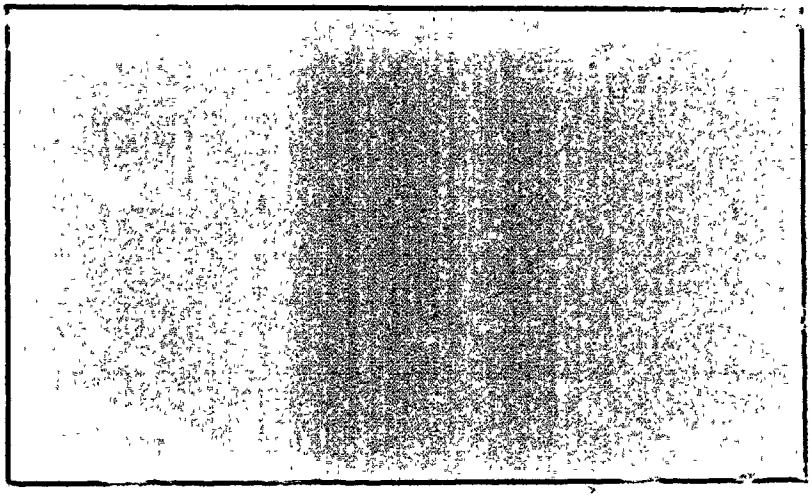


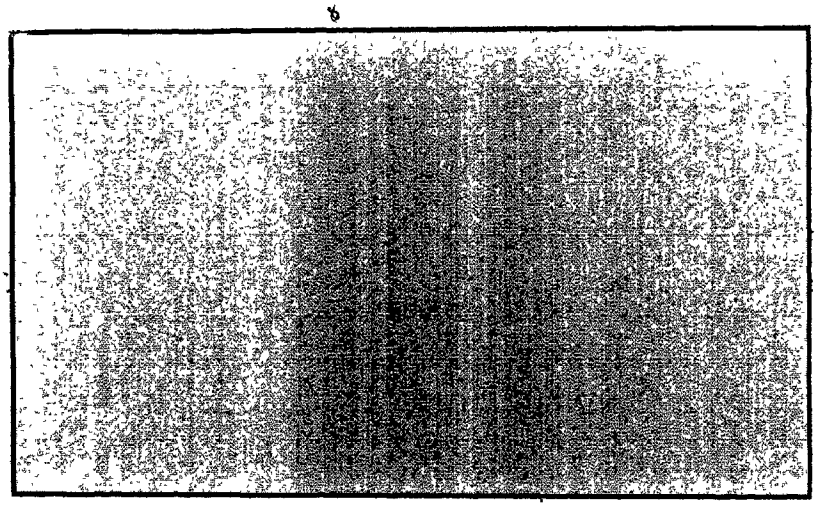
FIGURE 11

"Histological section of feet of a vesicular stomatitis virus-FP-inoculated MHA hamster". Haematoxylin-eosin stained section of right and left footpads of a MHA hamster 6 days after injection of the right footpad with $2x10^3$ pfu of vesicular stomatitis virus. A. Right foot showing the presence of a cellular infiltrate (312x). B. Uninfected left foot (312x). C. Magnification of right foot showing that the infiltrate consisted predominantly of mononuclear cells (1250x).

A



B

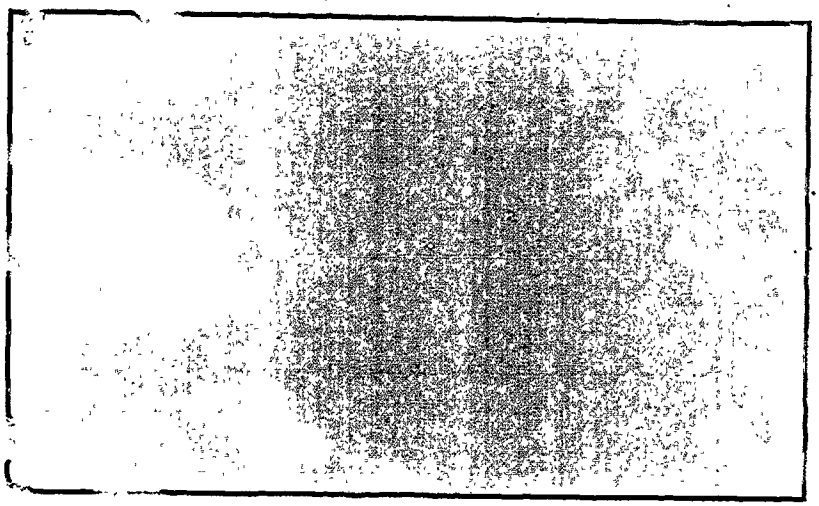


C

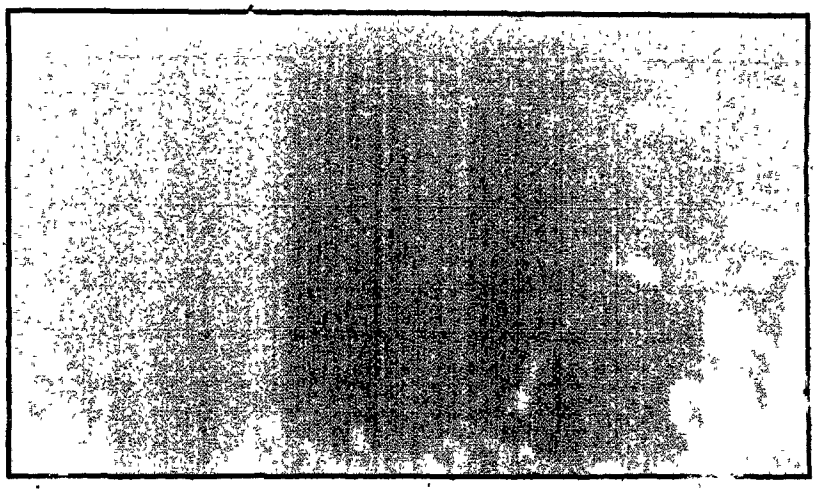
FIGURE 12

"Histological section of feet of a vaccinia virus-FP-inoculated MHA hamster". Haematoxylin-eosin stained section of right and left footpads of a MHA hamster 6 days after injection of the right footpad with $2x10^3$ pfu of vaccinia virus. A. Right foot showing the presence of a cellular infiltrate (312x). B. Uninfected left foot (312x). C. Magnification of right foot showing that the infiltrate consisted predominantly of mononuclear cells (1250x).

A



B



C

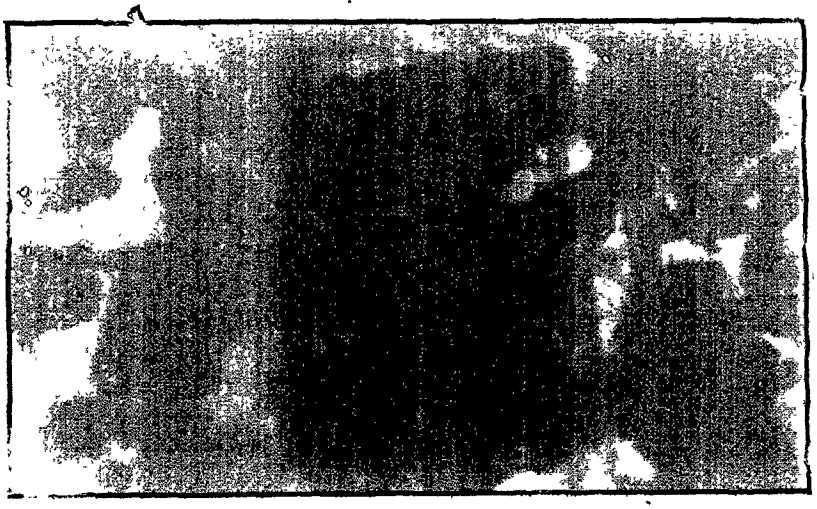
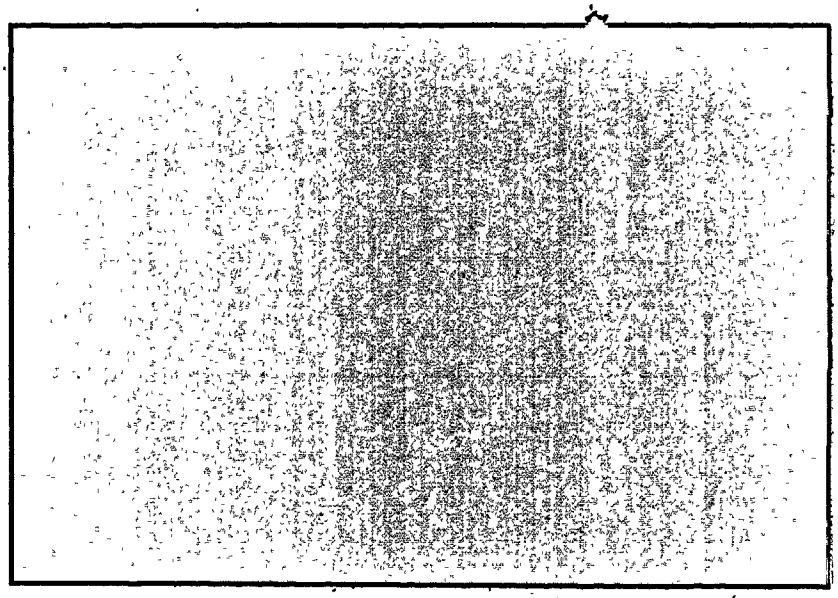


FIGURE 13

"Histological section of feet of a Pichinde virus-FP-inoculated MHA hamster". Haematoxylin-eosin stained section of right and left footpads of a MHA hamster 8 days after injection of the right footpad with $2x10^3$ pfu of Pichinde virus. A. Right foot illustrating the absence of inflammatory cells (312x). B. Uninfected left foot (312x).

A



B

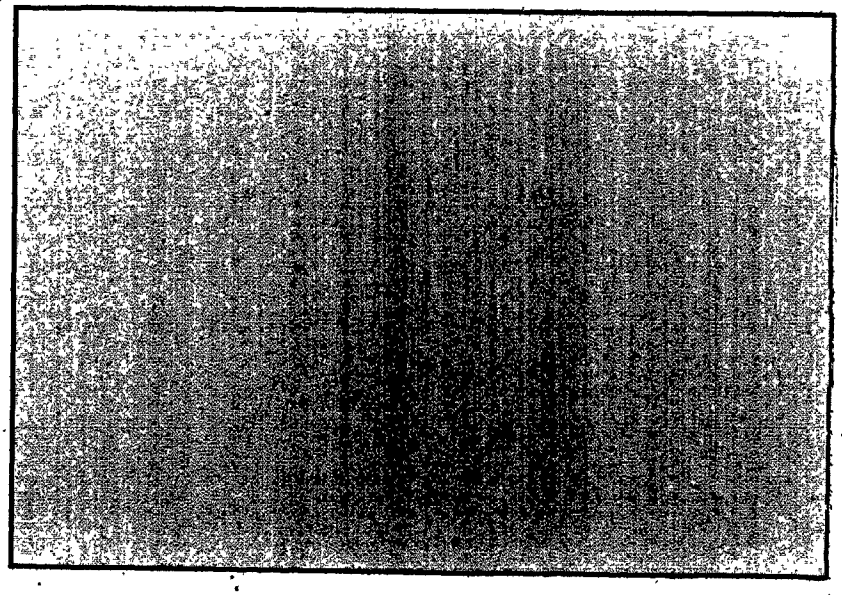


FIGURE 14

"Histological section of the right foot of a vaccinia virus and Pichinde virus-FP-inoculated MHA hamster". Haematoxylin-eosin stained section of the right footpad of a MHA hamster 5 days after injection with $2x10^4$ pfu of vaccinia virus together with $2x10^3$ pfu of Pichinde virus. A. Virus-injected footpad revealing the presence of a dense cellular infiltrate (312x). B. Magnification of footpad showing that the infiltrate consisted predominantly of mononuclear cells (1250x).

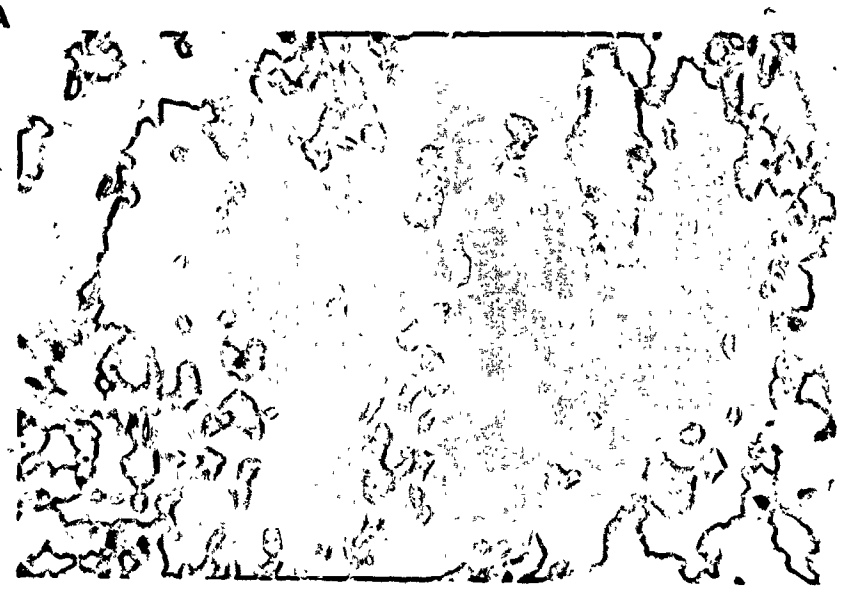
A

B

FIGURE 15

"Histological section of the right foot of a vaccinia virus and Pichinde virus-FP-inoculated MHA hamster". Haematoxylin-eosin stained section of the right footpad of a MHA hamster 5 days after injection with $2x10^4$ pfu of vaccinia virus followed one day later with $2x10^3$ pfu of Pichinde virus. A. Virus-injected footpad showing the presence of a cellular infiltrate (312x). B. Magnification of footpad showing that the infiltrate consisted mainly of mononuclear cells (1250x).

A



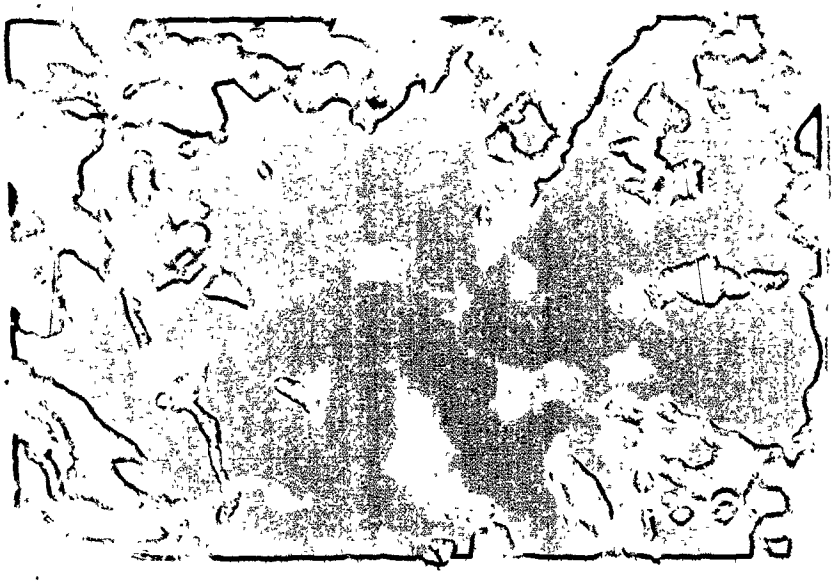
B

FIGURE 16

"Histological section of the right foot of a Pichinde virus and vaccinia virus-FP-inoculated MHA hamster". Haematoxylin-eosin stained section of the right footpad of a MHA hamster 5 days after injection with $2x10^3$ pfu of Pichinde virus followed one day later with $2x10^4$ pfu of vaccinia virus. A. Virus-injected footpad showing the presence of a cellular infiltrate (312x). B. Magnification of footpad revealing that the infiltrate consisted mainly of mononuclear cells (1250x).

A

B



degrees of footpad swelling were deemed to manifest the MHA phenotype. The results are shown in Table 4. The mean foot count ratio of F_1 (LSH X MHA) and F_1 X LSH progeny did not significantly differ from that of the responsive LSH parent but was significantly different from the MHA parent ($p<.0005$). Furthermore, foot count ratios of F_1 X MHA progeny did not differ from the values expected for a dominant characteristic controlled by a single autosomal gene ($p>.05$, χ^2).

Popliteal lymph nodes were also excised from these hamsters and the titres of Pichinde virus were determined for each animal (Table 5). Popliteal lymph nodes from MHA hamsters contained 3.75 ± 0.56 $\log_{10}$ pfu Pichinde virus compared to 2.65 ± 0.49 $\log_{10}$ pfu in the popliteal lymph nodes of footpad-inoculated LSH hamsters. Utilizing the mean $\pm$ 1 standard deviation as the criterion for classification, virus titres in F_1 and backcross progeny were designated as either LSH-like or MHA like. All F_1 (LSH X MHA) progeny and 20/22 F_1 X LSH progeny had titres comparable to their LSH parent. Of 37 F_1 X MHA animals, 26 had titres comparable to their LSH parent and 11 had MHA-like titres. No linkage by sex for either the footpad swelling or virus replication phenotypes was apparent.

These results suggested that a single autosomal dominant gene controlled Pichinde virus replication in the popliteal lymph node ($p>.01$, χ^2). Furthermore, this gene did not appear to be the same gene that controlled footpad swelling response, since of the 15 F_1 X MHA hamsters which showed the LSH footpad swelling phenotype, 11 had LSH-like titres of Pichinde virus and 4 had MHA-like titres. In addition, of the 22 F_1 X MHA hamsters that displayed the MHA footpad swelling phenotype, 15 had LSH-like titres of Pichinde virus and 7 had MHA-like titres (Table 5). From these genetic studies, it appeared that two

Table 4

Genetics of the Footpad Swelling Response in Syrian Hamsters

Background	n	Phenotype[a]	Foot-Count Ratio (mean ± SD)
LSH	25		1.93±0.52
MHA	28		1.23±0.22
F_1	8	8 LSH	2.00±0.16
F_1 X LSH	25	24 LSH	1.94±0.30
		1 MHA	1.31
F_1 X MHA	55	26 LSH	1.69±0.25
		29 MHA	1.20±0.11

[a]Animals received an inoculation of $2x10^3$ pfu of Pichinde virus in the right rear footpad on day 0. On day 7, 10^6 cpm ^{125}I-HaSA was injected IP. Animals were sacrificed 8 days after infection, and the hind feet were amputated and counted in a gamma counter. Animals were then divided into phenotypic classes using the mean foot-count ratio ± SD of the parents as a criteria. Means of the phenotypic groups were then calculated.

Table 5

Number of Genes Controlling Footpad Swelling and Pichinde Virus Replication

Background	n	Footpad Swelling Phenotype[a]	Virus Replication Phenotype[b]	Mean Virus Titre per Lymph Node Log_{10} pfu±SD
LSH	16	16 LSH	16 LSH	2.65±0.49
MHA	16	16 MHA	16 MHA	3.75±0.56
F_1	8	8 LSH	8 LSH	2.24±0.27
F_1 X LSH	22	21 LSH	20 LSH	2.59±0.41
			1 MHA	3.22
		1 MHA	0 LSH	
			1 MHA	3.29
F_1 X MHA	37	15 LSH	11 LSH	1.93±0.90
			4 MHA	3.58±0.15
		22 MHA	15 LSH	2.12±0.71
			7 MHA	3.57±0.32

[a] Animals received an inoculation of $2x10^3$ pfu of Pichinde virus in the right rear footpad on day 0. On day 7, 10^6 cpm ^{125}I-HaSA was injected IP. Animals were sacrificed 8 days after infection, and the hind feet were amputated and counted in a gamma counter. Animals were then divided into phenotypic classes using the mean foot-count ratio ± SD of the parents as a criteria. Means of the phenotypic groups were then calculated.

[b] Popliteal lymph node cells were excised from individual animals and assayed for total Pichinde virus pfu. Animals were classified as LSH-like or MHA-like using the criterion of mean virus titre ± SD; the means of the resulting phenotypic groups were then calculated.

independently segregating autosomal dominant genes controlled the footpad swelling response and Pichinde virus replication in the popliteal lymph node ($p>.05$, χ^2).

3.4 Cell-mediated suppression of footpad swelling of MHA hamsters

It has been demonstrated that MHA hamsters were capable of immune recognition to Pichinde virus since FP-inoculated animals produced antibodies to the virus. Furthermore, this hamster strain could manifest a footpad swelling response to vaccinia virus and vesicular stomatitis virus, although it failed to respond to Pichinde virus. These observations suggested that the absence of a footpad swelling response to Pichinde virus in the MHA hamster might have been caused by either a lack of T cell help which was necessary for the expression of delayed type hypersensitivity (DTH) or to the presence of cell-mediated suppression which may have interfered with the expression of DTH. It appeared unlikely that the unresponsive state was due to either a lack of antigen recognition or to a generalized defect in T effector cell function.

3.4.1 Effect of cyclophosphamide treatment on footpad swelling

Cyclophosphamide treatment of mice has been shown to augment DTH reactions in the animals, both in intensity and duration, by interacting with and inhibiting a suppressor cell population (Askenase, Hayden and Gershon, 1975; Lando, Teitelbaum and Arnon, 1979). Moreover, the time in which cyclophosphamide was administered appeared to be critical. The drug seemed only effective on the suppressor cell population when given 2 days before antigen (Askenase, Hayden and Gershon, 1975).

Cyclophosphamide, at concentrations of 25 mg/kg, 50 mg/kg and 100 mg/kg body weight was administered IP into MHA and LSH hamsters 2 days before the hamsters were FP-inoculated with Pichinde virus. A significant swelling response by micrometer measurements, was observed in the MHA hamster at all concentrations of cyclophosphamide tested ($p \leq .0005$) (Figure 17A). Interestingly, cyclophosphamde treatment of LSH hamsters also resulted in an augmented footpad swelling response (Figure 17A).

However, contrary to the micrometer measurments, when footpad swelling was determined by the radioisotopic method, an appreciable swelling response could not be demonstrated in the MHA hamster strain (Figure 17B). The discrepancy may have been due to the differences in the measurement methods. For example, the radioisotope assay assesses primarily soluble elements of the inflammatory response while micrometer measures swelling which is a result of the presence of inflammatory cells and soluble components. If footpad swelling was due primarily to an infiltration of mononuclear cells in drug treated animals, then the radioisotopic method might have been incapable of detecting this response. In order to determine if the footpad swelling response that occurred in FP-inoculated, cyclophosphamide treated MHA hamsters was accompanied by a mononuclear cell infiltrate, histological sections of the virus-injected and uninjected control feet were made after micrometer measurements of footpad swelling were completed. The histological sections of footpads of MHA and LSH hamsters that had received an IP injection of 25 mg/kg body weight cyclophosphamide 2 days before a FP-inoculation of Pichinde virus revealed an intense mononuclear cell infiltrate present

in the virus-injected footpad, as compared to the uninjected control foot (Figure 18 and Figure 19). Therefore, it appeared that the MHA hamster could manifest a footpad swelling response to Pichinde virus if treated with cyclophosphamide 2 days before the virus was inoculated into the footpad. Furthermore, the drug was also capable of enhancing the footpad swelling response in the LSH hamster strain. These results suggested that cyclophosphamide augmented the cellular component of delayed hypersensitivity possibly by blocking a cell-mediated suppressor mechanism that was present in both strains of hamster.

3.4.2 Adoptive transfer assay for serum and cell-mediated suppression of footpad swelling

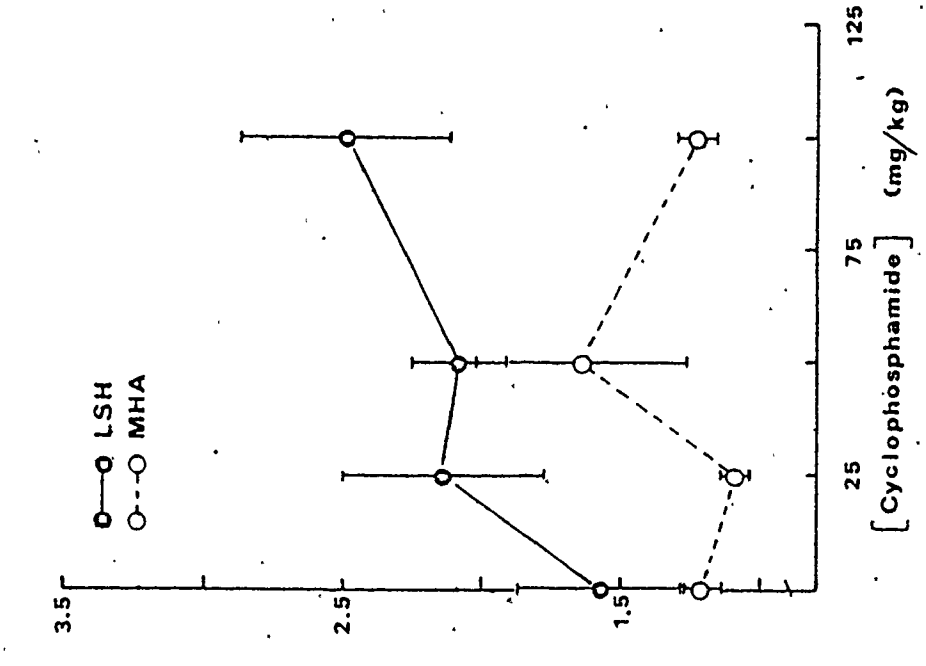
3.4.2.1 Lymphoid cell transfers

Augmentation of footpad swelling in the MHA hamster by cyclophosphamide suggested that a cell-mediated suppressor mechanism might be present in the MHA strain. To determine if such a suppressor mechanism was the underlying cause of the lack of footpad swelling in the MHA hamster, the effect of transfer of cells from the MHA hamster on the footpad response of LSH hamsters was tested. Spleen, mesenteric lymph node and popliteal lymph node cells from MHA hamsters that had been FP-inoculated 5 days previously, with Pichinde virus were injected IV into LSH recipients. Immediately after the transfer of cells, the LSH recipients were FP-inoculated with Pichinde virus and footpad swelling was measured 7 days later. Controls received sensitized or nonimmune spleen cells from LSH hamsters.

Table 6 shows that footpad swelling was significantly suppressed in those LSH recipients which received sensitized spleen, mesenteric

FIGURES 17A AND 17B

"Effect of cyclophosphamide on footpad swelling". LSH and MHA hamsters received an IP inoculation of 25 mg/kg, 50 mg/kg, and 100 mg/kg body weight of cyclophosphamide 2 days before $2x10^3$ pfu of Pichinde virus was injected into the right hind footpad. The hind feet were measured 8 days later using both micrometer (A. Micrometer measurement) and radioisotopic (B. Radioisotopic measurement) methods. Each point represents the mean of 5 animals $\pm$ SEM.


LSH
MHA
3.5
2.5
1.5
Foot Count Ratio
25
75
125
[Cyclophosphamide] (mg/kg)

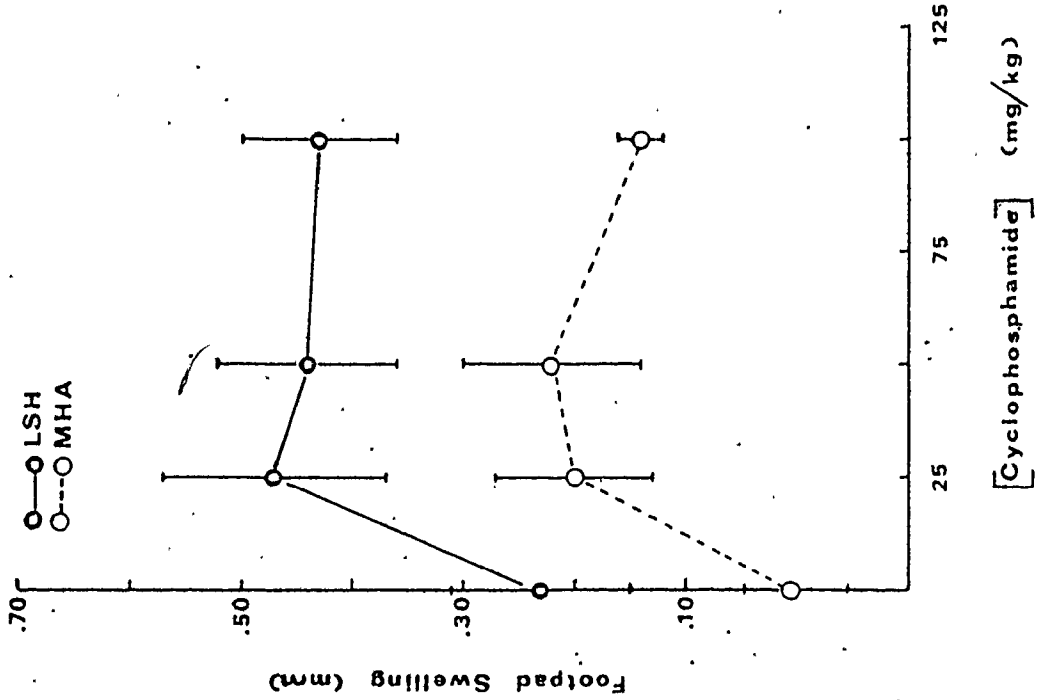
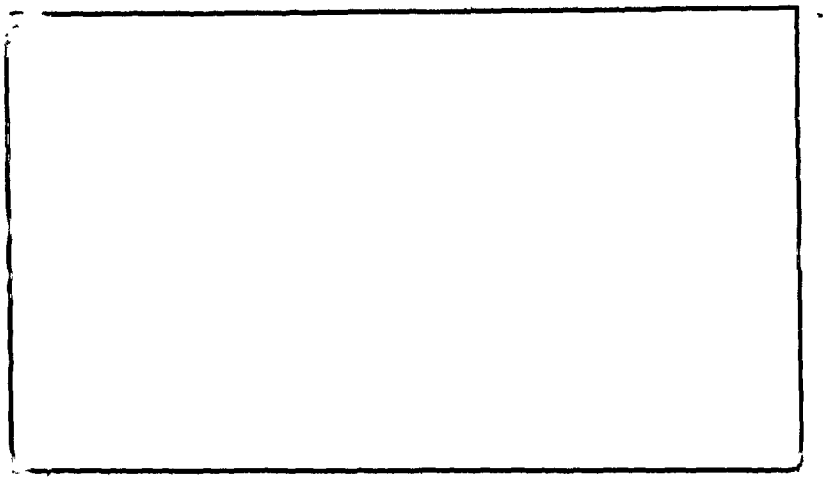

LSH
MHA
.70
.50
.30
.10
Footpad Swelling (mm)
25
75
125
[Cyclophosphamide] (mg/kg)

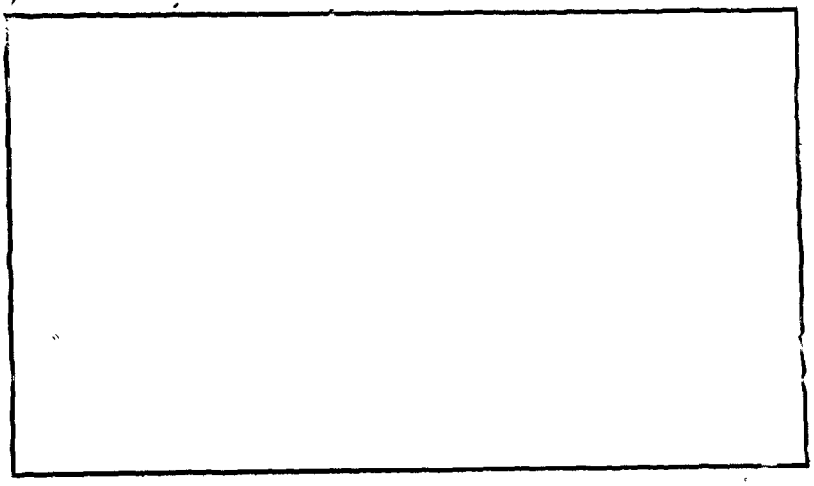
FIGURE 18

"Histological section of feet of a Pichinde virus-FP-inoculated, cyclophosphamide-treated MHA hamster". Haematoxylin-eosin stained section of right and left footpads of a MHA hamster 10 days and 8 days after treatment with 25 mg/kg body weight of cyclophosphamide and a right FP-inoculation of $2x10^3$ pfu of Pichinde virus, respectively. A. Right foot illustrating the presence of a cellular infiltrate (312x). B. Uninfected left foot (312x). C. Magnification of right foot showing that the infiltrate consisted predominantly of mononuclear cells (1250x).

A



B



C

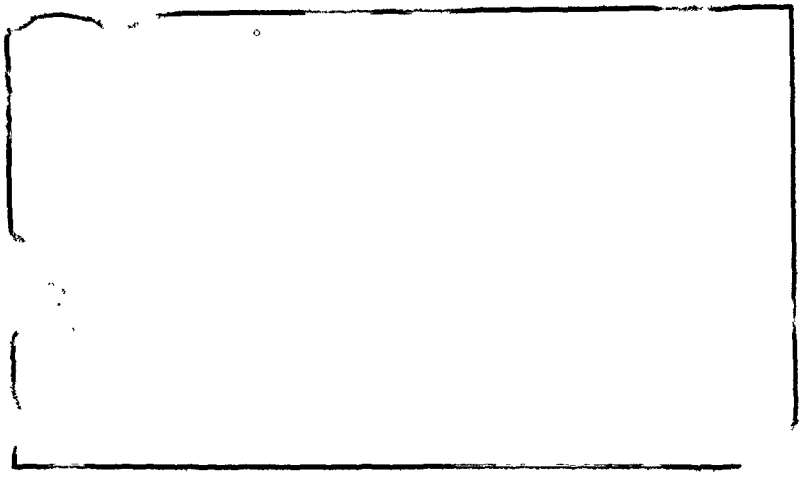
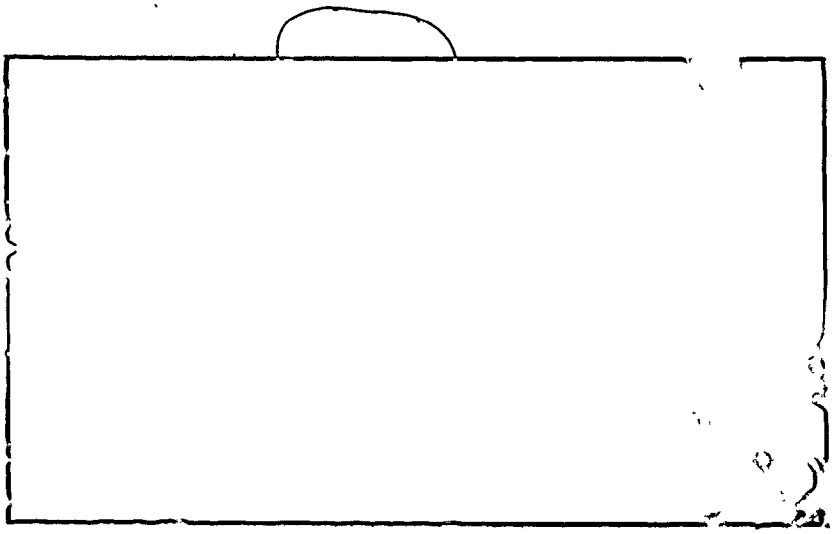


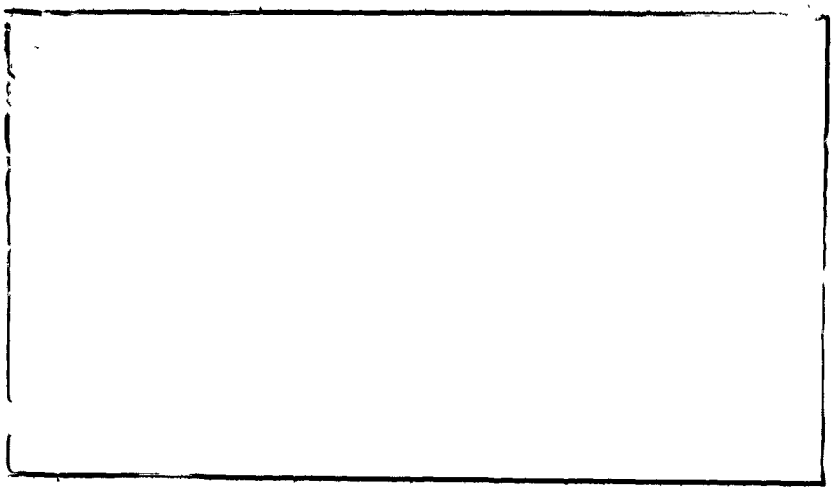
FIGURE 19

"Histological section of feet of a Pichinde virus-FP-inoculated, cyclophosphamide-treated LSH hamster". Haematoxylin-eosin stained section of right and left footpads of a LSH hamster 10 days and 8 days after treatment with 25 mg/kg body weight of cyclophosphamide and a right FP-injection of $2x10^3$ pfu of Pichinde virus, rspectively. A. Right foot illustrating the presence of a dense cellular infiltrate (312x). B. Uninfected left foot (312x). C. Magnification of right foot showing that the infiltrate consisted mainly of mononuclear cells (1250x).

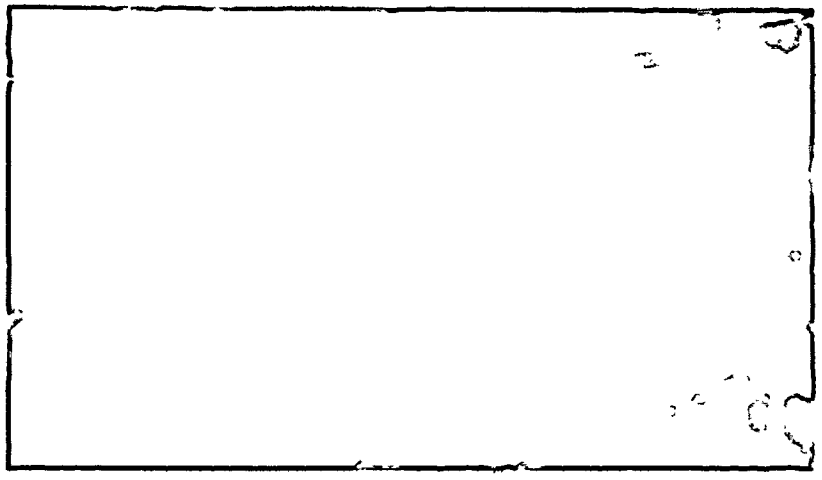
A



B



C



lymph node and popliteal lymph node cells from Pichinde virus-sensitized MHA hamsters when compared to LSH recipients which received LSH non-immune cells. The footpad swelling response of LSH recipients that had been injected with LSH sensitized spleen cells was also significantly less than the response of recipients of LSH nonimmune cells ($p<.05$) but was nevertheless greater than the response of recipients of either MHA spleen cells ($p<.005$) or MHA mesenteric lymph node cells ($p<.025$) (Table 6). Suppression was not observed in recipients that received $3x10^5$ pfu Pichinde virus IV prior to challenge with virus in the footpad. This dose of virus was comparable to that found in $5x10^7$ spleen cells from infected MHA hamsters.

Both the concentration of cells transferred and the time interval between sensitization of the donor and transfer of its cells into the recipient appeared to be critical for demonstrating suppression of the footpad swelling response in the LSH recipient. Varying concentrations of spleen cells from MHA hamsters that had been FP-inoculated 5 days previously, with Pichinde virus were injected into LSH recipients, as described above, and footpad swelling was measured 7 days later. As shown in Table 7, transfer of fewer than $5x10^7$ donor cells did not suppress the response of the LSH recipient. Unfortunately, it was not possible to transfer more than $5x10^7$ donor cells into the recipients due to volumes required. In a separate experiment, footpad swelling was monitored in LSH recipients that had received spleen cells from MHA hamsters that had been FP-inoculated 10, 5 and 3 days previously, with Pichinde virus. Control hamsters were injected with spleen cells from LSH and MHA hamsters that had been FP-inoculated with Pichinde virus on the day of transfer. Suppression of the footpad swelling response of the recipients was observed to be maximal when spleen cells were

obtained from MHA hamsters 5 days after FP-inoculation of the virus (Table 8).

In addition, the mode in which the donors were sensitized appeared to be important in the capacity of the immune cells to transfer suppression. Suppression of the footpad swelling response was not detected in LSH recipients that received $5x10^7$ sensitized spleen cells from either MHA or LSH donors which were injected with Pichinde virus IP rather than subcutaneously in the footpad (Table 9).

3.4.2.2 Serum transfers

To determine if MHA immune sera could also alter footpad swelling in LSH recipients, sera were collected from MHA hamsters either 8 days or 8 weeks after the animals had been FP-inoculated with Pichinde virus and injected IV into LSH recipients. In contrast to the results obtained in experiments that involved the transfer of MHA immune cells, transfer of MHA immune sera did not significantly diminish the footpad swelling response of the LSH recipients (Table 10). However, in this experiment, transfer of MHA nonimmune sera appeared to enhance footpad swelling in the recipients (Table 10).

Therefore, it appeared that the MHA phenotype could be bestowed upon a naive LSH recipient by the transfer of immune cells but not immune sera from MHA hamsters infected with Pichinde virus. Furthermore, successful transfer of the suppression was dependent on the number of MHA cells injected into the LSH hamsters and was maximal when cells were obtained from MHA hamsters 5 days after an injection of Pichinde virus into the footpad of the donors. The same pattern of results was observed in 2 other experiments. These findings strongly supported the concept that a cell-mediated suppressor mechanism was responsible for

the failure of the MHA hamster to elicit a footpad swelling response to a primary FP-inoculation of Pichinde virus.

3.4.2.3 Specificity of transferred cells

To determine if the cell-mediated suppressor mechanism had an influence on the footpad swelling response of LSH hamsters to an unrelated virus, spleen cells from MHA hamsters that had been FP-inoculated 5 days earlier, with Pichinde virus were injected as previously described, into LSH recipients. Other LSH hamsters received nonimmune MHA spleen cells. The LSH recipients were then FP-injected with either vesicular stomatitis virus or Pichinde virus.

When footpad swelling was measured in those LSH recipients that were FP-inoculated with vesicular stomatitis virus, no significant difference in footpad response could be detected in the recipients that received MHA immune cells as compared to the hamsters that received MHA nonimmune cells. Both groups displayed a pronounced footpad swelling response to vesicular stomatitis virus (Table 11). In contrast, a significant suppression in footpad response was observed in the hamsters that were FP-inoculated with Pichinde virus. Footpad swelling of recipients of MHA immune cells was significantly less than the swelling of those hamsters that received MHA nonimmune cells (Table 11).

These observations suggested that the action of the cell-mediated suppressor mechanism present in Pichinde virus-primed MHA hamsters was specific.

Table 6

Footpad Swelling Response of LSH Hamsters After Receipt of Immune Cells From FP-Inoculated Donors

Cells Transferred[a]	Number Tested	Foot Count Ratio (mean ± SEM)
MHA Immune SPL	6	1.28±0.06 $p<.0005$[b]
MHA Immune MLN	5	1.33±0.08 $p<.005$[b]
MHA Immune PLN	4	1.45±0.06 $p<.01$[b]
LSH Immune SPL	5	1.62±0.07
LSH Nonimmune SPL	5	1.94±0.13

[a] LSH hamsters received an IV inoculation of either $5x10^7$ spleen cells (SPL), $5x10^7$ mesenteric lymph node cells (MLN) or $5x10^7$ popliteal lymph node cells (PLN) from either MHA or LSH hamsters that had been inoculated 5 days earlier with $2x10^3$ pfu of Pichinde virus in both hind footpads. Other LSH recipients were injected IV with $5x10^7$ spleen cells from normal LSH hamsters. Immediately after the IV injection, all LSH recipients were inoculated with $5x10^6$ pfu of Pichinde virus in the right hind footpad. Footpad swelling was measured 7 days later using the radioisotopic method.

[b] The mean foot count ratio was significantly different from the mean ratio of LSH recipients that received LSH nonimmune cells.

Table 7

Footpad Swelling Response of LSH Hamsters After Receipt of Varying Concentrations of Immune Cells

Cells Transferred[a]	Concentration	Number Tested	Foot Count Ratio (Mean ± SEM)
MHA Immune SPL	$5x10^7$	5	1.14±0.19[b]
MHA Immune SPL	$2.5x10^7$	5	1.67±0.13
MHA Immune SPL	$1.25x10^7$	5	1.73±0.07
MHA Immune SPL	$6.25x10^6$	4	1.73±0.17
MHA Immune SPL	$3.0x10^6$	3	1.74±0.06
LSH Nonimmune SPL	$5x10^7$	3	1.68±0.06

[a]LSH hamsters were inoculated IV with either $5x10^7$, $2.5x10^7$, $1.25x10^7$, $6.25x10^6$ or $3x10^6$ spleen (SPL) cells from MHA hamsters that had been inoculated 5 days earlier, with $2x10^3$ pfu of Pichinde virus in both hind footpads. Other LSH recipients were injected IV with $5x10^7$ spleen cells from normal LSH hamsters. Immediately after the IV injection, all LSH recipients were inoculated with $5x10^6$ pfu of Pichinde virus in the right hind footpad. Footpad swelling was measured 7 days later using the radioisotopic method.

[b]The mean foot count ratio was significantly different from the mean ratio of LSH recipients that received LSH nonimmune cells ($p<.005$).

Table 8

Footpad Swelling Response of LSH Hamsters After Receipt of Immune Cells From Various FP-inoculated Donors

Cells Transferred[a]	Footpad Swelling (Mean ± SEM)			
	Experiment 1		Experiment 2	
	Difference in Thickness (mm)	Foot Count Ratio	Difference in Thickness (mm)	Foot Count Ratio
MHA Day 0	0.39±0.06 (3)[b]	2.05±0.30 (3)	0.41±0.05 (4)	1.54±0.03 (5)
MHA Day 3	0.38±0.06 (5)	1.80±0.06 (5)	0.24±0.03 (5)	1.93±0.10 (4)
MHA Day 5	0.09±0.04 (5)	1.53±0.15 (5)	0.09±0.02 (4)	1.14±0.19 (5)
MHA Day 10	0.46±0.08 (5)	1.76±0.08 (5)	0.31±0.04 (4)	1.45±0.28 (3)
LSH Day 0	N.D.	N.D.	0.38±0.10 (4)	1.68±0.06 (3)
LSH Day 5	0.32±0.03 (4)	1.71±0.13 (4)	N.D.	N.D.

Table 8 (Footnotes)

N.D. no data

[a]LSH hamsters were inoculated IV with $5x10^7$ spleen cells from MHA hamsters that had been inoculated 10, 5 and 3 days earlier, with $2x10^3$ pfu of Pichinde virus in both rear footpads. Other LSH recipients were injected IV with $5x10^7$ spleen cells either from MHA or LSH hamsters that had been FP-inoculated with Pichinde virus on the day of transfer or from LSH hamsters that had been FP-inoculated 5 days previously, with Pichinde virus. Immediately after the IV injection, all LSH recipients were inoculated with $5x10^6$ pfu of Pichinde virus in the right hind footpad. Footpad swelling was measured using both micrometer and radioisotopic methods 8 days later.

[b]Number of animals tested.

Table 9

Footpad Swelling Response of LSH Hamsters After Receipt of Immune Cells From IP-Inoculated Donors

Cells Transferred[a]	Number Tested	Foot Count Ratio (Mean ± SEM)
MHA Immune SPL	5	1.65±0.17
LSH Immune SPL	4	1.65±0.26
None	4	1.76±0.07

[a]LSH hamsters were inoculated IV with $5x10^7$ spleen (SPL) cells from either MHA or LSH hamsters that had been IP-inoculated 5 days earlier, with $2x10^3$ pfu of Pichinde virus. Other LSH recipients did not receive cells. Immediately after the IV injection, all LSH hamsters were inoculated with $5x10^6$ pfu of Pichinde virus in the right hind footpad. Footpad swelling was measured 7 days later using the radioisotopic method.

Table 10

Footpad Swelling Response of LSH Hamsters

After Receipt of MHA Immune Sera

Sera Transferred[a]	CF Ab Titre (log_{10})	Number Tested	Footpad Swelling (Mean ± SEM)	
			Difference in Thickness (mm)	Foot Count Ratio
MHA early (8d P.I.)	1.30	5	0.26±0.03	1.93±0.10
MHA hyper immune (8w P.I.)	2.50	5	0.22±0.05	1.72±0.10
MHA nonimmune	-	5	0.39±0.04	2.19±0.17
LSH nonimmune	-	5	0.27±0.04	1.98±0.13

P.I. post infection

[a]LSH hamsters received an IV inoculation of sera collected from MHA hamsters that had been injected 8 days or 8 weeks earlier, with $2x10^3$ pfu of Pichinde virus in the right hind footpad. Other LSH hamsters received an IV inoculation of sera collected from normal MHA and LSH hamsters. Immediately after the IV injection, all LSH recipients were inoculated with $5x10^6$ pfu of Pichinde virus in the right hind footpad. Footpad swelling was measured 7 days later using both micrometer and radioisotopic methods.

Table 11

Specificity of Transferred Cells

Cells Transferred[a]	Virus Challenge	Number Tested	Footpad Swelling (MEAN ± SEM) Difference in Thickness (mm)	Foot Count Ratio
Immune spleen	vesicular stomatitis	5	0.31±0.07	1.41±0.27
Normal spleen	vesicular stomatitis	5	0.29±0.09	1.26±0.06
Immune spleen	Pichinde	4	0.13±0.06	1.43±0.17[b]
Normal spleen	Pichinde	5	0.59±0.06	2.18±0.23

[a] LSH hamsters were inoculated IV with $5x10^7$ spleen cells from either MHA hamsters that had been inoculated 5 days earlier, with $2x10^3$ pfu of Pichinde virus in both rear footpads or from normal MHA hamsters. Immediately after the IV injection, the LSH recipients were inoculated with either $2x10^3$ pfu of vesicular stomatitis virus or $2x10^3$ pfu of Pichinde virus in the right hind footpad. Footpad swelling was measured 5 days later, using both micrometer and radioisotopic methods, in LSH recipients that were FP-inoculated with vesicular stomatitis virus. The LSH recipients that were FP-injected with Pichinde virus were treated in a similar manner 7 days after the inoculation of virus.

[b] Footpad swelling was significantly different from swelling of Pichinde virus-FP-inoculated LSH hamsters that received MHA nonimmune cells ($p<.025$).

3.4.3 *In vitro* cytotoxicity

3.4.3.1 Cytotoxic cell generation

Another parameter of cell-mediated immunity besides delayed hypersensitivity that was studied was the *in vitro* generation of cytotoxic cells. Previous attempts to demonstrate the presence of cytotoxic T lymphocytes in LSH and MHA hamsters after sensitization *in vivo* with Pichinde virus have been unsuccessful. Classically, cytotoxic T lymphocytes, which specifically lyse histocompatible targets bearing the antigenic determinant(s), become detectable after 3 days and peak 4-8 days after primary exposure to antigen.

When spleen cells from hamsters infected with Pichinde virus were tested for the presence of cytotoxic T lymphocytes in a ^{51}Cr release assay, using syngeneic and allogeneic tumour cells infected with Pichinde virus as targets, it was found that the syngeneic combinations of cytotoxic effectors and targets yielded no greater specific ^{51}Cr release than did the allogeneic combinations (Gee, Clark and Rawls, 1979). Furthermore, infection of the target cells with Pichinde virus did not increase their susceptibility to lysis and peak activity occurred on the third day of infection. These results suggested that this cytotoxic activity was not attributable to classic T lymphocyte-mediated lysis.

Since the presence of cytotoxic T lymphocytes could not be demonstrated in hamsters after primary exposure of antigen *in vivo*, efforts to detect their presence after a second exposure of antigen *in vitro*, were made. Spleen cells from hamsters that had been FP-inoculated 1 week previously with Pichinde virus, were incubated *in vitro* with syngeneic peritoneal exudate cells that had been exposed to UV-inactivated Pichinde virus. After 5 days, the lymphoid cells were tested

in a ^{51}Cr release assay, using syngeneic Pichinde virus-infected fibroblast cells as targets.

Spleen cells from primed hamsters exhibited cytotoxic activity against the target cells and maximum cytotoxic activity was obtained when the effector cells were incubated in vitro in the presence of both peritoneal exudate cells and virus (Table 12). A similar cytotoxic response pattern was also observed in spleen cells from hamsters primed with herpes simplex virus (Table 13). Note, however, that it was unnecessary to have herpes simplex virus-infected targets to show killing. Table 14 and Table 15 provide further evidence that the cytotoxic activity generated from in vivo primed, in vitro restimulated hamsters lacked specificity in killing. Spleen and mesenteric lymph node cells from MHA and LSH hamsters that had been primed in vivo and restimulated in vitro with either Pichinde virus or herpes simplex virus exhibited comparable lysis against Pichinde virus-infected, herpes simplex virus-infected and uninfected LEF targets.

This lack of specific killing by the effector cells was further substantiated in a ^{51}Cr release cold target inhibition assay that utilized combinations of ^{51}Cr-labelled and unlabelled targets. In Figure 20, cytotoxic activity of spleen (A) and mesenteric lymph node cells (B) from LSH hamsters that had been FP-inoculated in vivo and restimulated in vitro with Pichinde virus was measured using a fixed number of target cells comprised of a varying percentage of ^{51}Cr-labelled (hot) Pichinde virus-infected LEF cells and either unlabelled (cold) Pichinde virus-infected or herpes simplex virus-infected LEF cells. As the percentage of cold targets increased, the cytotoxic activity decreased. Furthermore, more of a decrease in cytotoxic activity was seen with cold herpes simplex

virus-infected targets than with cold Pichinde virus-infected targets. It should be noted that as the labelled target cell concentration decreases, N_{at} tends to increase (a behaviour that is not corrected for in the model, p. 34) and hence one sees a plateau as the percentage cold targets is increased. In Figure 21, cytotoxicity was measured using a fixed number of radiolabelled (hot) Pichinde virus-infected LEF cells to which was added either unlabelled (cold) Pichinde virus-infected or herpes simplex virus-infected LEF cells. The results showed that as the number of cold targets increased, cytotoxic activity decreased. Again, it was noted that herpes simplex virus-infected targets were more effective inhibitors.

These results confirmed the conclusion drawn from Tables 14 and 15, that cytotoxic activity generated from the spleen of hamsters after exposure to Pichinde virus or herpes simplex virus *in vivo* was not attributable to classic antigen-specific T lymphocyte-mediated lysis.

It was of interest to know if *in vivo* virus priming was essential for a secondary *in vitro* cytotoxic response. Table 16 shows the results of the study of the cytotoxic response of spleen and mesenteric lymph node cells from unprimed MHA and LSH hamsters following primary stimulation with Pichinde virus *in vitro*. The levels of cytotoxic activity generated from the unprimed hamsters were comparable to those generated from *in vivo* virus primed animals (Table 14 and Table 15). Moreover, although not statistically significant, the cytotoxic response of the unprimed hamsters appeared to show a tendency for some degree of specific killing.

3.4.3.2 *In vitro* suppression of cytotoxic activity

It should be recalled from section 3.4.2.1 that a cell-mediated suppressor mechanism which was present in the spleen of MHA hamsters 7

Table 12

Requirements for Generation of Maximum Cytotoxic Activity of Spleen Cells from Pichinde Virus-Primed LSH and MHA Hamsters

Effector[a]	PEC	Virus	Exp. No.	Cytotoxic Activity[b] (Nαt x10^3/culture)
LSH Spleen	+	+	1.	354±87 (4)
			2.	426±109 (4)
LSH Spleen	+	-	1.	82±35 (4)
			2.	<SR (4)
LSH Spleen	-	-	1.	180±40 (4)
			2.	151±70 (4)
MHA Spleen	+	+	3.	210±53 (4)
			4.	N.D.
MHA Spleen	+	-	3.	<SR (4)
			4.	<SR (4)
MHA Spleen	-	-	3.	58±40 (4)
			4.	<SR (4)

N.D. No data

SR Spontaneous Release

[a]LSH and MHA hamsters were FP-inoculated with 2x10^3 pfu of Pichinde virus in the right fear footpad. 7 days later, spleen cells were tested for the presence of cytotoxic activity in a ^{51}Cr assay after stimulation in vitro with UV-inactivated Pichinde virus.

[b]Cells were assayed for cytotoxicity against ^{51}Cr-labelled Pichinde virus-infected LEF targets, using an initial effector to target ratio of 100:1. Cytotoxic activity is expressed as the mean ± SEM of replicate cultures. The number of cultures is noted in parentheses.

Table 13

Cytotoxic Activity of Spleen Cells from Herpes Simplex Virus-Primed MHA Hamsters

Effector[a]	PEC	Virus	Cytotoxic Activity[b] (Nat x10^3/culture)
Spleen	+	+	1942±237 (4)
Spleen	-	+	863±129 (3)
Spleen	-	-	562±74 (4)
MLN	+	+	948±215 (3)
MLN	-	+	335±77 (3)
MLN	+	-	373±71 (3)
MLN	-	-	258±57 (2)

[a] MHA hamsters were FP-inoculated with 2x10^3 pfu of herpes simplex virus in the right rear footpad. 12 weeks later, spleen and mesenteric lymph node cells were tested for the presence of cytotoxic activity in a ^{51}Cr release assay after stimulation in vitro with UV-inactivated herpes simplex virus.

[b] Cells were assayed for cytotoxicity against ^{51}Cr-labelled uninfected LEF targets, using an initial effector to target ratio of 100:1. Cytotoxic activity is expressed as the mean ± SEM of replicate cultures. The number of cultures is noted in parentheses.

Table 14

Non-Specific Cytotoxic Activity of Spleen Cells from Pichinde Virus-Primed LSH and MHA Hamsters

Effector[a]	Exp. No.	Cytotoxic Activity[b] (Nαt x10^3/culture) LEF-Pichinde	LEF-KOS	LEF
MHA Spleen	1.	1070±110 (4)	1147±82 (4)	941±151 (4)
	2.	144±92 (1)	590±120 (4)	933±269 (4)
MHA MLN	1.	1252±119 (4)	1118±94 (4)	1270±206 (4)
	2.	1010±144 (2)	2406±351 (4)	1162±138 (4)
LSH Spleen	3.	1565±104 (4)	N.D.	N.D.
	4.	N.D.	N.D.	1296±325 (4)
LSH MLN	5.	258±76 (4)	278±90 (4)	N.D.
	6.	1068±183 (4)	577±38 (3)	N.D.

N.D. No data

[a] MHA and LSH hamsters were FP-inoculated with 2x10^3 pfu of Pichinde virus in the right rear footpad. 8-12 weeks later, spleen and mesenteric lymph node cells were tested for the presence of cytotoxic activity in a ^{51}Cr-release assay after stimulation in vitro with UV-inactivated Pichinde virus.

[b] Cells were assayed for cytotoxicity against various ^{51}Cr-labelled infected target cells, using an initial effector to target ratio of 100:1. Cytotoxic activity is expressed as the mean ± SEM of replicate cultures. The number of cultures is noted in parentheses.

Table 15

Non-specific Cytotoxic Activity of Spleen Cells from Herpes Simplex Virus-Primed LSH and MHA Hamsters

Effector[a]	Exp. No.	Cytotoxic Activity[b] (Nαt x10^3/culture) LEF-KOS	LEF-Pichinde	LEF
MHA Spleen	1.	1925±206 (2)	1563±242 (4)	1942±237 (4)
	2.	1104±127 (2)	1220±131 (3)	2449±253 (3)
MHA MLN	1.	1335±156 (2)	1634±195 (4)	2237±245 (4)
	2.	518±92 (1)	467±85 (2)	948±215 (3)
LSH Spleen	3.	367±23 (4)	292±72 (4)	357±43 (4)
LSH MLN	3.	471±76 (4)	373±95 (2)	603±144 (1)

[a] MHA and LSH hamsters were FP-inoculated with 2x10^3 pfu of herpes simplex virus in the right rear footpad. 8-12 weeks later, spleen and mesenteric lymph node cells were tested for the presence of cytotoxic activity in a ^{51}Cr release assay after stimulation in vitro with UV-inactivated herpes simplex virus.

[b] Cells were assayed for cytotoxicity against various ^{51}Cr-labelled infected target cells, using an initial effector to target ratio of 100:1. Cytotoxic activity is expressed as the mean ± SEM of replicate cultures. The number of cultures is noted in parentheses.

Table 16

Cytotoxic Activity of Spleen Cells From Normal Uninfected LSH and MHA Hamsters

Effector[a]	Exp. No.	Cytotoxic Activity[b] (Nαt x10^3/culture) LEF-Pichinde	LEF-KOS	LEF
MHA Spleen	1.	674±70 (4)	293±73 (2)	464±108 (4)
	2.	415±53 (3)	521±100 (3)	363±69 (3)
MHA MLN	2.	1530±331 (3)	681±187 (2)	260±93 (2)
	3.	2036±498 (4)	1125±233 (4)	744±108 (4)
LSH Spleen	3.	3926±877 (2)	1787±235 (4)	830±152 (4)
LSH MLN	2.	564±103 (2)	231±43 (3)	269±83 (2)
	3.	3248±605 (4)	2326±406 (4)	1648±490 (4)

[a]Spleen and mesenteric lymph node cells from uninfected MHA and LSH hamsters were tested for the presence of cytotoxic activity in a ^{51}Cr release assay, after stimulation *in vitro* with UV-inactivated Pichinde virus.

[b]Cells were assayed for cytotoxicity against various ^{51}Cr-labelled infected target cells, using an initial effector to target ratio of 100:1. Cytotoxic activity is expressed as the mean ± SEM of replicate cultures. The number of cultures is noted in parentheses.

FIGURES 20A AND 20B

"Inhibition of cytotoxic activity of spleen and mesenteric lymph node cells from Pichinde virus-primed LSH hamsters". The cytotoxic activity of spleen and mesenteric lymph node cells from LSH hamsters that had been FP-inoculated with $2x10^3$ pfu of Pichinde virus was assessed in a ^{51}Cr release assay after stimulation in vitro with UV-inactivated Pichinde virus. The cells were assayed against a fixed number (10^5 cells/ml) of target cells comprised of a varying percentage of ^{51}Cr-labelled (hot) Pichinde virus-infected LEF cells and either unlabelled (cold) Pichinde virus-infected (•) or herpes simplex virus-infected LEF cells (o). Each point represents the mean cytotoxic activity ± SEM of 4 replicate cultures. A. Cytotoxic activity of spleen cells. B. Cytotoxic activity of mesenteric lymph node cells.

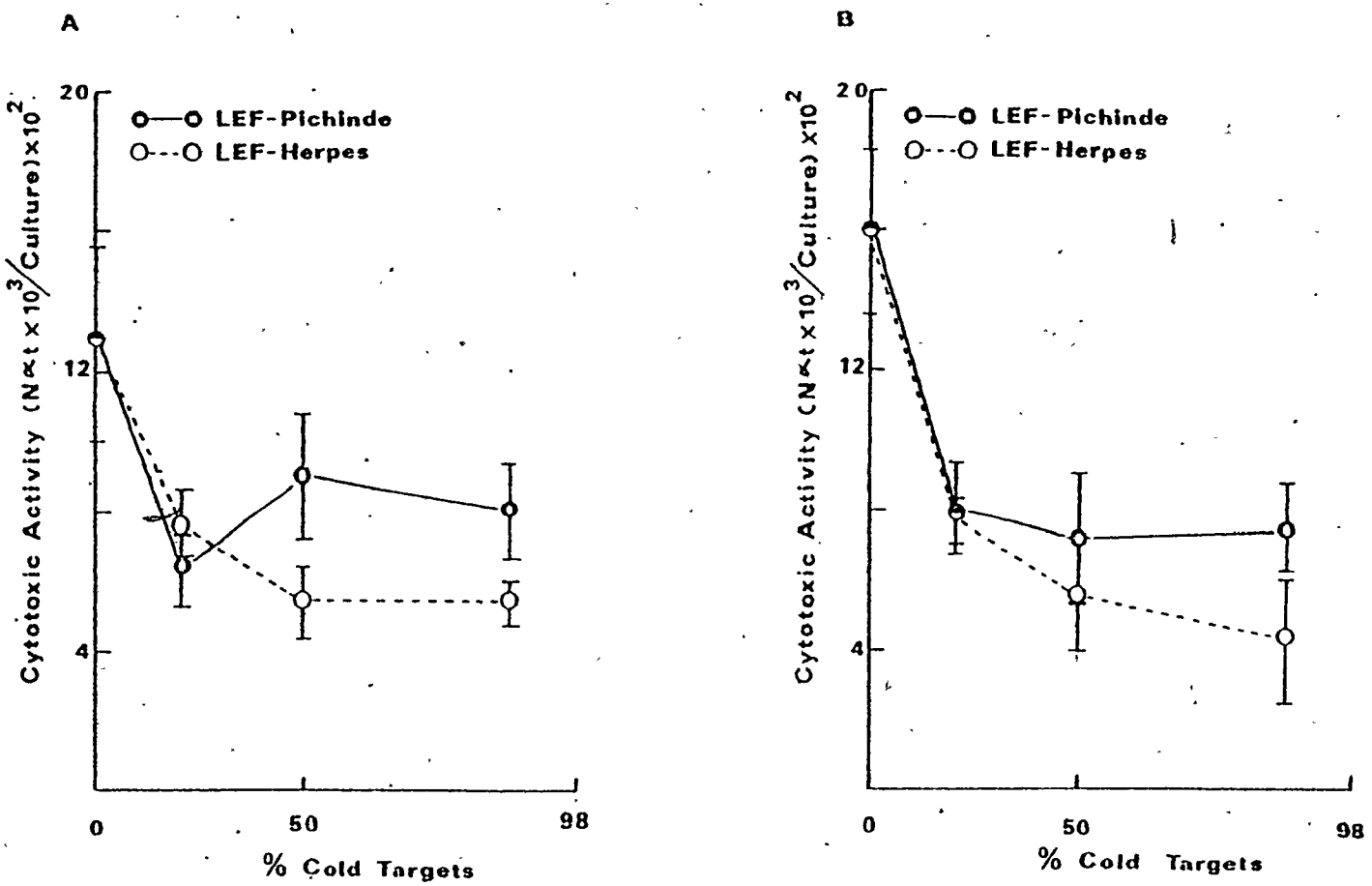
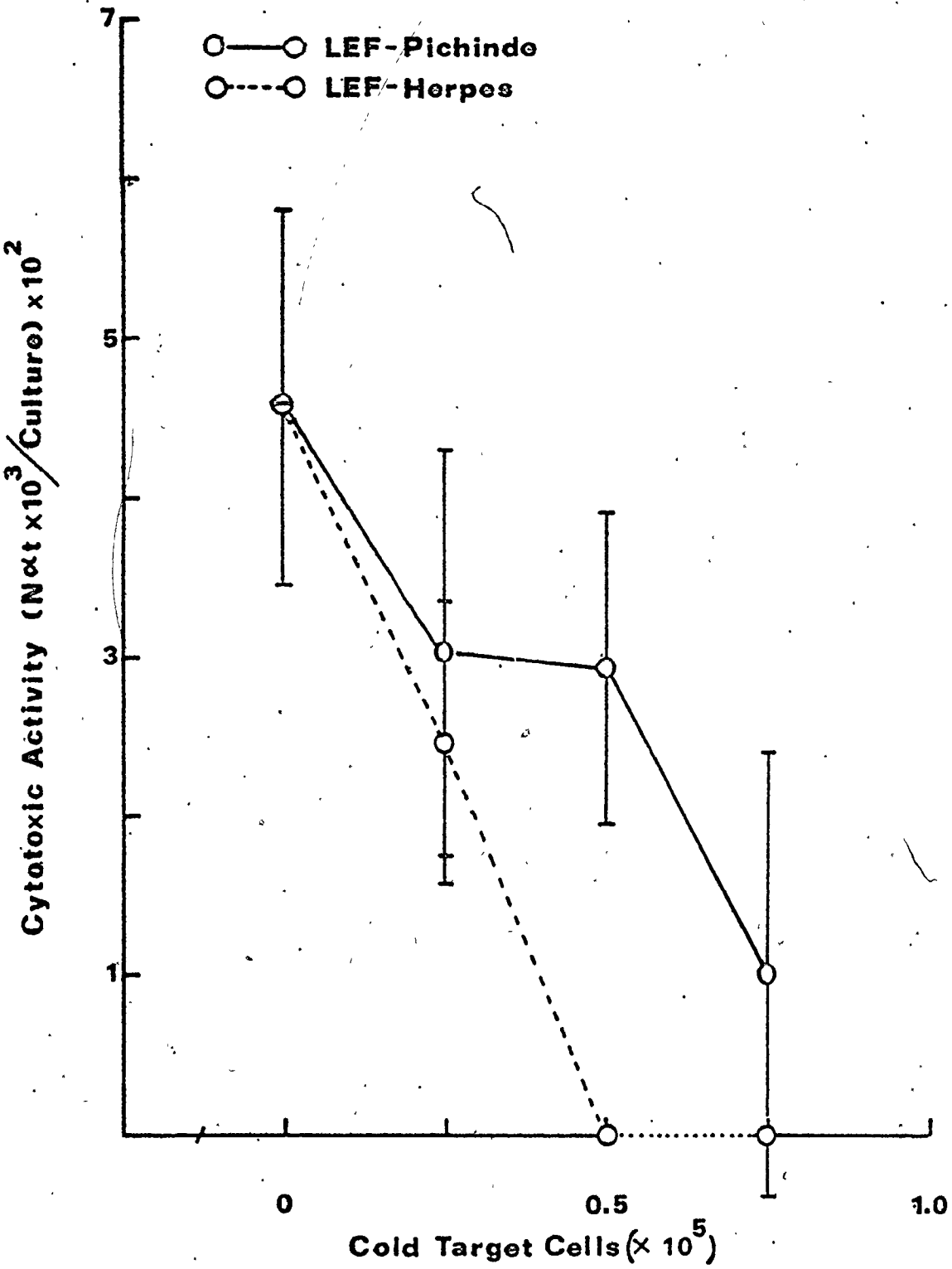

A
B
20
12
4
0
50
98
% Cold Targets
O—O LEF-Pichinde
O---O LEF-Herpes
Cytotoxic Activity (N∝t x 10^3/Culture) x 10^2

FIGURE 21

"Inhibition of cytotoxic activity of spleen cells from Pichinde virus-primed LSH hamsters". The cytotoxic activity of spleen cells from LSH hamsters that had been FP-inoculated with $2x10^3$ pfu of Pichinde virus was measured in a ^{51}Cr release assay after stimulation in vitro with UV-inactivated Pichinde virus. The cells were assayed against a fixed number (10^5 cells/ml) of ^{51}Cr-labelled (hot) Pichinde virus-infected LEF cells to which was added either unlabelled (cold) Pichinde virus-infected or herpes simplex virus-infected LEF cells. Each point represents the mean cytotoxic activity $\pm$ SEM of 4 replicate cultures.


LEF-Pichinde
LEF-Herpes
Cytotoxic Activity (N∝t x10³/Culture) x10²
7
5
3
1
0
0.5
1.0
Cold Target Cells (× 10⁵)

days after a FP-inoculation of Pichinde virus, appeared responsible for the lack of footpad swelling in this strain. To determine if the suppressor activity could also suppress another parameter of cell-mediated immunity, i.e. cytotoxic activity, spleen cells from MHA hamsters that had been FP-inoculated 7 days earlier, with Pichinde virus were mixed with spleen cells from unprimed LSH hamsters and the cytotoxic activity of the cell mixture was tested in a ^{51}Cr release assay after stimulation in vitro for 5 days with UV-inactivated, Pichinde virus-exposed peritoneal exudate cells from LSH hamsters.

In 3/3 experiments when Pichinde virus-primed MHA spleen cells were mixed in a 1:1 ratio with LSH spleen cells, a significant decrease in the cytotoxic activity of the LSH spleen cells was observed (Table 17). However, in only 1/2 experiments did Pichinde virus-primed LSH spleen cells show a similar effect. Therefore, the cell-mediated suppressor activity appeared to be expressed more consistently in the MHA hamster compared to the LSH strain.

3.5 Bone marrow transfer experiments

To try to determine the origin of the suppressor activity, LSH and F_1(LSHxMHA) hamsters were lethally irradiated and immediately reconstituted with bone marrow cells from either normal MHA or LSH donors. After 8 weeks, all survivors were FP-inoculated with $2x10^3$ pfu of Pichinde virus and footpad swelling was assessed 7 days later using the radioisotopic method. Two separate experiments were carried out and the data were pooled. Recipient hamsters were classified as having the LSH phenotype if their foot count ratios were within 1 standard deviation of the mean of irradiated hamsters that received LSH bone marrow cells.

Recipient hamsters with lesser degrees of footpad swelling were deemed to manifest the MHA phenotype. As shown in Table 18, the mean foot count ratio of the recipient hamsters classified as MHA was significantly different from that of the irradiated recipients of LSH bone marrow cells ($p<.0005$). These results suggested that the cell-mediated suppressor mechanism present in the MHA hamster strain was bone-marrow derived.

Table 17

In Vitro Suppression of Cytotoxic Activity of Uninfected LSH Hamsters

Cells Cultured[a]		Cytotoxic Activity[b] (Nαt x10^3/culture) Exp. 1	Exp. 2	Exp. 3[c]
LSH	$2.5x10^7$	721±137 (4)	506±149 (3)	354±87 (4)
LSH	$1.25x10^7$	702±166 (3)	492±72 (4)	N.D.
LSH + MHA-Pichinde	$1.25x10^7$ + $1.25x10^7$	268±72 (3)[d]	92±22 (2)[d]	127±55 (4)[e]
MHA-Pichinde	$1.25x10^7$	445±220 (3)	<SR	N.D.
MHA-Pichinde	$2.5x10^7$	N.D.	N.D.	210±53 (4)
LSH + LSH-Pichinde	$1.25x10^7$ + $1.25x10^7$	703±216	76±26	N.D.

N.D. No data

SR Spontaneous release

[a]Spleen cells from uninfected LSH hamsters were tested for the presence of cytotoxic activity in a ^{51}Cr release assay after stimulation in vitro with UV-inactivated Pichinde virus. In one case, spleen cells from MHA or LSH hamsters that had been FP-inoculated 7 days previously, with $2x10^3$ pfu of Pichinde virus were added to spleen cells from uninfected LSH hamsters prior to in vitro stimulation.

[b]Cells were assayed for cytotoxicity against ^{51}Cr-labelled LEF targets using an initial effector to target ratio of 100:1. Cytotoxic activity is expressed as the mean ± SEM of replicate cultures. The number of cultures is noted in parentheses.

[c]In experiment 3, spleen cells from LSH hamsters that had been FP-inoculated with $2x10^3$ pfu of Pichinde virus 7 days previously, were tested for cytotoxic activity.

Table 17 (Footnotes cont'd)

[d]The cytotoxic activity was significantly different from the activity of LSH spleen cells at a concentration of 1.25×10^7 ($p<.05$).

[e]The cytotoxic activity was significantly different from the activity if LSH spleen cells at a concentration of 2.5×10^7 ($p<.005$).

Table 18

Footpad Swelling Response of Bone Marrow Recipients

Background[a]	Bone Marrow	Number	Phenotype	Foot Count Ratio (Mean ± SD)
LSH	LSH	8		2.20±0.12
LSH	MHA	7	MHA	1.56±0.40
LSH	MHA	3	LSH	2.28±0.08
F_1(LSHxMHA)	MHA	10	MHA	1.41±0.36
F_1(LSHxMHA)	MHA	1	LSH	2.14

[a]Lethally irradiated LSH and F_1(LSHxMHA) hamsters were reconstituted with either normal LSH or MHA bone marrow cells. Eight weeks later, all survivors were inoculated with 2×10^3 pfu of Pichinde virus in the right hind footpad. On day 7, 10^6 cpm ^{125}I-HaSA was injected IP and 3 hr later, the hind feet were amputated and counted in a gamma counter. Recipient hamsters were then divided into phenotypic classes using the mean foot count ratio ± SD of the recipients of LSH bone marrow cells as a criteria. Means of the phenotype groups were then calculated.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

Pichinde virus causes a fatal infection in the inbred MHA strain of Syrian hamsters when injected intraperitoneally (Buchmeier and Rawls, 1977). Virus titres in the blood exceed 10^8 plaque-forming units per ml of serum and death appears to be caused by virus-induced necrosis of the cells of the reticuloendothelial system (Murphy et al., 1977). In contrast, the inbred LSH strain of Syrian hamsters survive an intraperitoneal injection of Pichinde virus and can limit viral replication.

During studies to determine the reason for the susceptibility of the MHA hamster to an intraperitoneal injection of Pichinde virus, it was discovered that the MHA hamster survived a footpad inoculation of the virus and was protected against a subsequent lethal intraperitoneal challenge with the virus. However, unlike the LSH strain hamster, the MHA hamster did not manifest a delayed hypersensitivity response to the footpad injection of virus. The current studies were initiated to determine some characteristics of footpad swelling and to establish the basis for the lack of swelling to a primary footpad inoculation of Pichinde virus in the MHA strain hamster.

4.1 Differences between hamster strains in footpad swelling response to viruses

LSH and LVG hamsters manifested an appreciable footpad swelling response that peaked 7 to 8 days after footpad injection of Pichinde virus (Figures 3A and 3B). Histological examination of the virus-injected feet revealed the presence of a dense mononuclear cell infiltrate (Figure 8). In contrast, no swelling was detected in the MHA strain

hamster at this time (Figures 3A and 3B) and the histological composition of the virus-injected foot was similar to the uninjected control foot (Figure 13).

Footpad swelling after intracutaneous injection of antigen in the footpad was devised by Gray and Jennings (1955) as an indicator of cell-mediated immunity in vivo and is currently used more widely than any other kind of test for detecting delayed-type hypersensitivity reactions in mice. For example, the footpad test has been employed to measure delayed hypersensitivity responses to several viruses, including, lymphocytic choriomeningitis virus (Tosolini and Mims, 1971), influenza virus (Webster and Hinshaw, 1977), rabies virus (Lagrange et al., 1978), St. Louis Encephalitis virus (Hudson, Wolff and DeMartini, 1979) and Semliki Forest virus (Kraaijeveld, Harmsen and Boutahar-Trouw, 1979).

In order for footpad swelling to occur in response to injection of antigen in the footpad, immune recognition of antigen, generation of effector mechanisms and factors that regulate the immune response are required. It follows, then, that the incapacity of the MHA hamster to develop footpad swelling after footpad injection of Pichinde virus could be due to the strain's inability to recognize Pichinde virus antigens. Alternatively, in the footpad, Pichinde virus may present itself as a weak immunogen, inadequate for development of delayed hypersensitivity. However, the observation that Pichinde virus replicated equally well in the footpad of both MHA and LSH strain hamsters (Figure 4) and that complement-fixing antibody titres to Pichinde virus produced by both strains were comparable, argues that Pichinde virus was a sufficient immunogen and that recognition of Pichinde virus antigens had occurred.

When two other viruses, vesicular stomatitis virus and vaccinia virus were used to test footpad swelling in the MHA hamster, it was found that this strain responded as well as the LSH strain. Footpad swelling was maximal 5 to 6 days after injection (Figure 5 and Figure 6) and a mononuclear cell infiltrate was present in all virus-injected feet (Figure 9, Figure 10, Figure 11 and Figure 12). Furthermore, when mixtures of vaccinia virus and Pichinde virus were injected into the footpad of MHA hamsters, a footpad swelling response accompanied by a cell infiltrate occurred in all MHA hamsters that received some combination of the two viruses (Figure 7, Figure 14, Figure 15 and Figure 16). Only MHA hamsters that received Pichinde virus alone did not manifest a footpad swelling response (Figure 7). Thus, the failure of the MHA hamster to respond to Pichinde virus was unlikely to be caused by a general defect in effector mechanisms responsible for the components of the footpad swelling response. Instead, the MHA unresponsive state appeared to be Pichinde virus-restricted and the mechanism by which Pichinde virus induced unresponsiveness could not nonspecifically suppress footpad swelling in the MHA hamster in response to other viruses.

4.2 Genetic control of footpad swelling to Pichinde virus

The genetic studies of F_1 (LSH X MHA) and backcross progeny indicated that the footpad swelling response was under genetic control. Footpad swelling was a dominant phenotype and the footpad swelling responses of the backcross progeny (F_1 X MHA) were consistent with results expected if a single gene or closely linked genes controlled this trait (Table 4). Similarly, the capacity to limit Pichinde virus replication in the popliteal lymph node was shown to be under genetic control.

The ability to limit virus titres in the node was a dominant trait and virus titres observed in the backcross progeny were also consistent with results expected if control of this phenotype was governed by a single gene or closely linked genes (Table 5). However, this gene did not appear to be the same gene that controlled footpad swelling response since the backcross progeny that showed the LSH footpad swelling phenotype and the MHA footpad swelling phenotype, each segregated into 2 groups (Table 5). Furthermore, no linkage by sex for either the footpad swelling or viral replication phenotypes was apparent. From these genetic studies, therefore, it appeared that two independently segregating autosomal dominant genes controlled footpad swelling response and Pichinde virus replication in the popliteal lymph node.

4.3 Mechanism(s) underlying the failure of the MHA hamster to respond to a footpad injection of Pichinde virus

After establishing that footpad swelling was under genetic control and that lack of footpad swelling in the MHA hamster was Pichinde virus-restricted, studies were undertaken to determine the reason for this unresponsiveness. The observation that footpad-inoculated MHA hamsters produced high titres of complement-fixing antibodies to Pichinde virus (Table 1) and that these hamsters were protected against a subsequent lethal challenge of virus (Gee et al., 1981b) demonstrated that lack of footpad swelling was not due to a lack of immune recognition of Pichinde virus antigens.

Suppressor cells have been implicated in the inhibition of delayed-type hypersensitivity reactions after virus injection. Liew and Russell (1980) found that antigen-specific suppressor cells of the

Lyt-1$^+$ phenotype were generated when mice were infected by aerosol with influenza virus. Similarly, antigen-specific suppressor cells for delayed hypersensitivity were produced in mice injected intravenously with UV-inactivated reovirus (Greene and Weiner, 1980). These cells were shown to be T cells since their function could be abrogated by treatment with anti-Thy 1.2 antiserum and complement. Thus, studies were begun to determine if footpad injection of Pichinde virus in the MHA hamster induced the generation of suppressor activity that was subsequently responsible for the lack of footpad swelling in this strain.

Cyclophosphamide has been shown to enhance delayed-type hypersensitivity responses in mice to influenza virus (Leung and Ada, 1980b) and to Semliki Forest virus (Kraaijeveld, Harmsen and Boutahar-Trouw, 1979). In addition, Maguire (1980) found that treatment of MHA hamsters with cyclophosphamide augmented the allergic contact dermatitis response of this strain to the hapten, dinitrochlorobenzene. My data have shown that cyclophosphamide treatment of MHA hamsters 2 days before Pichinde virus injection into the footpad resulted in a significant swelling response (Figure 17A).

The target of cyclophosphamide immunopotentiation has been reported in mice to be a suppressor cell population (Askenase, Hayden and Gershon, 1975; Lando, Teitelbaum and Arnon, 1979). In fact, Diamantstein and colleagues (1981), using a derivative of cyclophosphamide *in vitro* that reportedly mimiced the drug's action *in vivo* suggested that cyclophosphamide enhancement of delayed-type hypersensitivity to sheep red blood cells in mice was due to the selective inhibition of precursors of suppressor T cells. Thus, the observation that cyclophosphamide restored footpad swelling in the MHA hamster suggested that a suppressor mechanism

could be present in the MHA strain.

The discrepancy observed in the footpad swelling response of the MHA hamster, as measured by the two methods, after cyclophosphamide treatment (Figures 17A and 17B) was somewhat surprising. However, consideration of the components of the footpad swelling response could account for this disparity. In murine delayed-type hypersensitivity reactions, mast cell degranulation with the release of the vasoactive amine, serotonin was found to occur after challenge with antigen (Askenase et al., 1980). This appeared to be a T cell-dependent mechanism, since nylon-wool nonadherent, non-immunoglobulin-bearing lymphocytes were shown to transfer to recipients the capacity for mast cell activation. As a result of serotonin release, an increase in vascular permeability was observed which allowed the infiltration of cells from the blood to the site of antigen injection. These cells, predominantly comprised of macrophages and their precursors, were principally responsible for the swelling and induration of the hypersensitivity reaction. Therefore, manifestation of footpad swelling appeared to involve not only the infiltration of specifically sensitized T cells to the site of antigen injection but also the activation of local mast cells leading to an increase in vascular permeability.

Treatment of MHA hamsters with cyclophosphamide resulted in a footpad swelling response by this strain to Pichinde virus. Since this response was detected only by the micrometer measurements and not by the isotopic method, this suggested that an increase in vascular permeability was not associated with the swelling response. One explanation could be that Pichinde virus interfered with mast cell function or its activation by sensitized T cells. Alternatively,

Pichinde virus could have interfered with the function of the cells that migrate into the area following serotonin release. One of the major targets of Pichinde virus replication has been found to be the macrophage (Murphy et al., 1977). Since Pichinde virus has been shown to replicate in the MHA footpad (Figure 4), it is possible that one of the cell populations that supported Pichinde virus replication was involved in the swelling and induration aspect of the footpad reaction. Futhermore, histological examination of footpads of cyclophosphamide-treated MHA hamsters showed that a dense mononuclear cell infiltrate did occur in the virus-injected foot (Figure 18). Therefore, it appeared that MHA hamsters could respond with footpad swelling to a footpad injection of Pichinde virus after cyclophosphamide treatment. In addition, it was of interest to observe that treatment of LSH hamsters with cyclophosphamide resulted in an augmented footpad swelling response (Figure 17A). Thus cyclophosphamide seemed to enhance the cellular component of the delayed-type hypersensitivity reaction, possibly by blocking a suppressor mechanism that was present in both MHA and LSH strain hamsters.

A second approach that was used to demonstrate the presence of a suppressor mechanism in the MHA hamster was to study the effect of transfer of MHA immune cells on the footpad swelling response of LSH recipients. The observation that lymphoid cells but not immune sera from footpad-inoculated MHA hamsters had the capacity to suppress footpad swelling in LSH recipients (Table 6 and Table 10) constituted strong evidence in favour of a cell-associated suppressor mechanism. Cell concentration and the time interval between sensitization of the donor and transfer of the cells into the recipient were also found to be critical for demonstrating suppression. Suppression of footpad

swelling did not occur if fewer than $5x10^7$ cells were transferred (Table 7) and suppression was maximal when cells were taken from MHA hamsters that had been footpad-inoculated 5 days beforehand (Table 8).

Furthermore, the site where the initial suppressor induction event takes place appeared important in determining suppressor activation. Suppression of footpad swelling was not detected in LSH recipients of spleen cells from MHA hamsters that had been injected with Pichinde virus intraperitoneally rather than subcutaneously in the footpad (Table 9). In the murine system, route of inoculation of antigen has been found to be a major determinant in establishing whether a protective immune response ensues or active suppression occurs. The intravenous route of inoculation for example, has been shown to promote suppressor cell induction (Bach et al., 1978; Sherr, Benacerraf and Dorf, 1980). As suggested by Gershon (1980), a heterogeneous population of antigen-presenting cells might exist in the immune system, separate ones for separate circuits, such that antigen presented on one type of antigen-presenting cell may result in a qualitatively different response than if the antigen were presented on another type of antigen-presenting cell. It is quite conceivable then, that Pichinde virus encounters a different population of antigen-presenting cells when injected into the footpad as compared to the intraperitoneal route and that antigen-presenting cells in the footpad are part of a suppressor circuit.

The observation that LSH hamsters manifest an enhanced footpad swelling response after cyclophosphamide treatment suggests that LSH hamsters also possess a cell-associated suppressor mechanism. The presence of this suppressor mechanism was further demonstrated in the

cell transfer experiments. The footpad swelling responses of recipients of LSH sensitized spleen cells were found to be significantly less than the responses of recipients of LSH nonimmune cells (Table 6). However, the degree of suppression was more pronounced in recipients of cells from footpad-sensitized MHA hamsters (Table 6).

Characterization of the cell-mediated suppressor mechanism revealed that its action was Pichinde virus-specific. Suppression of footpad swelling was not observed in recipients of MHA sensitized cells that were challenged in the footpad with vesicular stomatitis virus. Only those recipients of MHA immune cells that were footpad-inoculated with Pichinde virus displayed depressed footpad swelling as compared to controls (Table 11). Furthermore, the cell-mediated suppressor mechanism appeared to be bone-marrow derived. Of the 10 lethally irradiated LSH recipients of MHA bone marrow cells, 7 had significantly reduced footpad swelling as compared to recipients of LSH bone marrow cells. Similarly, of the 11 F_1 (LSH X MHA) recipients of MHA bone marrow cells, 10 had significant reductions in footpad swelling.

These observations, therefore, support the concept that a Pichinde virus-specific cell-associated suppressor mechanism is responsible for the failure of the MHA hamster to manifest footpad swelling in response to a primary footpad-inoculation of Pichinde virus. Furthermore, the suppressor mechanism appears to be enhanced in this strain compared to the suppressor activity observed in the LSH strain hamster. Thus, it is possible that Pichinde virus preferentially enhances a suppressor mechanism in the MHA hamster, such that generation and expression of the effector mechansims involved in delayed hypersensitivty reactions are inhibited.

Streilein and colleagues (1981) have shown that hamsters can be rendered unresponsive to the haptens, trinitrochlorobenzene (TNCB) and dinitrofluorobenzene (DNFB) by, as has been demonstrated in the mouse, intravenous injection of either syngeneic cells derivatized with trinitrobenzene sulfonate or the soluble salt, dinitrofluorobenzene sulfonate. A cell-mediated suppressor mechanism was also found to be responsible for this unresponsiveness. However, the cells responsible for the suppression was unknown. Mediation of suppression by T cells appeared unlikely since pretreatment of lymphoid cells from unresponsive hamsters with goat anti-hamster thymocyte serum and complement did not abolish the ability of the cells to render recipients unresponsive in cell transfer experiments.

The manner in which Pichinde virus induced the production of suppressor cells is unknown. However, it could be perceived as either through a direct interaction with (i.e. replicating within) precursor T cells or through a mediator or inducer cell. In the latter case, Pichinde virus upon entry into the footpad, would encounter an antigen-presenting cell, probably of macrophage lineage. Through a series of events, the virus would be presented on the surface of the cell at which time an inducer cell would be recruited into the area. The inducer subsequently would relay signals to precursor T cells which have the potential to differentiate into different virus-specific T effector cells. These cells could include suppressor cells, cytotoxic cells and cells involved in delayed-type hypersensitivity reactions. However, when the immune system encounters Pichinde virus, precursor T cells could be activated in such a way that more suppressor cells are produced than other effector cell types. Consequently, an insufficient number of

virus-specific effectors of the delayed hypersensitivity response are generated to manifest an adequate hypersensitivity response. Futhermore, the suppressor cells might function in a feedback mechanism, such as has been described in the humoral response to sheep red blood cells (Eardley et al., 1978), in which the suppressor cells interfere with the further induction and proliferation of effectors of the hypersensitivity response.

In the murine system, suppressor cells have been classified into two basic groups (Asherson et al., 1980). Afferent suppressors are cells that suppress the induction of an immune reaction and efferent suppressors are those that interfere with the expression of the response. The major characteristic that appears to distinguish the two subsets is the sensitivity of the precursors of the afferent suppressor cells to cyclophosphamide. However, Gill and Liew (1978) in their study of delayed hypersensitivity to sheep red blood cells found that the precursors of the efferent suppressor cells were sensitive to the drug. In the hamster, it appears that Pichinde virus induces the production of an afferent suppressor mechanism in the MHA strain. This mechanism is sensitive to cyclophosphamide. In transfer experiments, only cells given within 24 hr of challenge could suppress footpad swelling in recipients 8 days later. Alternatively, since LSH hamsters appear also to possess a suppressor mechanism, it is possible that in the cell transfer experiments, LSH recipients are not receiving cells directly responsible for suppression but rather a population of cells that can induce a suppressor mechanism in the recipients, themselves.

It is unlikely that antibody is a mediator of suppression in the MHA strain either through an anti-idiotypic antibody mechanism or through a suppressor cell inductive process. Complement-fixing antibody titres were very low 8 days after a footpad injection (Table 1) and sera from footpad-inoculated MHA donors failed to suppress the 8 day footpad swelling response by LSH recipients (Table 10).

Thus, the data comply with the hypothesis that Pichinde virus induces the production of a virus-specific, cyclophosphamide-sensitive cell-mediated suppressor mechanism that suppresses the induction of a footpad swelling response in the MHA strain hamster following a footpad inoculation of Pichinde virus.

4.4 In vitro generation of cytotoxic cells

In addition to studying delayed-type hypersensitivity reactions in MHA and LSH strain hamsters, another parameter of cell-mediated immunity, the in vitro generation of cytotoxic cells was examined. In earlier studies, Gee, Clark and Rawls (1979) showed that spleen cells from hamsters infected with Pichinde virus lysed syngeneic and allogeneic tumour cells to the same degree and infection of the target cells with Pichinde virus did not increase their susceptibility to lysis. Therefore, it appeared that the cytotoxic activity of the spleen cells was not attributable to classic T lymphocyte-mediated lysis.

Since the presence of cytotoxic T lymphocytes could not be demonstrated in hamsters after primary exposure to viral antigen in vivo, an alternative approach to detect their presence by a second exposure to antigen in vitro was made. This approach was successful in that cytotoxic activity was generated and found to be maximal when the effector cells were incubated in vitro both in the presence of peritoneal

exudate cells and virus (Table 12 and Table 13). The presence of accessory cells, such as peritoneal exudate cells has been found to be an essential requirement, in the mouse, to generate virus-specific cytotoxic T cells to Sendai virus (Koszinowski and Gething, 1980) and to herpes simplex virus (Rouse and Lawman, 1980). The role of adherent cells was likely to provide a lymphocyte-activating factor (interleukin 1) that was necessary for the subsequent generation of cytotoxic effector cells.

However, despite the successful generation of cytotoxic activity of spleen cells after antigenic restimulation in vitro, the pattern of killing remained the same. Spleen and mesenteric lymph node cells from MHA and LSH hamsters that had been primed in vivo and restimulated in vitro with either Pichinde virus or herpes simplex virus killed Pichinde virus-infected, herpes simplex virus-infected and uninfected LEF target cells to the same degree (Table 14 and Table 15). This observation was further substantiated in cold target inhibition experiments. Cytotoxic activity of spleen cells from Pichinde virus-primed LSH hamsters decreased to a greater extent when unlabelled herpes simplex virus-infected target cells were used in competition experiments than when unlabelled Pichinde virus-infected target cells were used (Figure 20 and Figure 21). These observations are in keeping with the earlier conclusion of Gee, Clark and Rawls (1979) that antigen-specific cytotoxic T lymphocytes were not responsible for the cytotoxic activity generated in hamsters that were infected with either Pichinde virus or herpes simplex virus.

Several other investigators have also reported that hamsters do not develop cytotoxic T cells in response to acute viral infections.

Instead, natural killer (NK) cells appear to predominate as the major cytotoxic effector cell in hamsters. Yang and colleagues (submitted for publication) found that spleen cells from vaccinia virus-infected hamsters produced substantial levels of nonspecific cytotoxic activity and possessed characteristics of NK cells. Using a monoclonal anti-Thy 1.2 murine antiserum that detects a homologous Thy 1.2 antigenic determinant on hamster T cells, Yang and Tompkins (1982), showed that pretreatment of the splenic effector cells with this antiserum plus complement did not decrease the levels of cytotoxicity significantly on the target cells tested. Nelles, Duncan and Streilein (1981) also reported that the cytotoxic activity generated in vaccinia virus-infected hamsters was not mediated by cytotoxic T cells. However, these investigators suggested that the major cell-mediated cytotoxic mechanism operating in the vaccinia virus-infected hamsters was an antibody-dependent cell-mediated mechanism. Cytotoxic activity of the infected spleen cells was inhibited by pretreating the cells with heat-aggregated human gamma globulin. In contrast, Henderson (1979) and Kimmel, Wyde and Glezen (1982) reported that virus-specific cytotoxic T lymphocytes were generated in MHA and LSH strain hamsters infected with parainfluenza virus. Treatment of the effector cells with adsorbed goat anti-hamster thymus antiserum and complement or rabbit anti-hamster brain antiserum and complement significantly reduced their ability to lyse virus-infected target cells. However, unlike murine cytotoxic T lymphocytes, these virus-specific killer cells did not display major histocompatibility-restricted killing. The effector cells were capable of lysing different hamster cell lines infected with parainfluenza virus

but could not lyse xenogeneic L929 mouse fibroblasts.

Thus, it is possible that the cytotoxic effector cells that were generated in my in vitro system were NK cells. NK cells have been previously described in hamsters infected with Pichinde virus (Gee, Clark and Rawls, 1979). However, whether the NK cell that was produced after restimulation with Pichinde virus in vitro is the same killer cell that was present in the hamsters after Pichinde virus priming in vivo is not known. Fan, Yang and Tompkins (1982) found, in their system that unlike the in vivo NK cell, the in vitro NK cell generated in hamsters was sensitive to the murine monoclonal anti-Thy 1.2 antiserum plus complement.. Further characterization of the nonspecific killer cell cell generated in my in vitro system is required.

Chapes and colleagues (1981) showed, in the hamster, that bone marrow cells, presumably containing NK cell precursors, displayed enhanced cytotoxic activity when cocultured with immune macrophages. As previously mentioned, maximal cytotoxic activity in my system was obtained when the effector cells were incubated in vitro in the presence of peritoneal exudate cells that had previously been exposed to UV-inactivated virus (Table 12 and Table 13).

The cytotoxic effector cell that was generated from normal hamsters after primary stimulation with Pichinde virus in vitro was somewhat different from the effector cell produced from in vivo virus-primed, in vitro restimulated hamsters. Although comparable levels of cytotoxicity were obtained, the cytotoxic response of the unprimed hamsters showed a trend for some degree of specific killing. Pichinde virus-infected target cells were lysed to a greater degree than either

herpes simplex virus-infected or uninfected target cells (Table 16). Nevertheless, more lysis occurred on herpes simplex virus-infected than uninfected target cells. Since hamster NK cells have been shown to be preferentially cytotoxic for virus-infected target cells (Yang et al., submitted for publication; Lausch, Patton and Walker, 1981), it is possible that two populations of cytotoxic effector cells, an NK cell population and a virus-specific killer cell population were generated from unprimed hamsters after primary stimulation with Pichinde virus in vitro.

Alternatively, the nonspecific cytotoxic effector cell could be a macrophage. Chapes and Tompkins (1979) have described in vaccinia virus-immune hamsters, a population of adherent peritoneal exudate cells that were equally cytotoxic for vaccinia virus-infected and herpes simplex virus-infected target cells.

4.5 In vitro suppression of cytotoxic activity

Since a virus-specific cell-mediated suppressor mechanism appeared to be responsible for the inability of the MHA strain hamster to manifest a footpad swelling response to Pichinde virus, studies were undertaken to determine if the activity of the suppressor mechanism could extend to another parameter of cell-mediated immunity, the in vitro generation of cytotoxic activity. The observation that spleen cells from 7 day Pichinde virus-primed MHA hamsters depressed the cytotoxic activity of spleen cells from unprimed LSH hamsters indicated that a suppressor mechanism was present in the MHA hasmter that could suppress cytotoxic activity. However, whether the suppressor mechanism responsible for the absence of footpad swelling in the MHA hamster is

the same mechanism that suppressed the cytotoxic activity generated from unprimed LSH hamsters is not known.

Leung and Ada (1980c) reported that two T cell populations mediated the delayed-type hypersensitivity response to murine influenza virus infection. Using infectious influenza virus to sensitize and footpad challenge mice, these authors found that an I-region restricted Lyt-1$^+$ cell population and a K,D-region restricted Lyt-23$^+$ cell population, which was cytotoxically active were generated. Ertl (1981) also discovered that two different subsets of T lymphocytes were induced in the hypersensitivity reaction to Sendai virus. Similarly, an I-region restricted Lyt-1$^+$ cell population and a K,D-region restricted Lyt-23$^+$ were described. Thus, it is possible that two populations of effector cells, one with cytotoxic activity participate in the footpad swelling response to Pichinde virus. If so, then, suppression of footpad swelling in the MHA hamster and in vitro suppression of cytotoxic activity generated from unprimed LSH hamsters by spleens from Pichinde virus-primed MHA animals could be mediated by the same cell-associated suppressor mechanism. Further work is required to answer this question.

The suggestive observation that spleen cells from 7 day Pichinde virus-primed LSH hamsters suppressed the cytotoxic activity generated from unprimed LSH hamsters (Table 16) is in agreement with the concept that Pichinde virus-primed LSH hamsters also develop a cell-associated suppressor mechanism. However, as previously mentioned, the suppressor activity associated with the LSH strain is substantially less than in the MHA hamster after Pichinde virus injection. Thus, the difference between the two strains of hamsters may be quantitative rather than qualitative.

4.6 Statement of model and proposals for future work

The data that have been presented are consistent with the concept that a virus-specific, cell-associated suppressor mechanism was responsible for the failure of the MHA strain hamster to respond to a footpad-inoculation of Pichinde virus. It is possible that Pichinde virus preferentially enhances a suppressor mechanism resulting in increased suppressor activity in the MHA strain. However, the mechanism by which Pichinde virus generates suppressor activity is not known. Casali and colleagues (1981) have reported that glycoproteins from measles virus and from lymphocytic choriomeningitis virus, when inserted into artifical membranes or in soluble form could induce significant nonspecific cell-mediated cytotoxic activity in peripheral blood lymphocytes. Since Pichinde virus has been shown to replicate in macrophages (Buchmeier and Rawls, 1977), it is possible that the virus replicates in the antigen-presenting cells present in the footpad with the subsequent expression of the viral glycoproteins on the surface of the infected cells. As a result, this glycoprotein and cell surface antigen complex might be recognized specifically by cells involved in the induction of the suppressor circuit, leading to an increase in effectors involved in suppression.

A suppressor mechanism that could inhibit cytotoxic activity generated from unprimed LSH hamsters after primary stimulation with Pichinde virus *in vitro* was also found in the MHA hamster. Whether it is the same suppressor mechanism responsible for the lack of footpad swelling in the MHA hamster is not known at this time. However, if it were the same mechanism, then one would expect to observe a decrease in

footpad swelling in LSH recipients that receive spleen cells from 7 day Pichinde virus-primed MHA hamsters, that were incubated *in vitro* in the presence of peritoneal exudate cells and Pichinde virus.

Although the suppressor mechanism has been shown to be cell-associated, it is not known which cell populations are involved. Using the available cell separation techniques for hamster cells, it should be possible to determine which cell population is responsible for the suppression of footpad swelling.

The nonspecific cytotoxic effector cell generated after stimulation with Pichinde virus *in vitro* requires further characterization. It would be of interest to know if it is the same NK cell that is present in normal, uninfected hamsters. The NK cell appears to be a target cell for Pichinde virus replication in the MHA hamster and may be responsible for the death of this strain hamster to an intraperitoneal injection of the virus (Gee, Clark and Rawls, 1977). However, when Pichinde virus is injected by the footpad route, MHA hamsters survive (Gee *et al.*, 1981b). Since a footpad-injection of Pichinde virus also induces the development of a cell-associated suppressor mechanism, it is possible that the suppressor mechanism contributes to the survival of the MHA hamster by suppressing the putative target cell.

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