To the Graduate Council:

I am submitting herewith a thesis written by Carina E. Elfström Walder entitled "Peritoneal Macrophage Barrier to the Spread of Semliki Forest Virus in Mice." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

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PERITONEAL MACROPHAGE BARRIER TO THE SPREAD OF SEMLIKI FOREST VIRUS IN MICE

A Thesis

Presented for the

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Degree

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Carina E. Elfström Walder

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DEDICATIONS

To my mom and dad,

and to my husband, Tony.

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ABSTRACT

The intraperitoneal route for infection of mice with SFV was several thousand fold less efficient than the footpad, intravenous or intracerebral routes. Peritoneal resistance could be demonstrated in three strains of mice (C_3H/Ten, ICR, and C_3H/Hen), and increased with increasing age. Anti-viral factor(s) could not be found in the peritoneal washings of normal mice, as determined by pretreatment of the virus or L929 cells, or by inclusion of the washings in growth medium or agar overlays of infected cells throughout infection. In addition, anti-viral factor(s) were not produced by normal peritoneal cells **in vitro**. Very low amounts of IFN α/β could be detected in the peritoneal cavity of normal mice, which apparently increased slightly after viral infection. To determine the importance of the IFN for disease outcome in infected mice, anti-IFN α/β was administered intraperitoneally before SFV challenge. This treatment marginally reduced the mortality of the mice, and apparently played little role in the peritoneal resistance to SFV infection.

We next concentrated on the macrophages. LPS, which activates macrophages and induces IFN production (8), reduced the mortality of mice to SFV challenge (about 40%)

whereas thioglycollate did not. Silica-treatment to destroy had little, if any effect on the survival of mice to SFV infection, and peritoneal washings performed immediately before viral challenge did not remove the peritoneal barrier. Protein A, which stimulates lymphocyte proliferation (70), also had little or no effect on extent of mortality.

A very low percentage (<2%) of peritoneal macrophages could, by immunofluorescence, be shown to contain viral antigens after **in vivo** and **in vitro** infection with SFV. The antigens were, however, only seen on or below the plasma membrane as a thin rind. The fraction of cells containing viral antigens could not be increased by increasing the multiplicity of infection. The presence of viral antigens did not increase per cell or in the number of cells beyond what was seen immediately after exposure to virus, even after four days in culture. Furthermore, the macrophages that were initially resistant to the uptake of SFV remained so throughout the period of time tested (4 days). Both UV-inactivated and live virus appear to be taken up equally well by a small number of macrophages. Live virus could be detected by plaque assays of the culture supernanant fluids from "infected" macrophages, even though in very low titers, which decreased during the time of infection through three days. On the fourth day after infection, however, an increased titer of live virus, even though still low, (from

0.001/cell to 0.1/cell) could be detected. In addition, on the fourth day viral antigens were no longer detected in the macrophages by immunofluorescence. Furthermore, cytopathic effects were never seen in cultures of macrophages exposed to SFV.

In contrast to the well-known accepted systemic hematogenous route of spread to the CNS from the natural (blood vessel) and various experimental routes of inoculation, SFV was found to traverse the sciatic nerve from the site of footpad infection.

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LIST OF ABBREVIATIONS

CEF	chicken embryo fibroblasts
cpe	cytopathic effect
CTL	cytotoxic T lymphocytes
EEE	Eastere equine encephalitis
FBS	fetal bovine serum
FITC	fluorescein iso-thiocyanate
FP	footpad
HBSS	Hank's balanced salt solution
HSV	herpes simplex virus
IC	intracerebral
IFN	interferon
IgG	immunoglobulin G
IN	intranasal
IP	intraperitoneal
IV	intravenous
MEM	modified Eagle's medium
MHV	mouse hepatatis virus
MOI	multiplicity of infection
PBS	phosphate buffered saline
pfu	plaque forming units
PI	post infection
P-S	Penicillin-Streptomycin

RPMI	RPMI 1640 growth medium
SFV	Semliki Forest virus
VSV	vesicular stomatitis virus
WEE	western equine encephalitis

CHAPTER I

INTRODUCTION

In the natural setting, Semliki Forest virus (SFV) is believed to cause disease in susceptible hosts after transmission from infected mosquitoes (9). In humans, a wide variation in the disease outcome has been observed, ranging from encephalitis to fevers. A few cases of known human encephalitis appear to have resulted from laboratory exposure rather than from a natural infectious process (78).

In animals, the natural hosts are rodents or birds (9). A wide variability in the outcome of disease in animals can be produced in the laboratory, where the animal most often utilized has been the mouse (73). Interestingly, the wide variabilities in outcome (e.g., death by encephalitis or low grade fever) can be easily manipulated by choosing a particular route of infection (5),(7),(19),(50),(52),(64),(74),(80). For example, in order to kill half of the animals injected, Bradish et al. (1971) used 100 plaque forming units (pfu) when the virus was injected intraperitoneally (IP) into mice of 15-20 days in age, whereas only 1-3 pfu were required if the virus were injected intracerebrally (IC) (7). Furthermore, it has been reported that the infective dose required for an intravenous (IV) route is 100 times less than that required for a subcutaneous (SC) route of injection (50).

A wide range of doses necessary to produce encephalitis has also been reported to correlate with differences in the age or weight of the mice used (50),(74). For example, 3-6 day old suckling mice are equally susceptible to IP and IC infections, whereas weanling mice become more resistant to IP inoculation (50).

The variability in disease outcome that depends on the route of injection has also been observed with other viruses. For example, in 1941, Sabin suggested that pre-existing barriers to viral spread exist in mice, since Vesicular Stomatitis virus (VSV) infections always arrest at the same site (68). Mims suggested that macrophages may be critical for the outcome of viral infections, since these cells monitor most body tissues and compartments (50).

It would be logical to assume that macrophages play a role in determining the outcome in viral infections initiated by various routes, since different regions of the body contain different populations of macrophages (11). Indeed, it has been reported that the IP 50% infective dose could be decreased from 250 pfu to less than 20 pfu if the mice were pre-treated with Myocrisin or l-asparaginase, both of which impair macrophage function (6).

The main purpose of this thesis is to explain the observation that the dose of virus required to kill half of the animals after an IP infection is several thousand-fold higher than the dose required when infection is initiated

intracerebrally, intravenously or in the footpad. The basic question asked was: What is the protective barrier for the mouse in the peritoneal cavity?

CHAPTER II

AN HISTORICAL OVERVIEW

The Semliki Forest Virus

Classification

Semliki Forest virus (SFV) is an enveloped, positive-stranded RNA virus of the family Togaviridae (72),(77). It was first isolated in 1944, from a pool of 130 female Aedes abnormalis Theobald mosquitoes, in Uganda (84). Within this family of viruses, SFV belongs to the genus alphavirus. Among the 26 viruses and subtypes within this genus, all except one can be transmitted by mosquitoes. Owing to this, alphaviruses were earlier known as arthropod-borne viruses or arboviruses. Once the arthropod has become infected from a blood meal, it remains so for life, without signs of disease (77),(79).

The classification of the Togaviridae into genera is based on serological cross-reactivity (62) primarily in hemagglutination inhibition tests. The alphavirus genus is divided into three groups in which there is serological cross-neutralization of viruses within a group but not between groups (62).

Structure

The single strand of 42S RNA contained within the SFV is of positive sense, and is closely associated with the

nucleocapsid protein, in an isometric structure. A lipid envelope surrounds the capsid after budding of the virus from the host cell plasma, or cytoplasmic, membrane. Three virus glycoproteins, E1, E2 and E3, form spikes on the outer surface in an icosahedral array of 240 trimer clusters in which two of the glycoproteins, E1 and E2 cross the lipid bilayer and are attached to the capsid protein by electrostatic interaction (77),(81).

SFV in Natural Diseases

SFV and Human Disease

Although many of the Togaviruses can cause diseases such as encephalitis, arthritis, fevers and rashes in the human, SFV is not known to cause encephalitis under natural conditions. A few cases of SFV-induced diseases have been reported from various laboratory settings (78). For example, in 1978, one investigator was reported to have died from an SFV infection. In this instance, the virus had been aerosolized, and the apparent infection occurred via the intranasal (IN) route (94). Another case of a less severe infection was reported by Shope to cause the production of cross-reactive antibody, that aborted a later infection with the related Eastern Equine Encephalitis (EEE) virus (78).

SFV and Animal Diseases

SFV is endemic in Uganda and East Africa. In these tropical areas, SFV is believed to be maintained in a zootic cycle involving Aedes abnormalis mosquitoes, as the vector, and birds and rodents, acting as carrier-hosts. A mosquito cannot be infected unless a blood meal contains a minimum threshold dose of infectious virus. When the mosquito does become infected, the incubation period varies from a few days to weeks. During this period, the virus grows in stomach cells and then infects the salivary glands. The cycle is completed when the mosquito bites a susceptible host, passing virus-containing saliva into the wound (9). Little more is known about the natural infections caused by SFV, although much is known about artificially induced laboratory infections.

SFV Infection in Laboratory Animals

Course of Infection

Unlike the natural infection of SFV transmitted by mosquitoes in which the virus is injected into the blood of host animals (50), laboratory animals can be artificially infected by various routes. Depending on which route is used, susceptibility of the animal has been shown to vary widely (5),(7),(19),(50),(52),(64),(74),(80). For example, it has been shown in the mouse that the intravenous (IV) infectious dose is 100 times less than the subcutaneous (SC)

dose (50). It has also been shown that intraperitoneal (IP) injections of virus are relatively ineffective in causing encephalitis in the mouse. Bradish et al. (1971) reported that the 50% lethal dose (LD_{50}) for an IP injection in mice, which were 15-20 days of age, was 100 plaque forming units (pfu), whereas the dose for an intracerebral (IC) injection was only 1-3 pfu (7). Gates et al. reported that the brain titers found in mice after an IP injection of two SFV strains, M9 and M7, were 100 times less than the titers obtained after an IC inoculation (19). Bradish et al. (1972) also observed that in Porton random bred mice, 20% of the mice showed an abnormally slow and low response to IP infections (5). In 1967, Seamer et al. reported that IC or footpad (FP) injections of high doses of SFV ($10^{4.6}$ and $10^{1.6}$ pfu) in mice were fatal but a few mice infected IP survived. Survival was not attributed to specific immunity since subsequent IC inoculations killed those mice (74). SFV is not the only encephalitis-causing virus in which this phenomenon has been observed. EEE virus, which is another alphavirus (9), required $6.3x10^5$-$6.3x10^6$ times less virus for an IC LD_{50} dose as compared to an IP inoculation, in mice of 42 days in age (44). A more distantly related virus, the Vesicular Stomatitis virus (VSV), also shows variability in the infectious dose depending upon the route of infection. Olitsky et al. reported that mice were refractory to IP infections with VSV, but susceptible to IC

infections. No non-specific or anti-viral substances were identified in the serum (59). It appears that no matter how SFV is inoculated, it will eventually infect the brain, and cause encephalitis, if given in a high enough dose. Seamer et al. suggested three principal routes by which SFV can enter the central nervous system (CNS): 1. Via peripheral nerves; 2. Via the blood and across the endothelium of the cerebral and meningeal capillaries; 3. Via the olfactory nerve fibers to the olfactory bulb (74). The latter route was recently confirmed by Kaluza et al.(33). Murphy et al. wrote: "From sites of extraneural infection, particular arboviruses are disseminated and gain access to the CNS with widely varying efficiency" (52). One of the early events following an SFV infection is believed to be the replication of the virus to high titers in muscle cells. This has also been shown to be the case for infection with the Flavivirus, Murray Valley Encephalitis virus (MVE),in which increasing viral titers were found from 15 minutes through five days post infection (PI) following injection into the hindleg muscles (47). After IP inoculation of high numbers of SFV, virus could be found in the hind limb muscles by 12 hours PI (52). After IP inoculations of a virulent SFV strain (V13), the virus could be found replicating in the peritoneal wall and the hind limb muscles early in infection (64). The virus can be found in the blood within minutes after injection into the peritoneal cavity (64),(38). After IP

injection, virus can be found in the blood within one day PI, then increases for about a day, and subsequently decreases until the time of death (19),(52),(74). It is interesting to note that by 12 hours PI, the concentration of virus in the hindlimb muscles were 200 times higher than in the blood, and the viremia plateaud by 24 hours PI (52). The concentrations of virus in the liver and spleen has been found to compare with that in blood over most of the time following the infection (74). Virus can be recovered from the brain at one day PI, but at low titers (74). In general, the brain titers appear to increase until the time of death (19),(52),(74). Late in infection, virtually all brain cells were found to be infected (52). The signs of illness are: a ruffled coat, which usually precedes paralysis on day seven PI, and then death, by day 10 PI (7).

Age Dependence

It has long been known that young animals are more susceptible to viral infections than older ones (77). In 1936, the first reported study noted this difference in susceptibility with age (59). When VSV was injected IC, mice of two weeks in age were as susceptible as mice of one year in age. When the virus was injected IN, none of the old mice became sick, whereas all of the young ones died (59). The same phenomenon has been shown to be true for SFV since 3-6 day old suckling mice are equally susceptible to

IP and IC inoculations. However, mice become more resistant to IP inoculations. Increased resistance with age could also be correlated with an increase in body weight (7). In another report, mice of ages varying from three to 50 days were injected IC, FP or IP with SFV. The dose of virus was large enough that most animals died. There was a significant difference in the mean survival time in young versus old mice, particularly if infected IP or FP. The old mice survived 6-7 days, whereas the young ones only survived for approximately two days (74). Infection with the related EEE and Western Equine Encephalitis (WEE) viruses exhibited similar characteristics with respect to age and susceptibility of the host. When injected IC, young mice were as susceptible as old mice but when injected IN, older mice survived longer (59). In young mice the dose required for an IP LD_{50} is $2x10^6$-$2x10^7$ times less than that required in old mice. The dose required for an IC LD_{50}, however, remained $6.3x10^5$-$6.3x10^6$ times less than the IP LD_{50} dose in old mice (44). Not only in mice can this be seen, but even in an epidemic in humans caused by EEE. Seventy percent of the cases were in humans less than 10 years of age (68). In rats, Japanese B Encephalitis virus causes 100% mortality if inoculated IP in young animals; however, older animals are highly resistant. In addition, in the rat the same resistance also develops for the IC route with age (13).

Involvement of the Nervous System

The CNS is the target organ most directly affected by the encephalitis-inducing alphaviruses, in mice (73). In man, however, most infected individuals do not contract encephalitis, because the virus presumably cannot enter the CNS (73),(77). For those arthropod-borne viruses which do infect the human CNS, the cells of the gray matter are the target cells (32). Even for mice, where the target organ has been shown to be the CNS, there had been no direct evidence that SFV was capable of reaching the brain via nerves, until a paper by Kaluza et al. was published, in 1987. In this report, evidence was presented that SFV travels along neurons to the brain, after intranasal or subcutaneous infection. For example, after IN infection, no viremia was detected before mice died; however, viral antigens could nevertheless, be demonstrated early after infection in circumscribed areas of the nasal mucosa, the fibers extending to the olfactory bulbs, and the cerebrum. Also, IFN could not be detected in the serum until 30 hours PI. One conclusion of this report was that virus which spreads via the immunologically privileged nerves, would not be as efficient in stimulating IFN production (33). One might hypothesize that if the virus can escape immune surveillance, it would have an advantage over the infected host. Johnson and Mims have shown that aerosol infection of mice with West Nile virus leads to an initial infection of

the olfactory bulbs (31). Just the fact that laboratory workers have been infected with SFV (94) and West Nile virus (54) by aerosolization suggests that olfactory bulbs may also be infectable in humans. Another piece of evidence that might indicate the involvement of nerves after SFV infection is the fact that mice become paralyzed several days before death (7).

The involvement of the nerves in viral infections which affect the CNS has been shown for other viruses. When VSV is inoculated into the FP of mice, all survive; however, when the virus is inoculated into the sciatic nerve, fatal ascending myelitis occurs. The same sequence was seen in guinea pigs, and was interpreted to mean that the nerve endings posed a barrier to infection. This barrier could be overcome if the nerves were infected directly (69). For herpes simplex virus (HSV), it was shown that mice were largely protected if the sciatic nerve were resected prior to inoculation of the virus into the FP (57). With a virus such as SFV, which clearly is capable of invading and destroying the CNS of mice, it would appear possible that the virus might travel along the nerves to reach the brain, however, little evidence to support this exists.

Macrophage Involvement in Viral Infections

It has long been known that viral infections can elicit mononuclear cell reactions (1). It has also been considered

very likely that macrophages may be capable of limiting the spread of virus from the site of the primary infection to secondary locations (1),(50),(53),(68). In 1941, Sabin noticed that VSV infections always arrested at the same site, depending on the route of inoculation. He made the conclusion that there are pre-existing barriers to the spread of virus in mice, and that all tissues are not equally susceptible to viral infections (68). Insusceptible zones may form barriers to the further spread of virus (1),(68). According to Mims, macrophages monitor most body tissues and compartments, including the peritoneal cavity, where they remove microorganisms, inert particles and also virus, from the circulation. Thus, macrophages may be critical in determining the outcome of a viral infection. The effectiveness of viral clearance by macrophages differs greatly for different viruses. For example, poliovirus type I, could not be cleared from the blood at all. On the other hand, the CL strain of Vaccinia virus is taken up by liver macrophages within minutes after IV inoculation, and are quickly destroyed, as seen by fading immunofluorescence of the macrophages. SFV does not fall into either of these categories if injected IV. Apparently, macrophages likely to be encountered after IV inoculation do not clear the virus from the blood efficiently, i.e., SFV is different from Vaccinia (i.e., infects vascular endothelial cells) and/or peritoneal macrophages differ in function from others

(61). An "uncleared virus tail" (i.e., low numbers of virus) was always found. However, it is important to keep in mind that the SFV was inoculated IV. It is possible that virus introduced by other routes may be effectively blocked from further spread by macrophages. Another possibility, also proposed by Mims, is that the barriers for viral spread may be overwhelmed by large doses of virus (50). The following statement by Neighbour and Bloom in 1980 again emphasizes the role of the macrophage: "It would appear that macrophages can contribute towards natural resistance in the host by an intrinsic resistance to infection possessed by the macrophage itself..." (53). Another piece of evidence arguing for the involvement of macrophages in viral resistance is the fact that viruses which are capable of inducing disease in the host often are also capable of infecting and replicating in the macrophages of that host (1),(53).

The Macrophage

The macrophage is a phagocyte, which is capable of removing extraneous matter or organisms, sometimes with the aid of antibody through opsonization (87). The mature macrophage is derived from a committed stem cell (61), which develops into a monoblast. Monoblasts have only been identified in colonies grown **in vitro** (23). The monoblast then develops into a pro-monocyte, which is the most

immature cell of the mononuclear phagocytes identified **in vivo.** The promonocyte is also the immediate precursor of the non-dividing monocyte (90),(91). The monocyte is an antigen presenting cell, which differentiates into a small macrophage, that is Ia antigen positive (41), (42),(43). Ia is specified by the I-region of the major histocompatibility complex (MHC) (67). Maturation of the macrophage is accompanied by cell-enlargement (22). Large macrophages are usually Ia antigen negative (40),(41), (42),(43). Large macrophages are also known to be non-specific in their cytotoxic and cytostatic effects on proliferating lymphoid or tumor cells (40). It is also interesting to note that macrophages are a heterogeneous group of cells (11),(22),(39),(45),(92),(95). For example, peritoneal macrophages predominantly lack Ia (85%), whereas splenic macrophages predominantly express Ia.

The macrophage is involved in many different processes. It can act as a phagocyte (61), or as a scavenger cell (45); it can be involved in antigen presentation to T-cells (76),(88); and it can be induced to kill tumor cells after induction with interferon (IFN) (4).

Studies With Macrophages in Viral Infections

When Balb/c mice are infected IP with 10^4 pfu SFV, the average day of death is approximately seven. In an attempt to determine which cells are involved in protection, Rodda

et al. (65) performed a cell-mediated cytotoxicity assay, and found that peak cellular activities fell on day two and day six PI. The first episode was found to be non-specific, unaffected by anti-thymocyte serum treatment, did not involve Ia antigen, and could be removed by passage of all cells through a nylon wool column. The second episode of high cytotoxic activity showed the opposite effects to the already mentioned treatments. The conclusion drawn from these experiments was that peritoneal macrophages can destroy infected cells early in infection.

In several reports, Myocrisin treatment has been shown to elevate the numbers of SFV in the brains of infected mice (6),(20),(49),(58). Bradish et al. (1975) reported that the IP 50% infective dose (ID_{50}) could be decreased from 250 pfu to <20 pfu if the mice were pre-treated with Myocrisin or l-asparaginase, both of which impair macrophage function. In a study by Mehta et al. (49), Myocrisin treatment prior to SFV infection given IP was correlated with a suppression of the lysosomal enzymes, acid phosphatase, β-glucuronidase and N-acetyl-β-D glucosaminidase. This report suggested that suppression may be a result of competition for the cellular machinery by already replicating virus. Furthermore, after infection of normal mice with avirulent SFV, even at high doses, no virus could be seen in the brain. After Myocrisin treatment, mature virions could be found. The reason that no virus could be seen in normally

infected mice may be that the macrophages are capable of acting as a barrier for their further spread (49).

The efficiency of IP infection was unchanged after cyclophosphamide pre-treatment (6). It was also noted that efficient infection could only be enhanced during the first phase of infection, and that efficiency of infection is determined by the probability that potentially infective virus particles are not lost by phagocytosis, pinocytosis, interference or wasteful adsorption. When the virus can escape these barriers, the infection can be followed by replication in local tissues, and then by an exponential upsurge of viremia (6). In a similar study done by Oaten et al., it was found that treatment of the mice with Myocrisin had no effect on the development of neutralizing antibodies. Furthermore, the transfer of macrophages from adult, SFV-resistant mice, conferred some protection to otherwise susceptible baby mice (58).

Thorotrast (thorium dioxide) is another substance which impairs macrophage function (61). If it is given IV to mice at the same time as SFV is given SC, a significant increase in the blood titers of SFV can be seen (50). Van Der Groen et al. (89) found that SFV infection of peritoneal macrophages **in vitro** was ineffective, with yields much lower than from infected L929 cells. At two days PI the yields were 0.6 $\log_{10}$ pfu/ml using strain V13, and 3.1 $\log_{10}$ with strain A7. However, if the macrophages were first

stimulated with proteose peptone **in vivo**, higher yields of virus was found with the avirulent A7 strain, but not with the virulent V13 cultures. No obvious cytopathic effects (cpe) could be seen in infected macrophages 0-7 days PI. Intracellular virus could not be detected on either A7 or V13 infected macrophages 0-7 days PI, but there was a linear increase in the supernatant fluid of the $\log_{10}$ pfu/ml with time. The extracellular virus was determined to be newly made, since infected macrophages treated with anti-SFV serum, did not affect the viral titers found in the supernatant fluids (89).

Kraaijeveld et al. (38) found that very few SFV-sensitive cells could be found in the peritoneal cavity. Using the infectious center assay, the estimated number of viruses produced was less than one per 1000 cells. McFarland et al. found that after removing the adherent cells from Sindbis (SIN) virus sensitized lymph node cells **in vitro**, viral clearance was depressed (48). Other reports show that Sindbis virus can not grow in macrophages **in vitro** or **in vivo** (30).

Natural resistance to two Flaviviruses, Yellow Fever and West Nile virus, is expressed in macrophages of PRI and BRVR mice. Infected macrophages from these mice do not yield very much virus, whereas macrophages from susceptible mice (C_3H/He) do yield virus (21),(86). Mice exposed to aerosolized West Nile virus die from infection of the CNS.

Virus can be found first in the lungs, where it remains throughout the infection. No pathological damage is seen to the lungs, and the CNS is invaded via the olfactory bulbs rather than via the lungs. Interestingly, using immunofluorescence, alveolar macrophages in the lungs can be shown to contain virus (55).

Macrophages can also be infected with Dengue virus, with subsequent suppression of phagocytic and migratory functions. This has been shown to be due in part to a cytotoxic factor produced in the spleen, which suppresses macrophage functions and induces the production of a cytotoxin in macrophages. After IC infection of mice, a significant reduction in the numbers of macrophages present in the spleen and peritoneal cavity can be observed (10).

Infectivity with other viruses have been found to be influenced by macrophages. Zisman et al. found that IP infections of mice with herpes simplex virus (HSV) did not cause a lethal encephalitis, whereas IC infection did. The protective barrier in the peritoneal cavity could be overcome by treatment with silica or anti-macrophage serum (97). Hirsch et al. reported that peritoneal macrophages transferred from adult CBA mice protected suckling syngeneic otherwise sensitive mice from IP infections with HSV, and that macrophages stimulated with proteose-peptone showed enhanced resistance (29). In another report, it was found that HSV could not grow in mouse macrophages infected in

vitro (16). Johnson reported that HSV infected the peritoneal cavity 1000 times less efficiently than via the IC route, and that only one macrophage per 100,000 could be infected. Larger doses of virus (10^5-10^6 pfu) could overcome the resistance of the peritoneal cavity and spread to other parts of the body. It was also noted that macrophages from mice of increasing age were decreasingly capable of infecting surrounding cells after a primary infection (30).

Other reports show that Influenza A virus can infect human macrophages, but that no infectious virus is produced by the mononuclear cells, even though viral proteins are expressed on the surface of infected cells (56). Kowalch et al. have shown that only 5% of peritoneal macrophages can be infected with the Lactate Dehydrogenase-Elevating virus (37). Belardelli et al. found that only a small percentage of peritoneal macrophages can take up VSV at wide ranges of multiplicities, but that viral replication can not proceed (2). Hirsch et al. reported that infant mice pre-treated with anti-macrophage serum rather than normal serum died faster after VSV IP infections (28). VSV has also been found to grow better in cultures of macrophages several days old rather than in those freshly harvested (16). It has also been shown that there is a difference in the IP LD_{50} dose for Encephalomyocarditis virus (EMCV) of $>10^5$ after whole body x-irradiation, accompanied by a shorter

incubation period and enhanced mortality. With IC infections, however, the irradiation did not have an effect (51). Mouse Hepatitis virus (MHV) is also known to be inefficient in infecting macrophages from mice resistant to infection. Less than 2% (83) or 3% (36) of the macrophages have been shown to be infectable. The resistance is thought to be due in part to a genetic, inherited resistance expressed in macrophages, and encoded on chromosome 7 (75),(83),(85),(93).

As the above reports strongly suggest, macrophages are indeed involved in protection from viral infections, not only with SFV, but also with many other viruses.

Involvement of Interferon

Although alphaviruses have been shown to induce the production of interferon (IFN) during the course of a normal infection (46), it is doubtful that IFN is important for the outcome of the infection. While studying a virulent and avirulent strain of SFV, Bradish et al. found that 70% of young as well as old mice had <100 units/ml of IFN in the blood during the critical period before 36 hours when benign or lethal responses developed. Eighty percent or more of the mice failed to show detectable activity in the blood or brain during the critical two days PI. A few mice, however, showed IFN levels of >100 units/ml blood. These were also positively correlated with high blood infectivities.

Therefore, the induction of IFN was interpreted as a secondary factor of infection, that is, after the virus had multiplied to high numbers in muscle tissues, rather than a primary expression in infection (5).

Smillie et al. also studied a virulent and avirulent strain of SFV, and found no significant difference between the two strains when mouse IFN was given **in vitro** or **vivo**. Yet, the virulent strain caused encephalitis whereas the avirulent did not (82). Finter et al. (1966) showed that increasing doses of IFN (up to 32,000 units) protected mice against SFV infection. It was noted, however, that the dose of IFN became less potent as the dose of challenging virus was increased (17). Although IFN could protect L929 cells from SFV infection (less virus was produced, and the growth was slower), cells could not be protected from high doses of virus. The proposal was made that the high dose of virus would infect all cells, such that IFN production would be too late to provide significant protection (18). In light of the fact that SFV has been found to replicate to very high levels in tissues outside of the CNS before invading it (19),(52),(64),(74), it seems reasonable to assume that IFN is not a major factor in determining the outcome of infection.

Injecting mice with anti-IFN enhanced the infection (25) and increased the multiplication of VSV in the macrophages, even though the uptake of virus by the

macrophages remained unchanged (2). Proietti et al. also found that macrophages could be infected after giving large doses of anti-IFN **in vivo**, but could nevertheless not detect any IFN in peritoneal washings or culture supernatant fluids (63). **In vivo** injections of anti-IFN have been shown to enhance the diseases in mice caused by Influenza A (26), HSV, Moloney Sarcoma virus (25) and EMCV (24). The resistance of macrophages to MHV could not be duplicated in susceptible mice by IFN treatment (36). Cell cultures from Flavivirus resistant mice have been shown to be more sensitive to the effects of IFN than were cells from susceptible mice (27). In conclusion, the role played by IFN is quite variable among different viruses, and little evidence supports the notion that IFN plays an important role in SFV infections.

CHAPTER III

MATERIALS AND METHODS

Cell Cultures

Chicken Embryo Fibroblasts

Primary chicken embryo fibroblast (CEF) cultures were prepared from 10-11 day old fertilized eggs (Truslow Farms, Maryland). The eggs were incubated in a humid egg-incubator at 38^{o}C. After incubation, eggs were sprayed with 70% ethanol, placed in a laminar flow cabinet, and then wiped with an iodine-ethanol egg wash. Next, the egg shell above the air-sac was chipped with a sterile pair of forceps, the chorioallantoic membrane peeled away, and the embryo removed from the egg. The embryos were decapitated, and placed in a 100 x 15 millimeter (mm) plastic Petri dish (Fisher Scientific Products, Norcross, Georgia) containing 15 ml Hank's Balanced Salt Solution ([HBSS], Gibco Laboratories, Grand Island, New York). The embryos were eviscerated, washed five times in HBSS (Gibco), and fragmented by forcing them through a 50 ml syringe (Becton Dickinson Labware, Rutherford, New Jersey) into a solution of Saline A (0.8% NaCl, 0.1% dextrose, 0.04% KCl, 0.4 ml phenol red, pH 7.4) with 0.025% trypsin (Gibco). After one hour of incubation at 37 ^{o}C with constant stirring, the cell suspension was filtered through sterile gauze into 50 ml centrifuge tubes

(Corning Glass Works, Corning, New York). The cells were centrifuged at 800 x g for 15 minutes, and the trypsin-solution decanted. Growth medium 199 (Gibco) supplemented with 5% fetal bovine serum ([FBS], Gibco), 50 units (u) per ml Penicillin-Streptomycin ([P-S], Gibco), at pH 7.4, was then added to the cell pellet. The pellet was vortexed lightly, such that the CEF separated from the underlying red blood cells. The cell suspension was decanted and the number of viable cells determined by the trypan blue dye exclusion method. Cells were seeded into six-well microtiter plates (Corning) for plaque assays, or into roller bottles (Corning) for virus propagation. At confluence, each square centimeter (cm^2) contained 2.5×10^5 cells.

L929 Cells

In 1940, W.R. Earle established the cell-line Strain L (14). These cells were derived from normal subcutaneous areolar and adipose tissue from a 100-day old male mouse of the strain C_3H/An. In 1948, after 95 generations of subculture of this cell line, the first cloned strain was developed from it. This clone is what is now commonly known as the L929 cell line (71). The L929 cells used for this project were obtained from the National Culture Type Collection, and cultured routinely in plastic tissue culture

flasks (Corning). The growth medium used for these cells was McCoy's Modified 5A Medium ([McCoy's], Gibco) supplemented with 10% FBS (Gibco), 50 u/ml P-S (Gibco), at pH 7.4. At confluence, each cm^2 contained 2.5 x 10^5 cells.

Mouse Peritoneal Cells

Female C_3H/Ten mice (University of Tennessee [UT] Hospital, Knoxville, Tennessee), seven to ten weeks in age, or female ICR mice (Harlan Sprague Dowley, Indianapolis, Indiana) of the same age were used as sources for peritoneal cells. Naive animals were killed by cervical dislocation, immersed in 70% ethanol, and placed on alcohol saturated paper. A segment of skin and the outer peritoneal membrane was cut on the abdominal side. The exposed abdominal membrane, was sprayed vigorously with 70% ethanol to remove any adherent fur. The animals were then transferred to a laminar flow cabinet, and five ml of cold RPMI Medium 1640 ([RPMI], Gibco) supplemented with 10% FBS (Gibco), 50 u/ml P-S (Gibco), at pH 7.4, was injected into the abdominal cavity. This medium was used routinely for peritoneal cells. With the needle ([21 gauge, 1 1/2 inch], Becton Dickinson) from the injection maintained in position, the abdomen was gently massaged. The fluid was withdrawn into the syringe, and expelled into a 50 ml centrifuge tube (Corning). The peritoneal washings were centrifuged at 800 x g for 10 minutes, at $4^{o}C$. The supernatant fluid was

removed, and the cell pellet was suspended in RPMI medium. The number of viable cells was determined by trypan blue dye exclusion using a hemocytometer. Cells were seeded into six- or 24-well plates (Corning) for immunofluorescence experiments, into six-well plates (Corning) for Semliki Forest virus (SFV) infection experiments, or into 96-well flat bottomed plates (Becton Dickinson) for neutral red uptake experiments. After three hours of incubation at 37°C with 5% CO_2, the non-adherent cells were pipetted off the adherent cells. The non-adherent cells were either collected and incubated separately, or discarded. The adherent cells were washed three times with cold RPMI medium, and incubated for 18-20 hours at 37°C with 5% CO_2. The cells were washed at least three times in RPMI (Gibco) before use.

Semliki Forest Virus (SFV) Propagation

The SFV strain used routinely in this laboratory was provided by Dr. A. Brown. SFV was propagated on CEF monolayers, which had been grown to confluence in roller bottles. First, the medium was decanted from the roller bottles, and 10 to 20 ml HBSS was added to each, which were then rolled for 40 minutes at 37°C. The HBSS was removed, and the cells infected with 2-5 ml HBSS containing SFV to a multiplicity of infection (MOI) equal to 0.01. After 45-60 minutes of constant rolling at 37°C to allow adsorption of

the virus, the fluid was removed and 20-40 ml Medium 199 (Gibco) supplemented with 2% FBS (Gibco) and 50 u/ml P-S (Gibco) was added. The roller bottles were incubated at 37°C for 14 hours, at which point the supernatant fluid was harvested. Medium was added back to the cells, and additional harvests were performed two and four hours later. These latter supernatant fluids were pooled with the first one. To clarify the fluids, they were centrifuged at 1000 x g for 15-20 minutes, at 4°C. The virus-containing medium was aliquoted into 12 x 35 mm glass vials (American Scientific Products, Stone Mountain, Georgia) and stored at -70°C as a laboratory **in vitro** stock. For **in vivo** stocks, the same virus was passaged twice through brains of C_3H/Ten mice that were six-eight weeks old. The titer of the viral suspensions were determined in plaque assays on CEF. This procedure is described in detail under "Infectivity of SFV on CEF versus L929 Cells," found on page 52.

Animal Models

The animals used in this project were female mice of the following strains: C_3H/Ten (UT Hospital); C_3H/Hen (Charles Rivers, Wilmington, Massachusetts); ICR (Harlan Sprague Dowley), and ICR (Hilltop Lab Animals Inc., Scottdale, Pennsylvania). The ages used are specified in each experiment. All animals were housed in the Life

Sciences Animal Facility, with a maximum of ten animals per pan. The animal facility is accredited by the American Association for Accreditation of Laboratory Animal Care.

Determination of The 50% Lethal Dose (LD_{50}) of SFV in Mice

Injection Via the Intraperitoneal (IP) Route

C_3H/Ten mice. Animals of 6-8 or 16-18 weeks in age, were injected with $3x10^3$, $4x10^3$, $5x10^3$ or $6x10^3$ pfu of SFV via the IP route. The virus was given in 0.2 ml BHI. Control animals received 0.2 ml BHI without virus via the same route. All animals were observed two times per day, for 21 days.

C_3H/Hen mice. Animals of six weeks in age, were injected with four, 40, $4x10^2$, $4x10^3$, $4x10^4$ or $4x10^5$ pfu of SFV. The virus was given in 0.2 ml BHI, and injected via the IP route. Control animals received 0.2 ml BHI without virus. All animals were observed two times per day for 21 days.

ICR mice (Harlan Sprague Dawley). Animals, of 6-8 weeks in age, were injected with $2x10^3$, $3x10^3$, $4x10^3$, $5x10^3$ or $6x10^3$ pfu of SFV. The virus was given in 0.2 ml BHI, and injected via the IP route. Control animals received 0.2 ml

BHI without virus via the same route. All animals were observed two times per day, for 21 days.

Injection Via the Footpad (FP) Route

C_3H/Ten mice. Animals of 6-8 or 16-18 weeks in age, were injected with one, five, 10, 50, or 100 plaque-forming units (pfu) of SFV, suspended in 0.1 ml Brain Heart Infusion medium ([BHI], Gibco). A viral dilution (0.05 ml) was injected into each of the two hindleg footpads. Control animals received 0.1 ml BHI (Gibco) without virus via the same route. All animals were observed two times per day, for 21 days.

ICR mice (Harlan Sprague Dawley). Animals of 6-8 weeks in age were injected with 0.05 ml BHI containing two, four, eight, 32, 64, 200 or 500 pfu of SFV, into each of the two hindleg footpads. Control animals received 0.1 ml BHI without virus via the same route. All animals were observed two times per day, for 21 days.

Injection Via the Intravenous (IV) Route

C_3H/Ten mice. Animals of six weeks in age, were injected with one, five, 10, 30, or 500 pfu's of SFV via the IV route. The virus was suspended in 0.1 ml BHI. Control animals received 0.1 ml BHI without virus via the same

route. All animals were observed two times per day, for 21 days.

ICR mice. Animals of 7-9 weeks in age were injected with two, eight, 16, 200 or 500 pfu of SFV via the IV route. The virus was suspended in 0.1 ml BHI. Control animals received 0.1 ml BHI without virus via the same route. All animals were observed two times per day, for 21 days.

Assay of the Peritoneal Cavity Contents For Possible Anti-Viral Factor(s)

Peritoneal Cavity Lavage

The procedure for rinsing the peritoneal cavity was similar to that described under "Mouse Peritoneal Cells," found on page 26. The rinse medium was HBSS, and the **supernatant fluid** was saved, rather than the cells, after the centrifugation.

Assay of the Peritoneal Lavage Fluid - Short Pretreatment

L929 cells were prepared and seeded into three six-well plates as described under "L929 Cells," found on page 25. The cells were grown to confluence in two ml of McCoy's Medium. One plate was washed with HBSS,and two ml of the cell-free peritoneal rinse was added to each of the six wells. The plate was incubated at $37^{o}C$ with 5% CO_2 for 1.5 hours. SFV was diluted to contain 50 pfu per 0.2 ml of cell-free peritoneal rinse, and incubated at room

temperature for 30 minutes. Additional virus was diluted to the same concentration in BHI. All six-well plates were washed with HBSS. One un-treated plate was inoculated in triplicate with 0.2 ml of SFV diluted in peritoneal cell-free rinse. Three wells were inoculated with SFV diluted in BHI.The wash was removed from the three pre-treated wells, which were then inoculated with 0.2 ml of SFV diluted in BHI. In addition, one un-treated well each was inoculated with 0.2 ml of McCoy's Medium, cell-free peritoneal rinse, or BHI. All plates were incubated for one hour at 37°C with 5% CO_2. The plates were shaken every 10 minutes to spread the inocula. Fluids were removed and replaced with two ml per well of Modified Eagle's Medium (MEM) agar overlay (one part MEM [Gibco], one part 1.8% noble agar [Difco], 100 u/ml P-S [Gibco], 2% FBS [Gibco]). After two days of incubation at 37°C with 5% CO_2, the monolayers were stained with two ml of a 1:11,100 dilution of neutral red (Gibco). Plaques were counted after four hours of incubation at 37°C with 5% CO_2.

Assay of the Peritoneal Lavage Fluid - Long Pretreatment

L929 cells were again prepared and seeded into six-well plates as described under "L929 Cells," found on page 25. All wells were washed with HBSS. Three wells were inoculated with two ml McCoy's Medium, additional wells with two ml McCoy's Medium containing 25% cell-free peritoneal rinse, or McCoy's Medium with 25% rinse medium - HBSS. All

plates were incubated for 18 hours at 37°C with 5% CO_2. All plates were then washed with HBSS three times. The wells were then inoculated with 0.2 ml BHI containing SFV. The plates were incubated for one hour, with rocking every ten minutes. The inocula were removed, and replaced with two ml MEM-agar overlay. After three days of incubation, the cells were stained with two ml neutral red diluted to 1:11,100. After four hours of incubation at 37°C with 5% CO_2, plaques were counted.

Virus Yields From SFV-Infected L929 Cells in the Presence of Peritoneal Fluid

Preparation

Cell-free peritoneal rinse fluid was harvested from female C_3H/Ten mice of 18 weeks in age as described under "Assay of the Peritoneal Cavity Contents for Possible Anti-Viral Factor(s)," found on page 31. The peritoneal rinse was diluted to a 10% concentration in McCoy's Medium. One six-well plate with confluent monolayers of L929 cells was prepared as described under "L929 Cells," found on page 25. The monolayers were washed with two ml McCoy's Medium per well. SFV was diluted on ice to contain 2.4×10^3 pfu per 0.2 ml McCoy's Medium with this dose representing an MOI equal to 0.001. The medium was removed from the monolayers and two wells were inoculated with 0.2 ml McCoy's Medium while four different wells were inoculated with 0.2 ml of SFV

diluted in McCoy's Medium. The plate was incubated at 37°C with 5% CO_2 for 40 minutes. Every 10 minutes the plate was rocked, to spread the inocula. After the incubation, five ml McCoy's Medium was added to each of the two wells inoculated with medium only, and to two of the wells inoculated with virus. To the two remaining wells inoculated with virus, five ml of medium containing 10% peritoneal rinse, was added. The plate was incubated at 37°C with 5% CO_2 for approximately 46 hrs. Duplicate 190 µl samples were removed from each well at the following timepoints:

(a) +0 hrs (immediately after adsorption)
(b) +5 hrs
(c) +8 hrs
(d) +10 hrs
(e) +22.25 hrs
(f) +27.25 hrs
(g) +30.25 hrs
(h) +46.00 hrs

All samples were stored at -70°C until the final assay.

Assay of the Samples

Six-well plates containing confluent monolayers of CEF were prepared as described under "CEF," found on page 24, and washed with Medium 199. The samples were diluted with serial 10-fold dilutions in McCoy's Medium, on ice. The

medium on the target monolayers was removed, and each sample dilution was inoculated onto three wells in a volume of 0.2 ml. All plates were incubated at 37°C with 5% CO_2 for 40 minutes. All inocula were removed and replaced with two ml per well MEM agar overlay as described. All plates were incubated at 37°C with 5% CO_2 for two days. Thereafter, the cells were stained with two ml per well of a 1:11,000 dilution of neutral red. After four hours of incubation at 37°C, plaques were counted.

Assay of Possible Anti-Viral Factor(s) Released From Peritoneal Cells During Two Hours of Culture

Preparation of Peritoneal Cell Culture Supernatant Fluids

Peritoneal cells and fluid was harvested from two female C_3H/Ten mice of six weeks in age. The method has been described under "Assay of the Peritoneal Cavity Contents For Possible Anti-Viral Factor(s)," found on page 31. The medium used for the peritoneal rinse was MEM. After obtaining the peritoneal rinse, the total harvest of cells and liquid was placed in a 100x15 mm plastic Petri dish (Fisher) and incubated at 37°C with 5% CO_2 for two hours. The culture supernatant fluid was pipetted off and placed in a 50 ml centrifuge tube (Corning). Two washes of three ml each of MEM was used to rinse the Petri dish with and the washes were added to the culture supernatant fluid.

Cells were removed by centrifugation at 800xg for 10 minutes.

Assay of Peritoneal Cell Culture Supernatant Fluid

Four six-well plates containing confluent monolayers of L929 cells were prepared as described under "L929 Cells," found on page 25. All monolayers were washed with McCoy's Medium. SFV was diluted in BHI at a concentration of one, 10 or 100 pfu per 0.2 ml and six wells were inoculated with each of the viral dilutions. As controls, one well was inoculated with 0.2 ml McCoy's Medium, HBSS, BHI, peritoneal cell culture supernatant or MEM. All plates were incubated at 37°C with 5% CO_2 for 40 minutes. The inocula were removed and replaced with one of three agar overlays as follows: Two wells each inoculated with one, ten or 100 pfu, and control wells, were overlaid with two ml of MEM-agar as described before. Two additional wells each inoculated with one, ten or 100 pfu were overlaid with two ml of a modified MEM-agar overlay, containing one part 2x concentrated MEM, one half part peritoneal cell culture supernatant fluid (which is a 1x MEM), and one part 2.25% Noble agar. The last set of monolayers inoculated with virus received two ml of the modified MEM-agar overlay in which the 1x concentrated MEM was the medium used to rinse the peritoneal cavity. All plates were incubated at 37°C with 5% CO_2 for three days. The cells were stained with two

ml per well of neutral red diluted 1:11,100. After four hours of incubation at 37°C, the stain was removed, and the plaques counted.

Assay of the Peritoneal Cavity for Anti-Viral Factor(s) After SFV Infection

Female C_3H/Ten mice of eight weeks in age or female ICR mice were injected IP with one LD_{50} SFV ($4x10^3$ pfu and $5x10^3$ pfu, respectively) suspended in BHI. Additional mice were left un-infected. L929 cells were prepared and seeded into wells on 6-well microtiter plates. One day PI the peritoneal fluid was harvested from the infected and normal mice as described. The peritoneal cells were removed by centrifugation. The infected peritoneal fluid was then placed in Petri dishes, and exposed to UV light for three minutes. Interferon (IFN) α/β (Sigma) was diluted in growth medium to contain 100 International Reference Units (IRU) per ml. A portion of the IFN was also UV-treated for three minutes, as a control. The L929 cells were washed with McCoy's Medium, and three wells each inoculated with two ml normal peritoneal fluid, UV-treated infected peritoneal fluid, IFN α/β , or UV-treated IFN α/β . The same samples were also pre-incubated one hour with anti-IFN α/β (Lee Biomolecular Research Laboratories, Inc.) diluted 1:20 in medium before adding to the L929 cells. In addition, several wells were inoculated with growth medium. All

plates were then incubated at 37°C with 5% CO_2 for 24 hours. At 24 hours, all monolayers were washed three times with McCoy's Medium. SFV was then diluted in BHI. All pre-treated wells were then inoculated with 0.2 ml SFV. Three normal wells also received 0.2 ml SFV. In addition, some of the viral suspension was exposed to UV light for three minutes, and inoculated in triplicate (0.2 ml) on the cells. As controls, normal L929 cells were also inoculated with 0.2 ml McCoy's Medium, BHI, IFN α/β , UV-treated IFN α/β, normal peritoneal fluid, UV-treated infected peritoneal fluid, anti-IFN α/β diluted 1:20 in medium, and un-treated infected peritoneal fluid. After one hour of incubation with rocking every ten minutes, the inocula were removed, and replaced with two ml MEM-agar overlay. Three days later, the monolayers were stained with two ml neutral red diluted 1:11,100. The cells were incubated at 37°C with 5% CO_2 for four hours, and plaques counted.

Effect of Anti-Interferon α/β on the LD_{50} of SFV Given IP

Female ICR mice (Harlan Sprague Dawley), 6-8 weeks in age, were divided into three groups. Each group contained 10 animals. On day zero, one group was injected IP with 0.2 ml of anti-IFN α/β (Lee Biomolecular Research Laboratories, Inc.). This dose contained at least 1.25×10^3 standard

neutralizing units. The second group of mice was injected IP with $8.6x10^3$ standard neutralizing units of the anti-IFN α/β. The third group received 0.2 ml IP of a 1:10 dilution of normal mouse serum. All dilutions were made in BHI. The third group was left un-inoculated. On day one, all mice were injected IP with $4x10^3$ pfu of SFV, suspended in 0.2 ml of BHI. All animals were observed two times per day, for 21 days.

Effect of Indomethacin on the LD_{50} of SFV Given IP

Female ICR mice (Harlan Sprague Dawley), 6-8 weeks in age were divided into three groups with 10 mice per group. On day zero, two of the groups were injected IP with 0.2 ml of a 1% solution of $NaHCO_3$ (Sigma) containing enough Indomethacin (Sigma) to represent five mg per kilogram (kg) of body weight. The solution was made by dissolving the Indomethacin (Sigma) in 1/5 of the required volume in a 5% solution of $NaHCO_3$. This was heated at 50^oC until the Indomethacin had dissolved completely, and 4/5 of the required volume was then added in the form of distilled water. One additional group was injected IP with 0.2 ml of a 1% solution of $NaHCO_3$. On day one, 24 hours after the first injection, all three groups were injected again with the same solutions. One hour later, one of the groups treated with Indomethacin and the group treated with $NaHCO_3$,

were injected IP with $4x10^3$ pfu of SFV diluted in 0.2 ml BHI. On day three, 24 hours later, all groups were again injected the same way as on day zero. All mice were observed two times per day, for 21 days.

Effect of Lipopolysaccharide on the LD_{50} of SFV Given IP

Female C_3H/Ten mice, 10-11 weeks in age, were divided into eight groups with 10 mice per group. On day zero, four of the groups were injected IP with 25 micrograms (μg) LPS ([**Escherichia coli**, Strain W], Difco, Detroit, Michigan) which was diluted in 0.1 ml of HBSS. Four additional groups were left un-inoculated. As controls, two mice were injected IP with 0.1 ml of the LPS solution alone, two mice were injected IP with 0.1 ml of the HBSS alone, and two mice were left as naive. On day one, one group from each of the two categories was injected IP with $5x10^2$ pfu of SFV suspended in 0.1 ml of BHI. A second group from each of the two categories was injected IP with $5x10^3$ pfu of SFV, a third group with $5x10^4$ pfu of SFV, and the last group in each of the two categories was injected with $5x10^5$ pfu of SFV. The control animals were left un-inoculated. All mice were observed two times per day, for 21 days. This experiment was also repeated in female ICR mice (Hilltop Lab, Scottdale, Pennsylvania) of six weeks in age. In this experiment, only mice challenged with $5x10^3$ and $5x10^5$ pfu of SFV were used.

Effect of Protein A on the LD_{50} of SFV Given IP

Female C_3H/Ten mice, 9-14 weeks in age, were divided into nine groups with 10 mice per group. On day zero, three of the groups were injected IP with 0.1 ml of a 10% suspension of Protein A (Sigma Chemical Co., St. Louis, MO), diluted in HBSS. The Protein A was washed three times in HBSS (Gibco) before use. Three additional groups were injected IP with 0.1 ml of HBSS. The remaining groups were left un-inoculated. As controls, two naive animals were left un-inoculated throughout the experiment, three mice each received 0.1 ml IP of the Protein A solution alone, and two animals received 0.1 ml IP of HBSS alone. On day one, one group each from the three categories was injected IP with $5x10^3$ pfu of SFV diluted in 0.2 ml HBSS. The second group from each of the three categories was injected IP with $5x10^4$ pfu of SFV. The last group from each of the three categories was injected with $5x10^5$ pfu of SFV. The control animals were left un-inoculated. All mice were observed two times per day, for 21 days.

Effect of Thioglycollate on the LD_{50} of SFV Given IP

Female C_3H/Ten mice, six weeks in age, were divided into six groups, with seven mice per group. On day zero, two of the groups were injected IP with two ml of a 3% solution of Thioglycollate (Becton Dickinson) diluted in

HBSS. Two other groups were injected with two ml of HBSS alone, and two additional groups were left un-injected. On day one, one of the two groups in each category was injected IP with $5x10^3$ pfu of SFV suspended in 0.2 ml BHI. The second of the two groups in each category was injected IP with $5x10^4$ pfu of SFV suspended in 0.2 ml BHI. In addition, two naive animals were left uninjected as controls throughout the experiment. The mice were observed two times per day, for 21 days.

Effect of Silica on the LD_{50} of SFV Given IP

Female ICR mice (Harlan Sprague Dawley), of 6-8 weeks in age, were divided into three groups with 10 mice each. On day zero, two of the groups were injected IP with 0.3 ml BHI containing 0.3 mg silica (Whittaker, Clark and Daniels, Inc., So. Plainfield, New Jersey) with an average particle size of 1.5 μm in diameter. The remaining group received 0.3 ml BHI without silica. On day one, one of the groups injected with silica plus the group injected with BHI, were injected IP with $4x10^3$ pfu SFV. The virus was diluted in 0.2 ml BHI. The remaining silica treated group was injected IP with BHI alone. All mice were observed two times per day, for 21 days.

Immunofluorescence of SFV Infected Peritoneal Cells

SFV Infection in Vitro

Preparation of monolayers. Peritoneal cells were harvested from female C_3H/Ten mice of 7-10 weeks in age, or female ICR mice (Harlan Sprague Dawley), of 6-8 weeks in age. The procedure for obtaining peritoneal cells is described in detail under "Mouse Peritoneal Cells," found on page 26. The bottoms of wells in 24-well microtiter plates (Falcon, Becton Dickinson Labware, Oxnard, CA) were etched by pipetting two drops of acetone (Fisher Scientific Company) into the wells. Sterile, round glass microscope coverslips (Fisher Scientific Company) were placed in each of the etched wells and the wells were washed three times with Phosphate Buffered Saline ([PBS], pH 7.4). After the peritoneal cells had been centrifuged, they were suspended in enough RPMI Medium 1640 to contain the equivalent number of cells harvested from two mice per ml medium. One ml of the cell suspension was then added to each of the etched wells. In addition, three ml of the cell suspension was added to each of several normal six-well microtiter plate wells (Corning Glass Works), for the purpose of pre-adsorbing antisera. The cells were incubated at 37°C with 5% CO_2 for 18-20 hours. The adherent cells were then rinsed three times with RPMI 1640 to remove any additional non-adherent cells.

Determination of cell numbers. Coverslips already holding adherent cells were removed from their culture wells. This was best accomplished by inserting a bent Tuberculin needle attached to a one ml syringe (Becton Dickinson) between the coverglass and the bottom of the well. The coverslip could then be secured with a small pair of forceps, removed from the well, and placed cell-side down on a hemocytometer. The cells were then counted on a small area of the hemocytometer. The total number of adherent cells in the original well could be determined by multiplying the number of cells on one mm^2 of the hemocytometer by the area of the microtiter well (201 mm^2).

Infection of the monolayers. SFV was diluted to contain the desired MOI in 0.2 ml cold BHI. The growth medium was removed from the rinsed monolayers, and replaced with 0.2 ml of the viral suspension or BHI alone, in duplicate. The cells were then incubated for one hour at 37°C with 5% CO_2, with rocking of the plates every 10 minutes. The inocula were removed, and the monolayers rinsed three times with RPMI 1640. Finally, one ml of medium was added to each well, which was incubated at 37°C with 5% CO_2 for the desired time before staining the cells with fluorescent antibody.

Antibody staining of the monolayers. At the desired time point post infection, the growth medium was removed

from the monolayers, which were then air-dried. Half of the duplicate monolayers were fixed for 10 minutes in absolute methanol (American Scientific Products) and all monolayers were washed three times in PBS. The sera used were rabbit pre-immune, rabbit hyper anti-SFV IgG and Fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA). These sera were diluted 1:20 in PBS, and incubated with normal monolayers of adherent cells for one hour, to remove any cell-reactive globulin. Then, 60 μl of each rabbit serum was added to one fixed and one unfixed monolayer for each time point. The same was done for mock-infected cells. In addition, 60 μl PBS was added to fixed and unfixed infected and un-infected cells. All cells were incubated for one hour at 37^{o}C with 5% CO_2. The cells were then washed three times in PBS for three minutes per wash. The coverslips were air-dried, and 60 μl FITC-conjugated goat anti-rabbit IgG was added to each of them. The cells were incubated in a humid incubator for one hour, at 37^{o}C with 5% CO_2, then washed three times in PBS, for three minutes per wash. The coverslips were air-dried, and mounted on glass slides (Fisher Scientific Company) with one drop of a 1:1 mixture of glycerin (Fisher Scientific Company) and PBS. The cells were viewed under a Nikon UV microscope. Photographs were taken with Echtachrome film, 400 ASA (Kodak).

SFV Infection **in Vivo**

SFV was diluted in BHI on ice to contain $4x10^3$ pfu (one LD_{50}) in 0.2 ml. Female C_3H/Ten mice of 6-8 weeks in age, were injected IP with 0.2 ml of the viral suspension, or BHI alone. Thirty minutes later, the peritoneal cells were harvested from the SFV infected and mock infected mice, as described in "Mouse Peritoneal Cells," found on page 26. Subsequent steps were the same as for the **in vitro** immunofluorescence. The adherent cells were reacted with the sera directly after the 18-20 hrs of incubation.

Assay of SFV in the Culture Fluids and Associated with Macrophages After One, Three and Four Days of Culture Post Infection

Preparation of Adherent Cell Monolayers

A peritoneal lavage was performed on 14 female C_3H/Ten mice, of seven weeks in age. This proceedure is explained under "Mouse Peritoneal Cells," found on page 26. The medium used for the lavage was RPMI 1640. After centrifugation of the peritoneal rinse, the cell pellet was suspended in RPMI 1640, and counted on a hemocytometer. Two ml of this cell suspension was inoculated into each of 18 wells of six-well plates. The cells were incubated in a humid incubator at 37^oC with 5% CO_2 for three hours. To remove non-adherent cells, the wells were washed at least three times with RPMI 1640. The washed monolayers were

viewed under an inverted microscope. If large numbers of non-adherent cells remained, the adherent cells were washed again. The adherent cells were further incubated in RPMI 1640 at 37°C with 5% CO_2 for 18-20 hrs. The cells were washed three times with RPMI 1640, and the cells from two wells were scraped off with a disposable Cell Scraper (American Scientific Products), pooled, and counted on a hemocytometer.

SFV Infection of Adherent Cells

SFV was diluted to contain enough virus to give multiplicities of infection (MOI) equal to 20, 0.2 and 0.002 in 0.2 ml of BHI on adherent cells. Each viral dilution was inoculated in triplicate onto the prepared adherent cell cultures. As a control, 0.2 ml of BHI was inoculated in triplicate onto adherent cell cultures. All cells were incubated in a humid incubator at 37°C with 5% CO_2 for one hour, with rocking every 10 minutes. Then two ml of cold RPMI 1640 was added to all wells, and duplicate samples of 0.5 ml were taken from each well and frozen at -70°C until assayed. The monolayers were washed three times with RPMI 1640, and then two ml of medium was added, followed by incubation at 37°C with 5% CO_2 for various lengths of time. At 24 hours post-infection, duplicate samples of 0.5 ml from each of the four triplicate samples were collected and frozen at -70°C. The same monolayers from which the samples

were taken were washed three times with RPMI 1640 and the cells were scraped off with disposable Cell Scrapers in two ml of medium. Duplicate cell-samples of 0.5 ml were collected from each well and frozen at -70°C until assay. The remaining cells from each of the four scraped wells were counted on a hemocytometer. The same procedure for collecting culture supernatant fluids and adherent cells was repeated on day three and day four post infection.

Assay for SFV Content in the Collected Samples

Six-well plates were prepared to contain confluent monolayers of CEF. This procedure is described under "CEF," found on page 24. The collected virus samples were thawed and diluted serially in BHI. The monolayers were washed with Medium 199 and 0.2 ml of each virus dilution was inoculated in duplicate. The cells were incubated in a humid incubator at 37°C with 5% CO_2 for one hour, with rocking every 10 minutes. The inocula were then removed and replaced with two ml per well of MEM-agar as described. After two days of incubation, the cells were stained with two ml per well of neutral red diluted 1:11,100, and incubated at 37°C with 5% CO_2. Four hours later, the stain was removed from the wells, and plaques counted.

Neutral Red Uptake of Infected and Mock-Infected Adherent Cells

Peritoneal adherent cells were harvested from normal, female C_3H/Ten mice of six weeks in age. This procedure is described under "Mouse Peritoneal Cells," found on page 26. After the centrifugation, the cells were counted, and suspended in enough RPMI 1640 to contain the equivalent of cells harvested from one mouse per ml. Then, 0.1 ml of the cell suspension was added to each of 20 wells on six separate flat-bottomed 96-well microtiter plates (Falcon, Becton Dickinson Labware). An additional 0.1 ml was added to four wells each on a seventh 96-well microtiter plate. Two ml were added to two wells each on a six-well microtiter plate (Corning Glass Works) and all cells were incubated at $37^{o}C$ with 5% CO_2 for three hours. All 96-well plate monolayers were washed carefully with PBS using a Pasteur pipet (American Scientific Products). The monolayers in the six-well plate were rinsed three times to remove any non-adherent cells and the adherent cells were then scraped off with a disposable Cell Scraper, and counted. This was done in order to estimate the number of adherent cells in the 96-well microtiter plates. SFV was diluted to contain MOI equal to 20, two and 0.002 in 0.1 ml BHI. L929 supernatants, containing colony stimulating factor (CSF), were diluted to 10% in RPMI 1640. On each of the 96-well

microtiter plates, four wells each were inoculated with 0.1 ml BHI, 0.1ml 10% L929 supernatant, 0.1 ml viral suspension at an MOI of 20, an MOI of two, and an MOI of 0.002. The seventh plate received RPMI 1640 only. All plates were incubated at 37°C with 5% CO_2 for 30 minutes. All wells were washed once with PBS, using a Pasteur pipet. No additional washes could be performed without losing significant numbers of the cells. RPMI 1640 (0.2 ml) was added to five of the six replicate plates, and these plates were then incubated for one, two, three, four or five days. The two plates left in PBS were treated as follows: The PBS was removed with pipet and 0.1ml neutral red diluted 1:11,100 was added to all wells containing cells, plus four empty wells. These plates were incubated at 37°C with 5% CO_2 for 40 minutes. The monolayers were then washed carefully with PBS and 0.1 ml lysis buffer (25 ml of 0.2 M monobasic sodium phosphate, 25 ml distilled water, 50 ml 95% EtOH, pH 5.7) was then added to all wells. The plates were frozen at -70°C until all plates had been collected. One plate each on day one, two, three, four and five post infection was stained with neutral red as just described. For the determination of neutral red uptake by the cells, all plates were thawed, centrifuged at 500xg for 10 minutes, and 50 µl of each supernatant was transferred to new 96-well microtiter plates. These plates were then read on an ELISA-reader using the 540 wavelength filter.

Effect of Rinsing the Peritoneal Cavity Immediately Before SFV Injection

Abdominal hair was removed from female C_3H/Ten mice of 6-8 weeks in age by applying Neet lotion hair remover (Whitehall Laboratories Inc., New York, NY) for ten minutes. The abdomen was washed with soap and warm water. SFV **in vivo** stock was diluted in BHI on ice to contain $4x10^3$ and $4x10^2$ pfu per 0.2 ml. One ml Tuberculin syringes with 26 gauge 3/8 inch needles (Becton Dickinson and Co.) were loaded with the viral suspensions or BHI alone. Five ml syringes with 21 gauge 1 1/2 inch needles (Becton Dickinson and Co.) were loaded with RPMI 1640. One mouse at a time was anesthetized by placing it in a beaker containing paper towels and a few drops of Metofane inhalation anesthetic (Pitman-Moore, Inc., Washington Crossing, NJ). The hair-free area was swabbed with 70% isopropyl alcohol (American Scientific Products) and the mouse was tilted forward in one hand to allow the internal organs to fall forward. The five ml of RPMI 1640 was injected into the peritoneal cavity. The mouse was placed on the benchtop, its abdomen massaged, and the medium drawn back up into the syringe. Immediately thereafter, 0.2 ml of the viral dilution or the BHI, was injected IP. Ten mice received $4x10^3$ pfu of SFV, ten mice $4x10^2$ pfu, and five mice received 0.2 ml BHI. As controls, ten mice each were injected IP

with $4x10^3$ and $4x10^2$ pfu SFV. The mice were observed two times per day, for 21 days.

Effect on Viral Infectivity Via the Footpad Route Following Resectioning of the Sciatic Nerve

Hair was removed from the right hindleg on female C_3H/Ten mice of 6-7 weeks in age, by applying Neet lotion hair remover for ten minutes. The limb was then washed with soap and warm water. One mouse at a time was anesthetized by placing it in a beaker containing paper towels and a few drops of Metofane inhalation anesthetic (Pitman-Moore Inc.). The mouse was placed on the bench with its abdominal side facing upwards. A small paper cone lined with plastic tape measuring approximately three cm in diameter, and six cm in height, was placed over the mouse's head. The cone contained a small piece of paper towel with one drop of Metofane. A sharp incision approximately one cm long was made on the hairless area, right above the knee, pointing towards the base of the tail. Only the skin was penetrated. A pair of sterile, sharp, pointed scissors was inserted into the wound, the muscle fibers were teased apart by opening the scissors and care was taken not to damage any blood vessels. When an opening had been formed all the way to the sciatic nerve, a pair of sterile, small forceps was inserted to secure the nerve. The nerve was pulled out slightly, and cut on both sides of the forceps with a 2-3 mm long piece

being removed. A few drops of Penicillin-Streptomycin (Gibco) was placed directly into the wound, which was stitched together with Ethicon absorbable surgical suture (Ethicon, Inc., Somerville, NJ). At 24 hrs, half of the mice were injected into the right footpad with 20 pfu SFV (10 LD_{50}) diluted in 0.05 ml BHI on ice. The remaining half of the mice was injected into the left footpad. The mice were observed two times per day, for 21 days.

Infectivity of SFV on CEF Versus L929 Cells

Six six-well plates were prepared to contain confluent monolayers of CEF as described under "CEF," found on page 24. Another six six-well plates were prepared to contain confluent monolayers of L929 cells as described under "L929 Cells," found on page 25. SFV was diluted serially in BHI, on ice. All monolayers were washed with their respective growth medium and 0.2 ml of each virus dilution was inoculated in triplicate on CEF and L929 cells. The plates were incubated for 40 minutes at 37°C with 5% CO_2 and every 10 minutes the plates were rocked to spread the inocula. Finally, the inocula were removed, and replaced with two ml per well of MEM-agar overlay. After two days of incubation at 37°C with 5% CO_2, the CEF were stained with two ml per well of neutral red diluted 1:11,100. The L929 cells were stained after three days of incubation. After four hours of

incubation at 37°C with 5% CO_2, the stain was removed, and plaques counted.

Preparation of Rabbit Anti SFV Serum

Hyperimmune rabbit anti-SFV serum was obtained from New Zealand White rabbits (Graves Rabbitry, Corryton, TN) by injecting purified SFV emulsified in Freund's complete adjuvant (Gibco) intramuscularly. A week later, the virus was given in Freund's incomplete adjuvant (Gibco). Further injections were given IV without adjuvant. After at least several months of continued injections, the rabbits were bled from the ear vein, the serum collected, heat-inactivated at 56°C for 30 minutes, and gamma globulin fractionated with 37% ammonium sulfate precipitation. The precipitate was reconstituted with distilled water, dialyzed overnight versus PBS and brought to the original volume of serum.

CHAPTER IV

RESULTS

LD_{50} of SFV in Mice

C_3H/Ten Mice

The reason for proceeding with this project developed when mice were immunized with alphaviruses in order to isolate and characterize cytotoxic T lymphocytes (CTL) from the poplieteal lymph node. A preliminary experiment for that project was to determine the LD_{50} for SFV administered IP. The results were as expected with the 50% lethal endpoint being at $4x10^3$ pfu, and the average day of death (DOD) at 8.9 (figure 1). The LD_{50} was then determined when the virus was given in the hindleg footpads. The maximum volume that could be administered into one footpad was 0.05 ml and this volume of viral suspension was injected into each footpad. The results of this LD_{50} titration were surprising. As seen in figure 2, the LD_{50} for SFV in the FP is about two pfu, with the average DOD being 10.3. This finding was unusual because of the wide difference in susceptibility between IP and FP injections. In effect, the LD_{50} for FP was almost the same as the LD_{50} for the IC injection, i.e., approximately one pfu. The titration was repeated several times, with the same results.

The question was asked: Is it possible that the virus

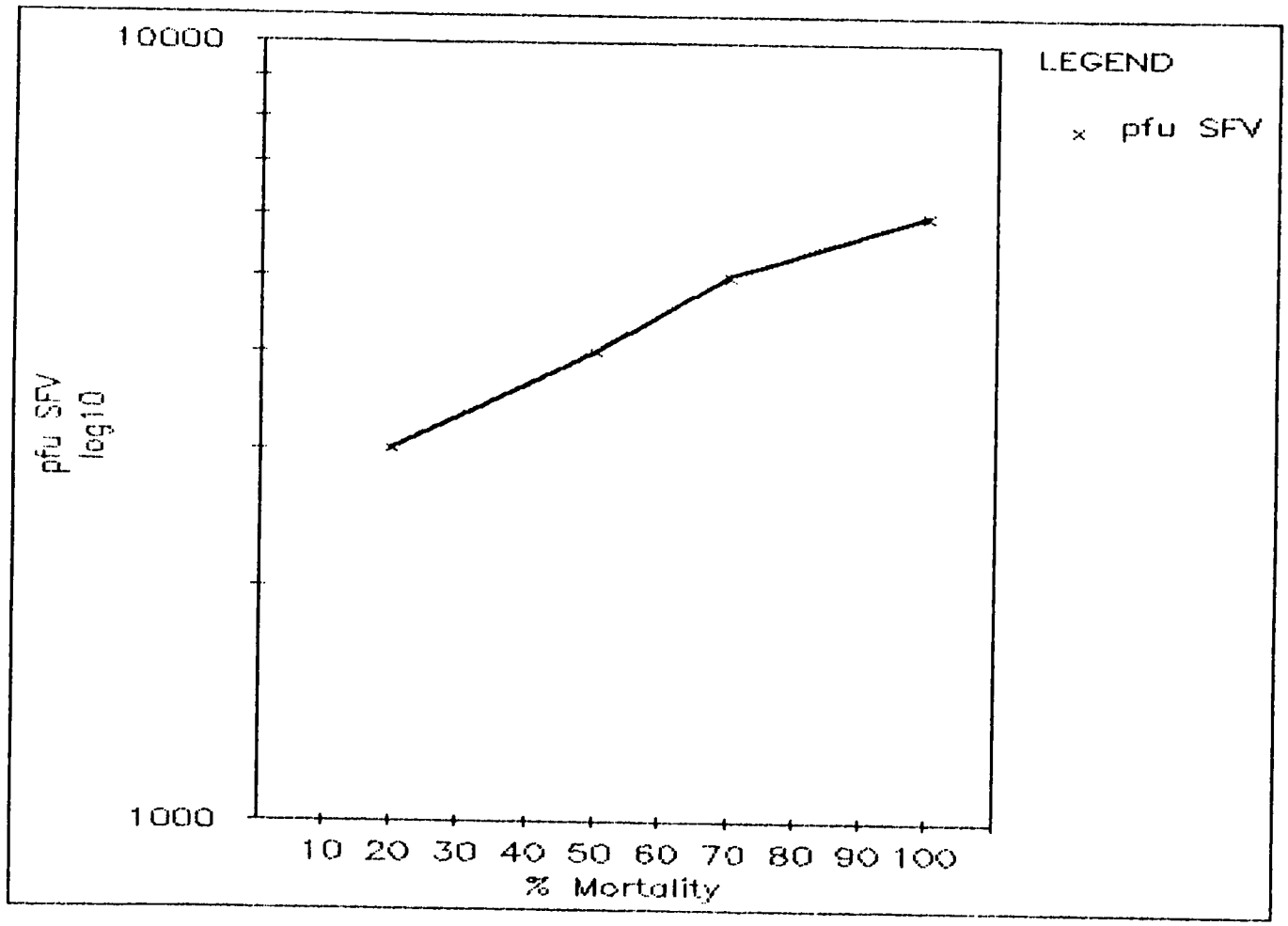


Figure 1. Mortality of C_3H/Ten mice as a function of dose of SFV given intraperitoneally.

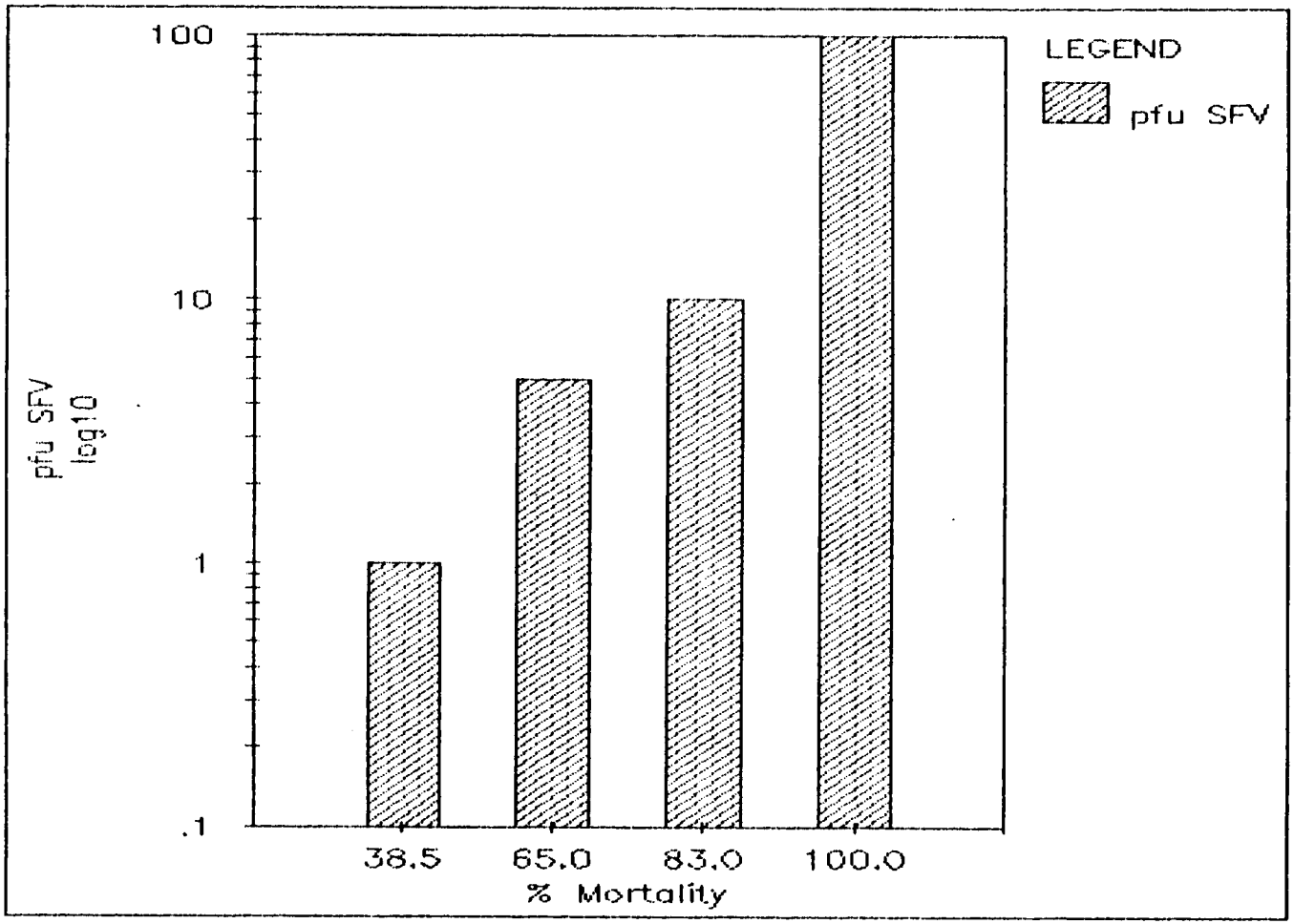


Figure 2. Mortality of C_3H/Ten mice as a function of dose of SFV given in footpads.

is indirectly infecting the nerve in the footpad rather than entering the vascular system? If it were entering the vascular system, one would have expected a titration similar to that for IP injections. Consequently, the LD_{50} for the IV route of inoculation was done. This LD_{50} (i.e., IV route) was determined to be one pfu, with the average DOD of about 10.3. These data are found in figure 3. At this time, the original project dealing with CTL was set aside, in order to answer the following question: Why is the peritoneal cavity of the mouse so refractory to SFV infection?

ICR Mice

Since the above LD_{50} titrations had been done in C_3H/Ten mice, it was of interest to determine if the same phenomenon could be seen in another strain of mouse. ICR mice were chosen because they had been routinely used in previous work in this laboratory and because they represent an outbred line of mice. It was found that the LD_{50} was at a somewhat higher value than for the C_3H/Ten mice, i.e., between 4 and $5x10^3$, with and average DOD of 7.6 (figure 4). The FP titration indicated that with 64 pfu, 20% of the animals died. These data can be seen in figure 5. The IV LD_{50} was found to be slightly >200 pfu, (figure 6) with an average DOD of 8.2. Although the FP and IV LD_{50} are higher than those observed with the C_3H/Ten mice, the mice are

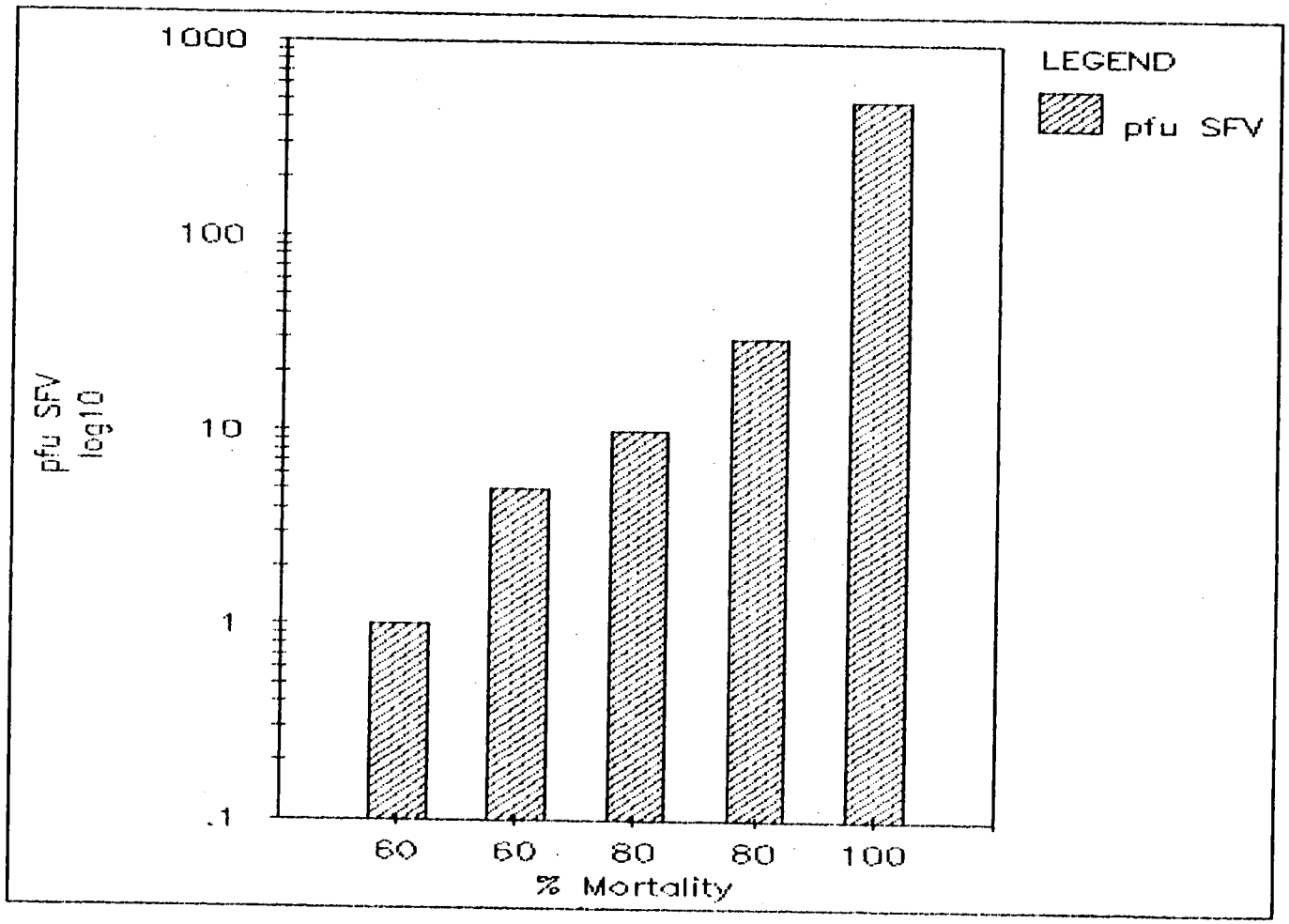


Figure 3. Mortality of C_3H/Ten mice as a function of dose of SFV given intravenously.

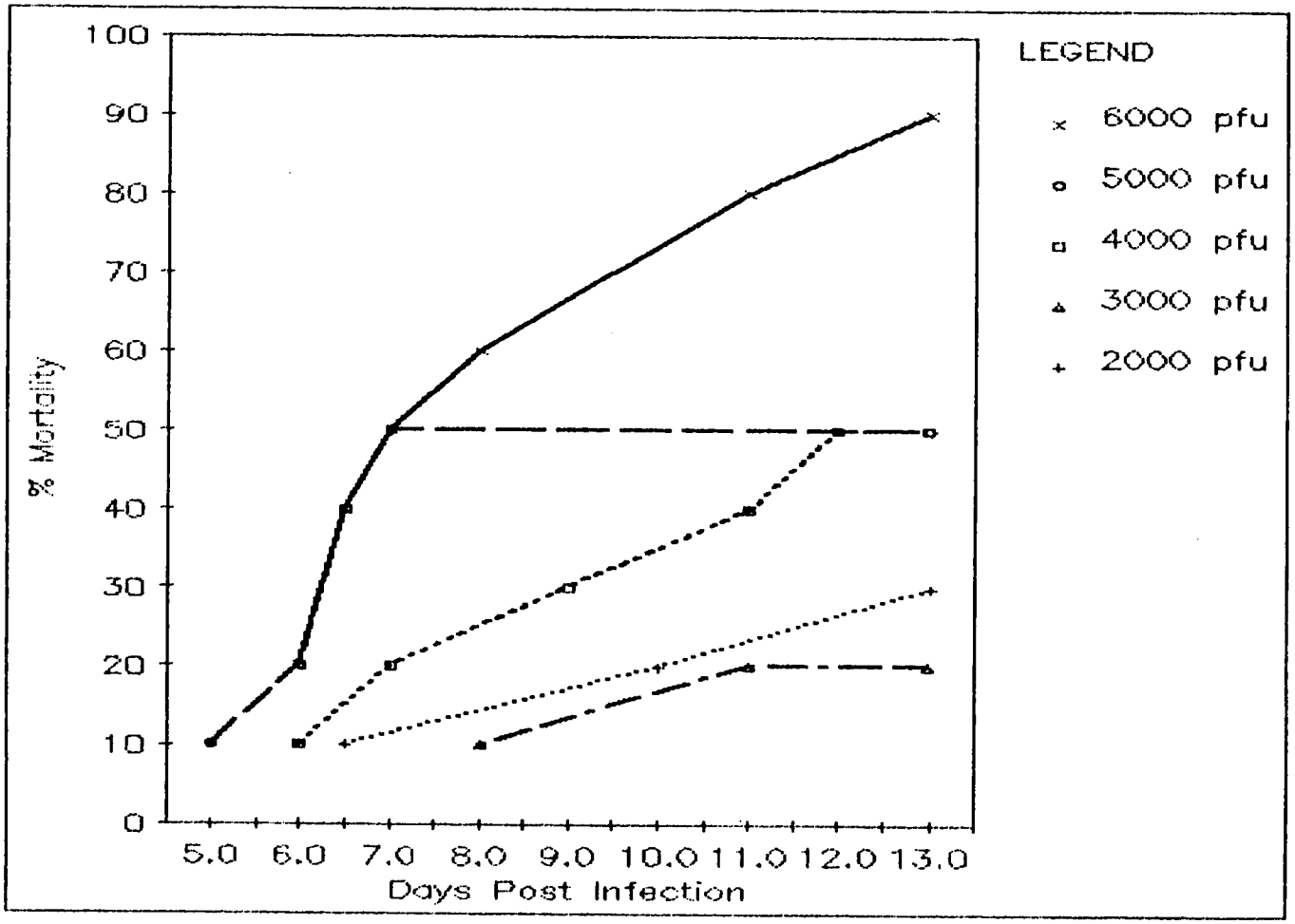


Figure 4. Mortality and day of death of ICR mice as a function of dose of SFV given intraperitoneally.

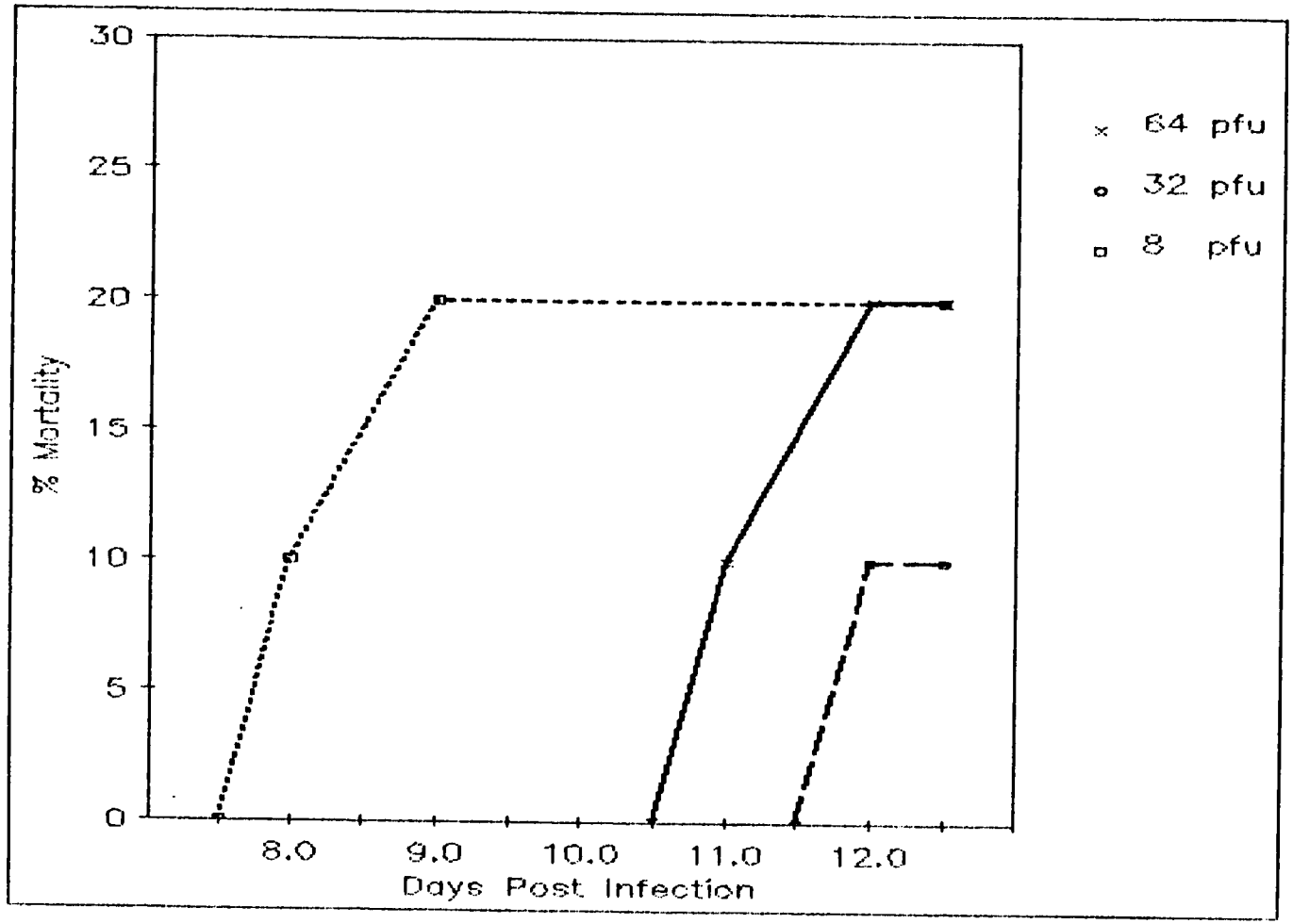


Figure 5. Mortality and day of death of ICR mice as a function of dose of SFV given in footpads.

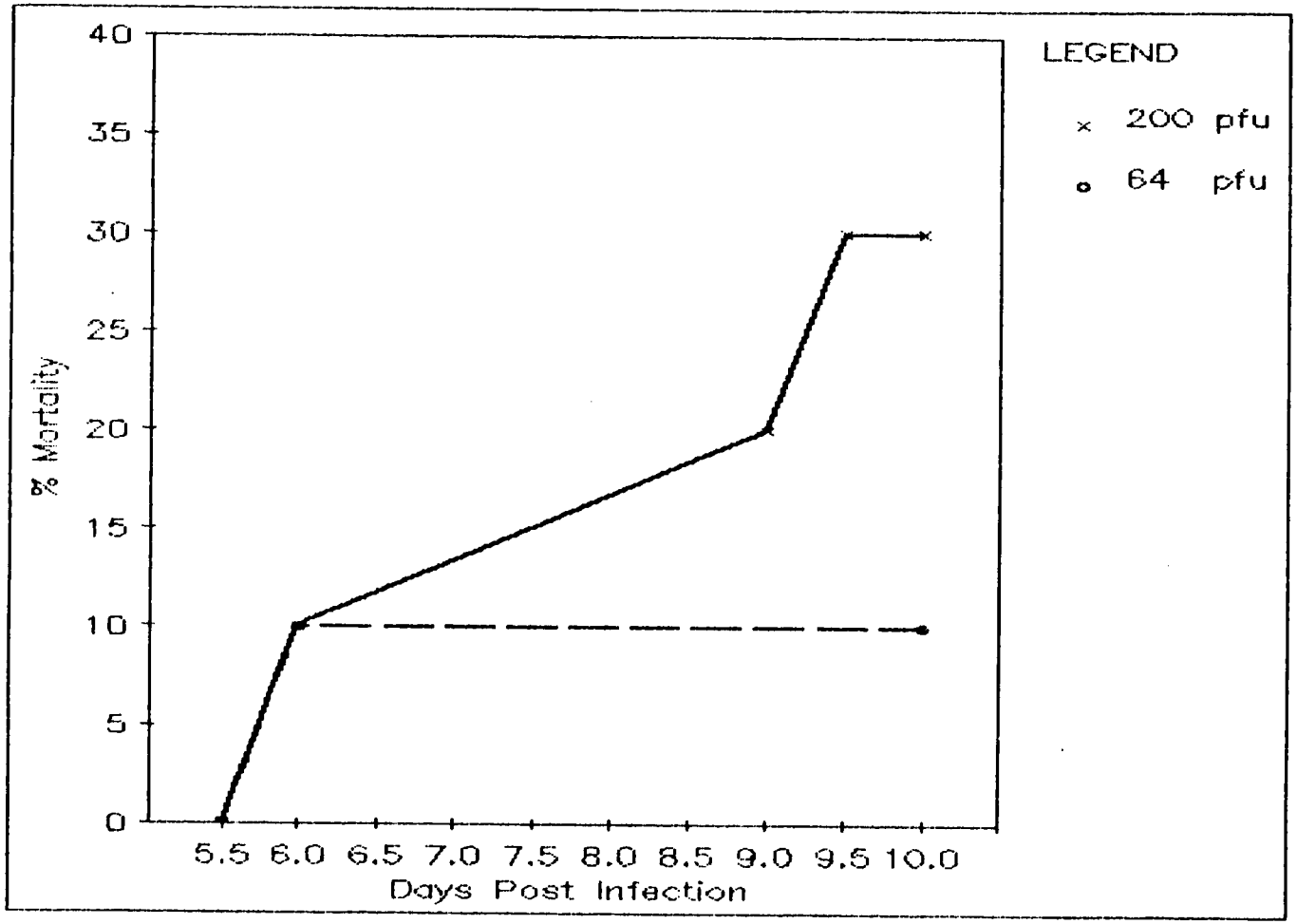


Figure 6. Mortality and day of death of ICR mice as a function of dose of SFV given intravenously.

markedly more susceptible to infection by these routes than by the IP route, which was, in fact, higher than that found in C_3H/Ten mice.

C_3H/Hen Mice

An IP LD_{50} titration was also performed in C_3H/Hen mice, and was found to be between 4 and $5x10^3$ pfu, with an average DOD of 8.0 (figure 7).

Correlation of Increasing Age with Increasing Resistance to SFV

During the course of the above-mentioned experiments, it was observed that old mice were more resistant to SFV than were younger mice. This became apparent when C_3H/Ten mice believed to be 6-8 weeks of age (but in fact were younger) showed an IP LD_{50} of <40 pfu! This is shown in figure 8. During this experiment it was noted that the mice appeared small. The weights of these mice are shown in figure 9. The growth of the mice as a function of age suggested that the mice used were in the middle of a growth spurt. The C_3H/Ten mice are supposed to be identical to C_3H/Hej mice. When the curve for C_3H/Ten mice was compared to one provided by Jackson Laboratory (Bar Harbour, ME) for C_3H/Hej mice (figure 9), our curve appeared to be shifted to the right, i.e., the mice gained weight at a time later than expected. Indeed, when the IP LD_{50} titration was done in

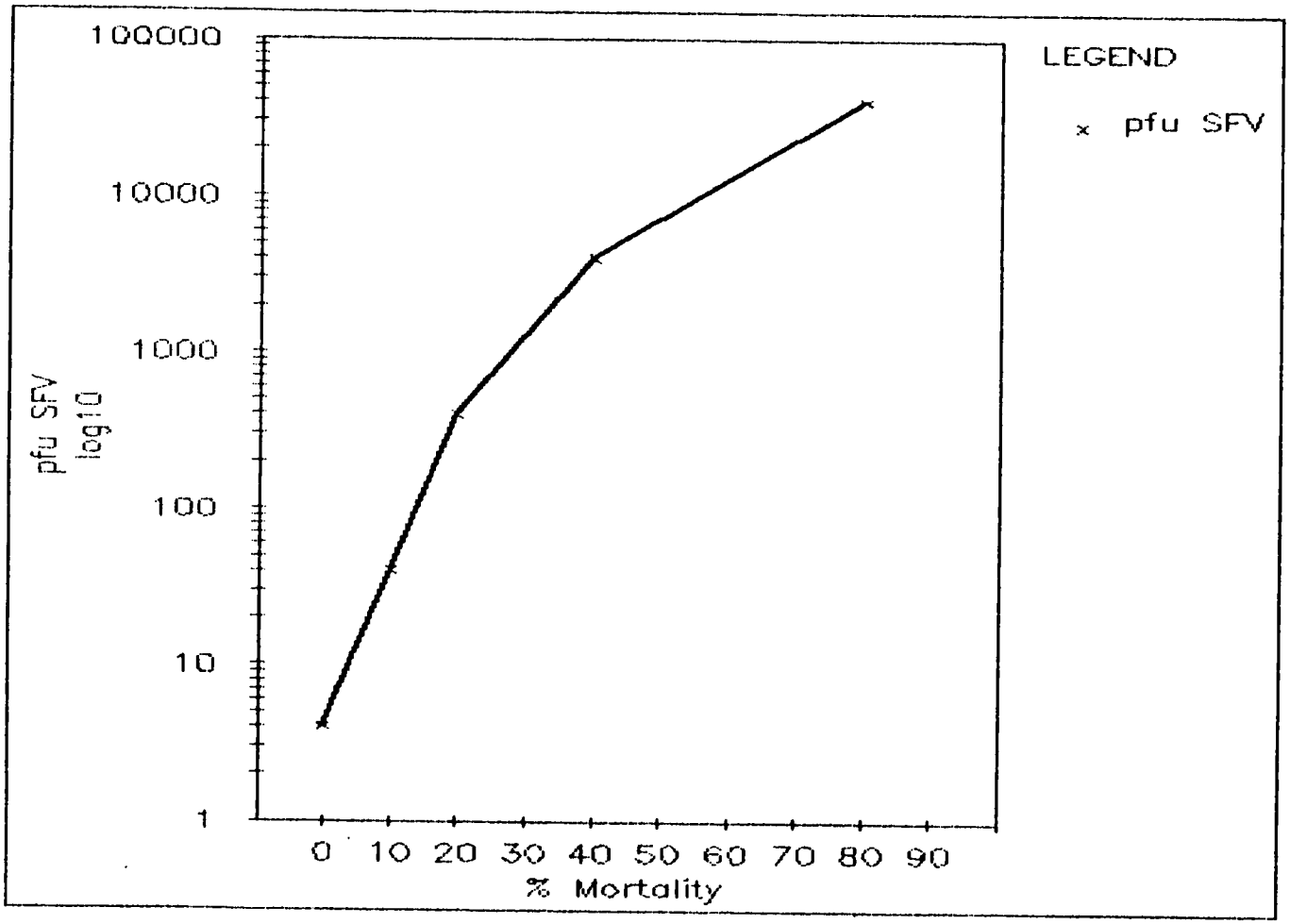


Figure 7. Mortality of C_3H/Hen mice as a function of dose of SFV given intraperitoneally.

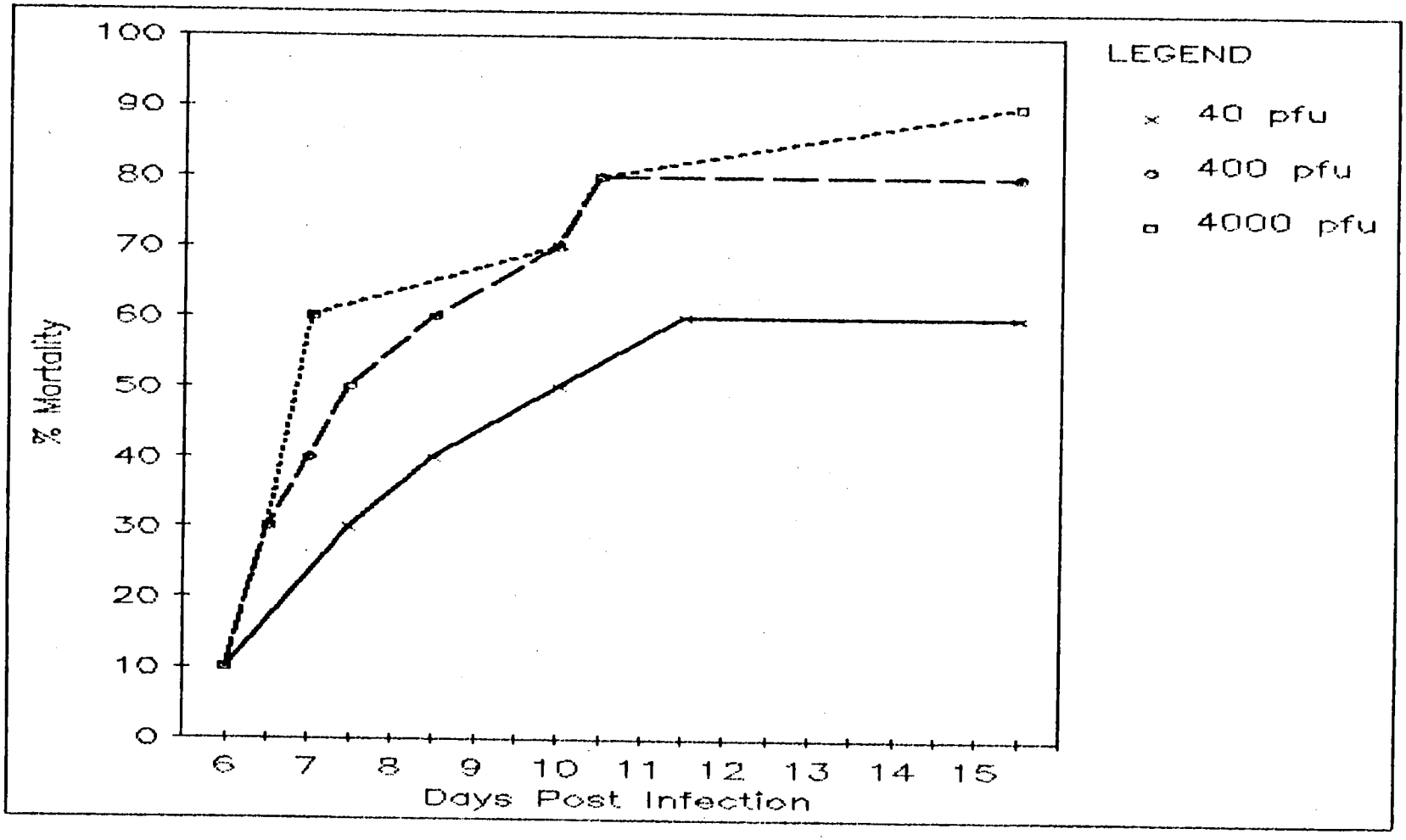


Figure 8. Mortality and day of death of young C_3H/Ten mice as a function of dose of SFV given intraperitoneally.

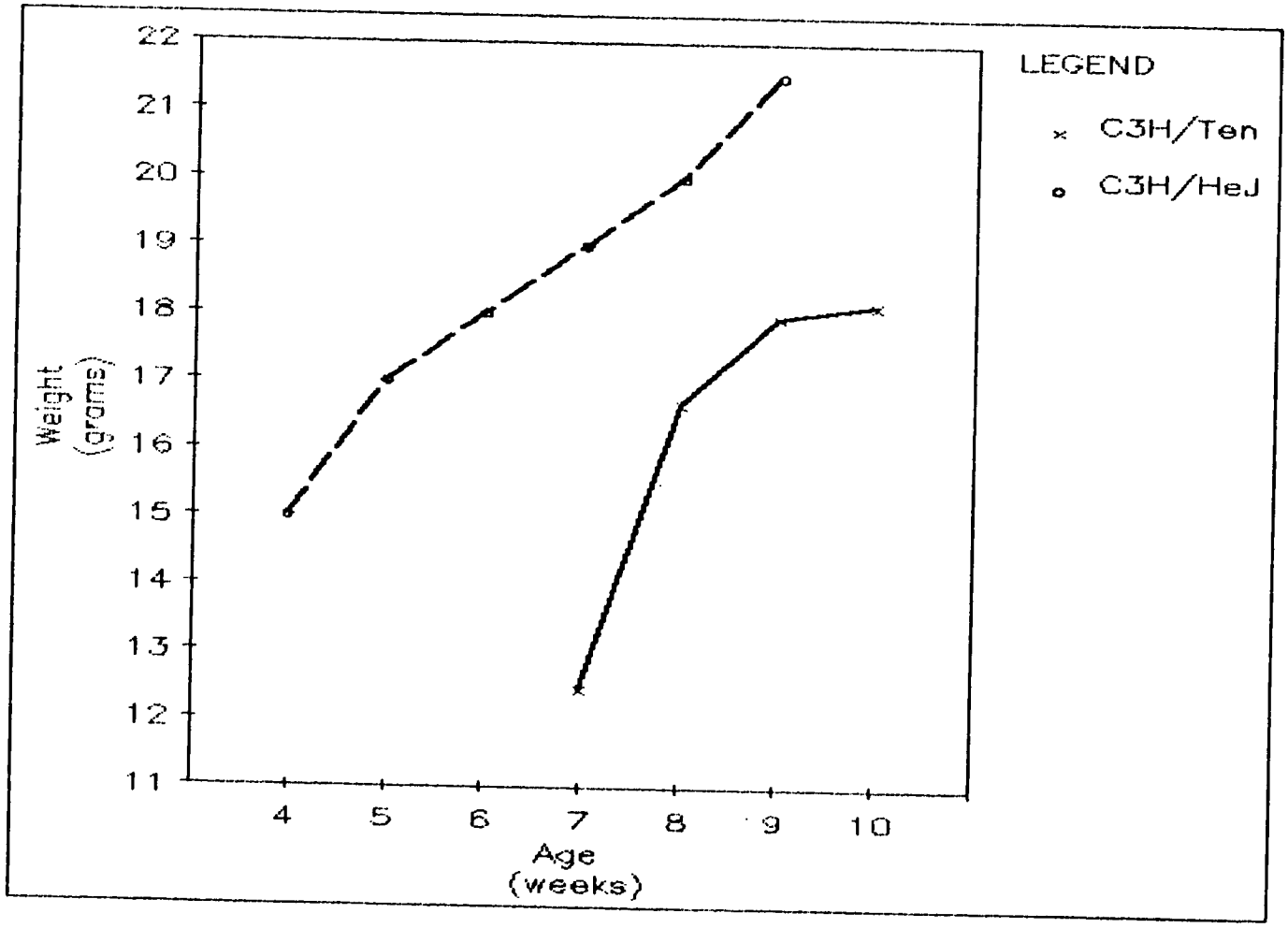


Figure 9. Weight of C_3H/Ten and C_3H/HeJ mice as a function of age. Source of C_3H/HeJ data: Jackson Laboratory, Bar Harbour, Maine.

older mice (16-18 weeks) compared to younger adult mice (6-8 weeks, at mature weight), the older mice were found to be more resistant to SFV infection (figure 10). A similar comparison was made for the FP route of infection, and the older mice were again found to be more resistant to SFV infection (figure 11). Owing to this increasing viral resistance with age, all animals were weighed and used only if they were between 17.5 and 19.0 grams.

Assay of the Peritoneal Cavity For Anti-Viral Factors

Since the IP LD_{50} was much higher than that by other known routes, it was assumed that some property of the peritoneal cavity rendered the mice resistant to SFV. It is important to note that the resistance could be overcome by increasing the dose of virus. The first attempt to identify the feature responsible for providing resistance in the peritoneal cavity was to assay for possible anti-viral factors present in the peritoneal cavity.

Short Pre-Treatment of Cells and Virus with Cell-Free Peritoneal Fluid

Virus inoculated into the peritoneal cavity is known to escape into the circulation within minutes after inoculation (38),(64). Therefore, any anti-viral factors present must inactivate the virus or protect the cells very rapidly,

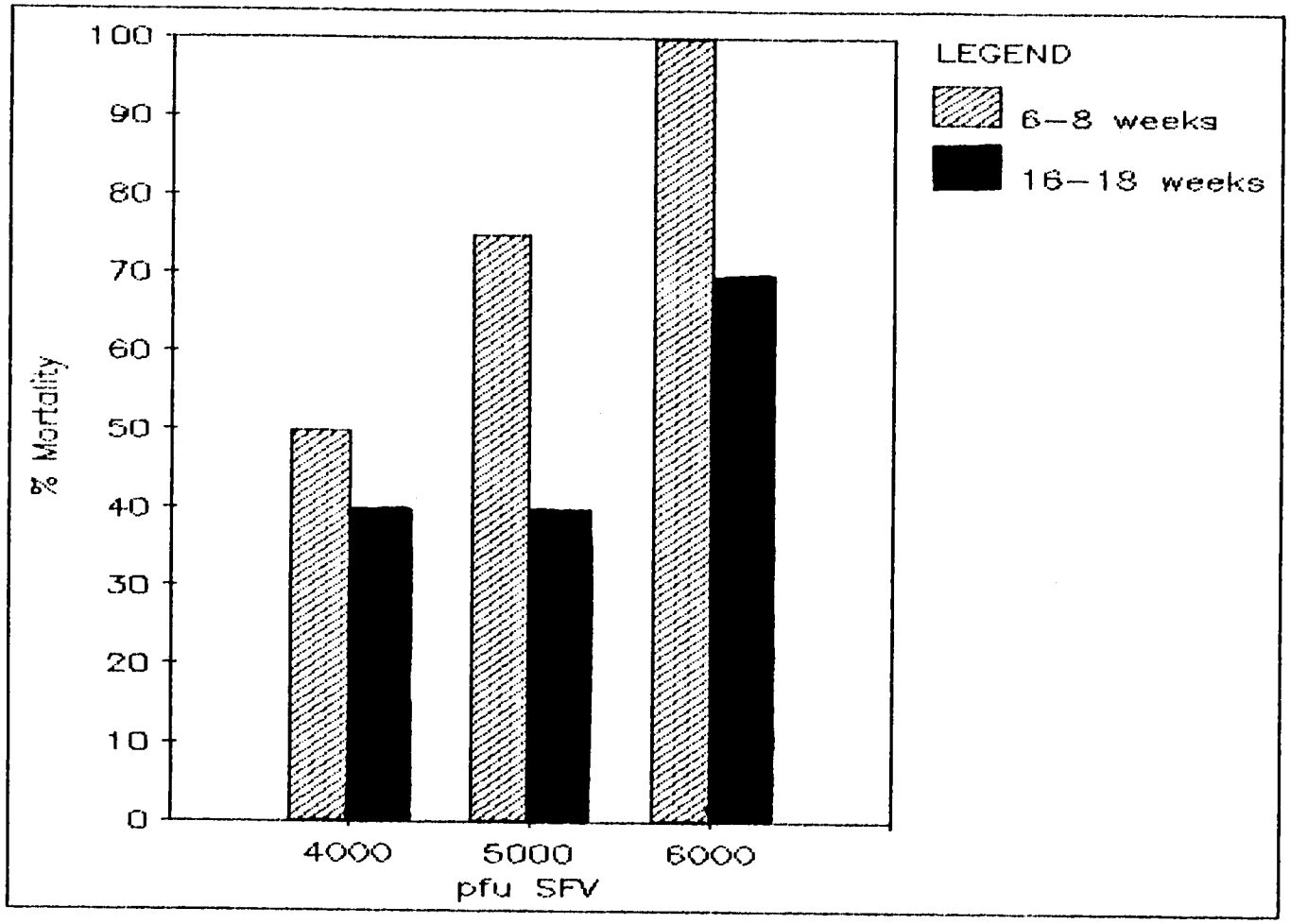


Figure 10. Mortality of 6-8 week old versus 16-18 week old C_3H/Ten mice as a function of dose of SFV given intraperitoneally.

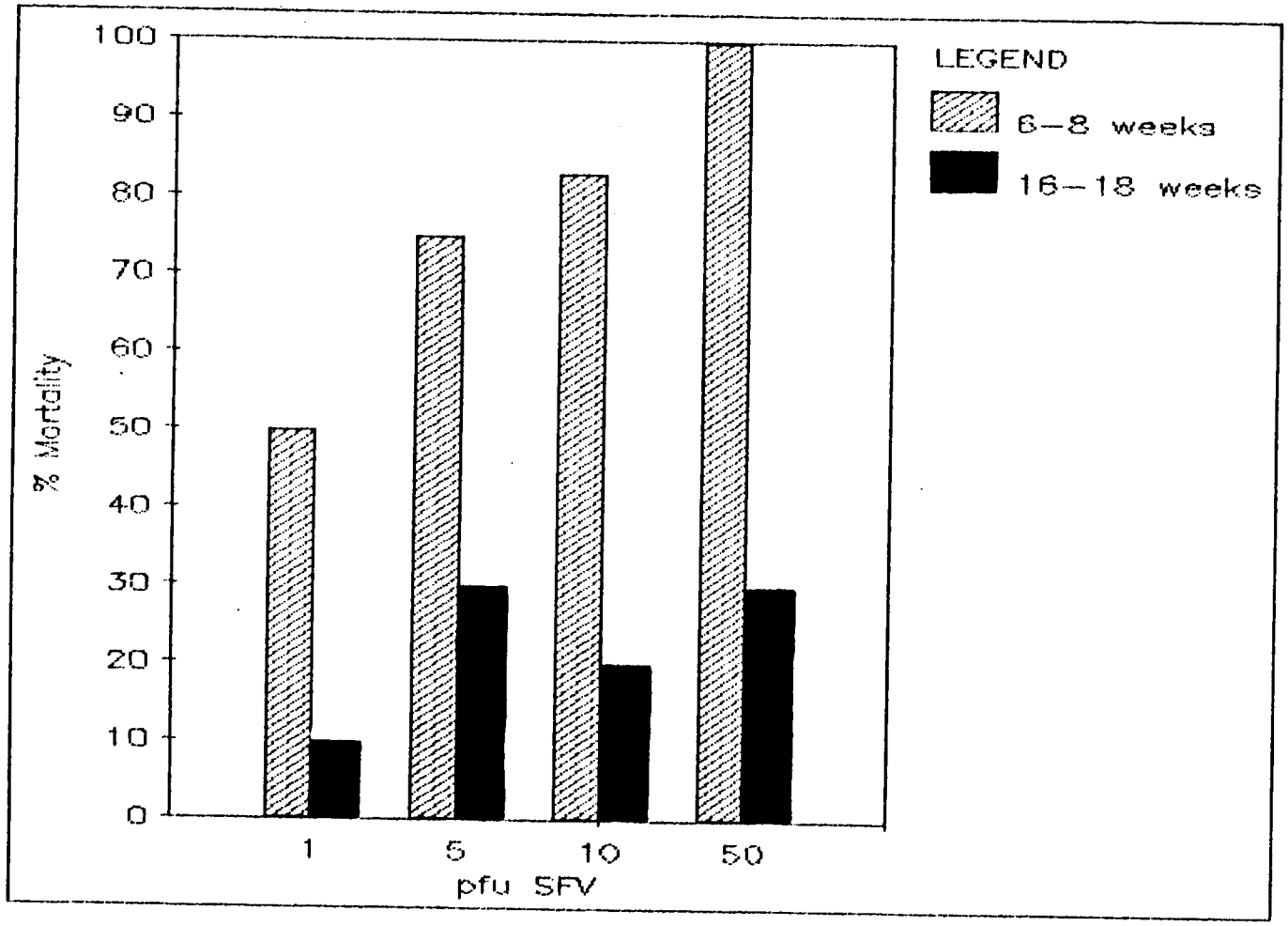


Figure 11. Mortality of 6-8 week old versus 16-18 week old C_3H/Ten mice as a function of dose of SFV given in footpads.

otherwise, virus should escape into the circulation, and cause death with only a few pfu. To test for the presence of anti-viral factors, the peritoneal cavity was rinsed with injected medium, the medium collected, the cells removed from the fluid by centrifugation, and the supernatant fluid assayed for anti-viral properties. The results are presented in figure 12. Pre-treatment of L929 cells for 1.5 hours with the peritoneal wash before infection with SFV, or pre-treatment of the virus itself for 30 minutes before addition to L929 cells, did not significantly protect the cells from virus infection, nor did it inactivate the virus itself. It was possible, however, that the cells in the peritoneal cavity might be protected after longer exposure to the presumptive factor(s).

Extended Pre-Treatment of L929 Cells with Cell-Free Peritoneal Fluid

In this experiment, the L929 cells were exposed to the cell-free peritoneal fluid for 18 hours before the addition of SFV. It is important to note that the fluid of the peritoneal wash was at a concentration of 25% in the cell growth medium. This dilution was considered appropriate since the LD_{50} between the IP and FP or IV injections was so great, that the concentration of any anti-viral factor must be relatively high, or very potent. However, as presented in figure 13, pre-treatment of L929 cells with peritoneal

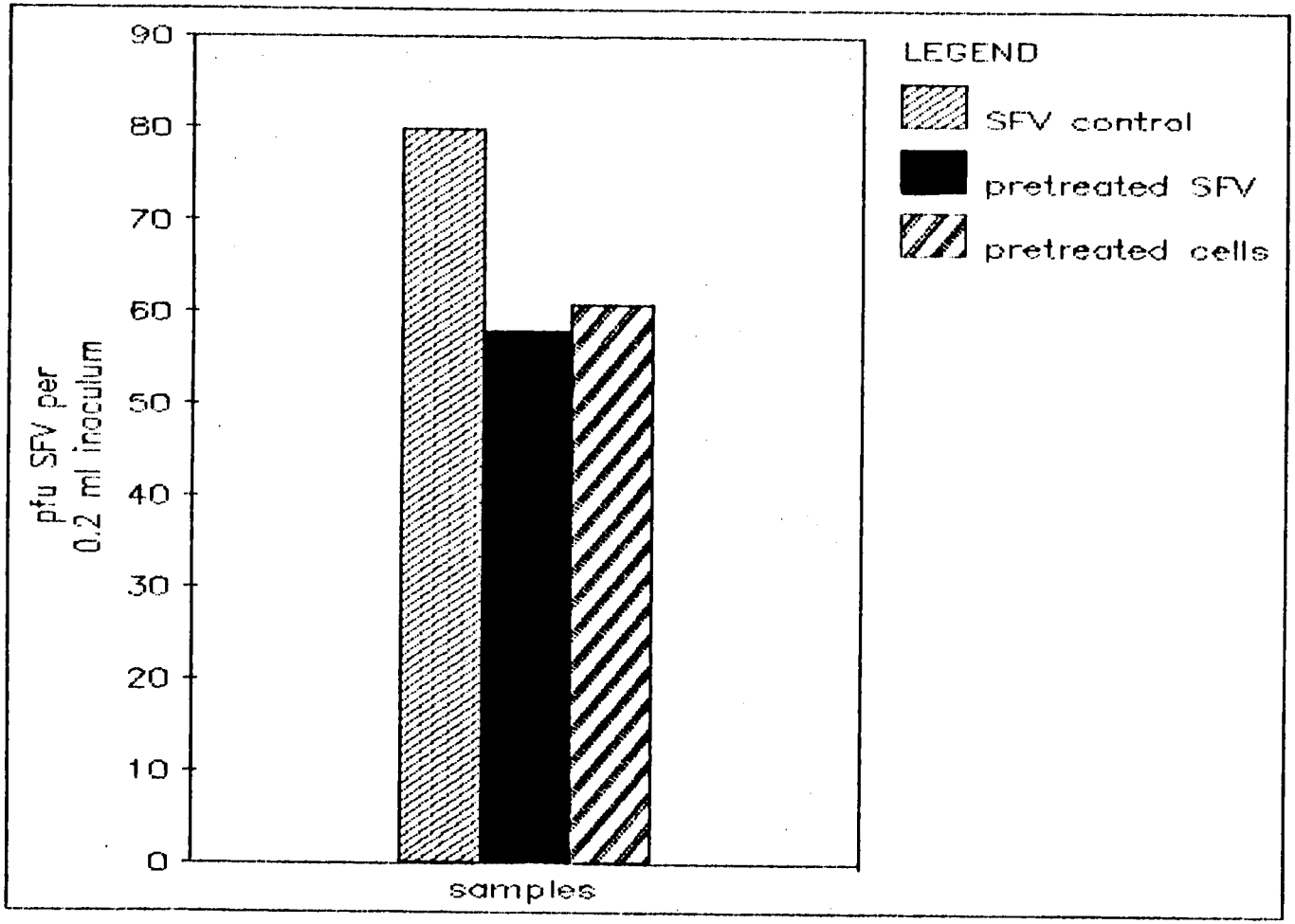


Figure 12. Number of plaques of SFV on L929 monolayers pretreated with cell-free peritoneal wash for 1.5 hours, or SFV pretreated with cell-free peritoneal wash for 30 minutes.

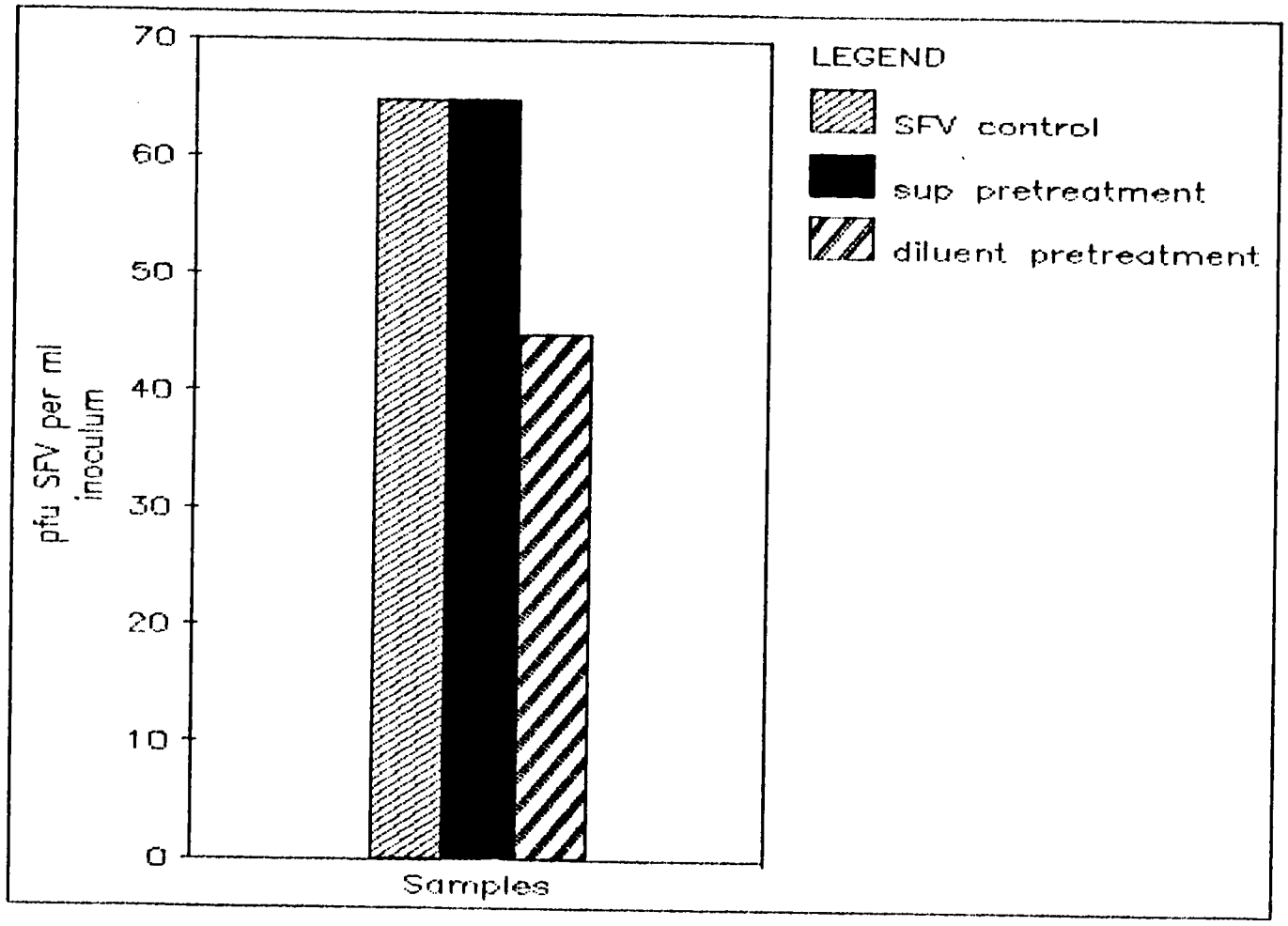


Figure 13. Number of plaques of SFV on L929 monolayers pretreated with a 1:4 dilution of cell-free peritoneal wash for 18 hours.

wash for 18 hours did not significantly protect the cells from SFV infection. Cells pre-treated with the supernatant fluid diluent alone had no effect on viral infectivity. Again, no protection of the L929 cells against SFV could be seen (figure 13).

Viral Yields from SFV-Infected L929 Cells in the Presence of Peritoneal Wash

In another attempt to detect anti-viral properties in the peritoneal wash, growth medium containing 10% cell-free peritoneal wash was added to infected L929 cells. At various time intervals, samples were taken from the supernatant fluid of the infected cells. As shown in figure 14, the viral yields from the cells grown in the presence of peritoneal wash is virtually the same as from cells without the wash. It was concluded that anti-viral factors are not constitutively present in the peritoneal cavity.

Assay of Peritoneal Resident Cells for Production of Anti-Viral Factor(s) **In Vitro**

It was still a possibility that anti-viral factors could be produced by peritoneal cells, and this had to be investigated. Briefly, normal peritoneal cells were harvested, cultured **in vitro** for two hours, centrifuged to remove the cells from the growth medium, and the fluid was added to agar overlays of infected L929 cells. Figure 15

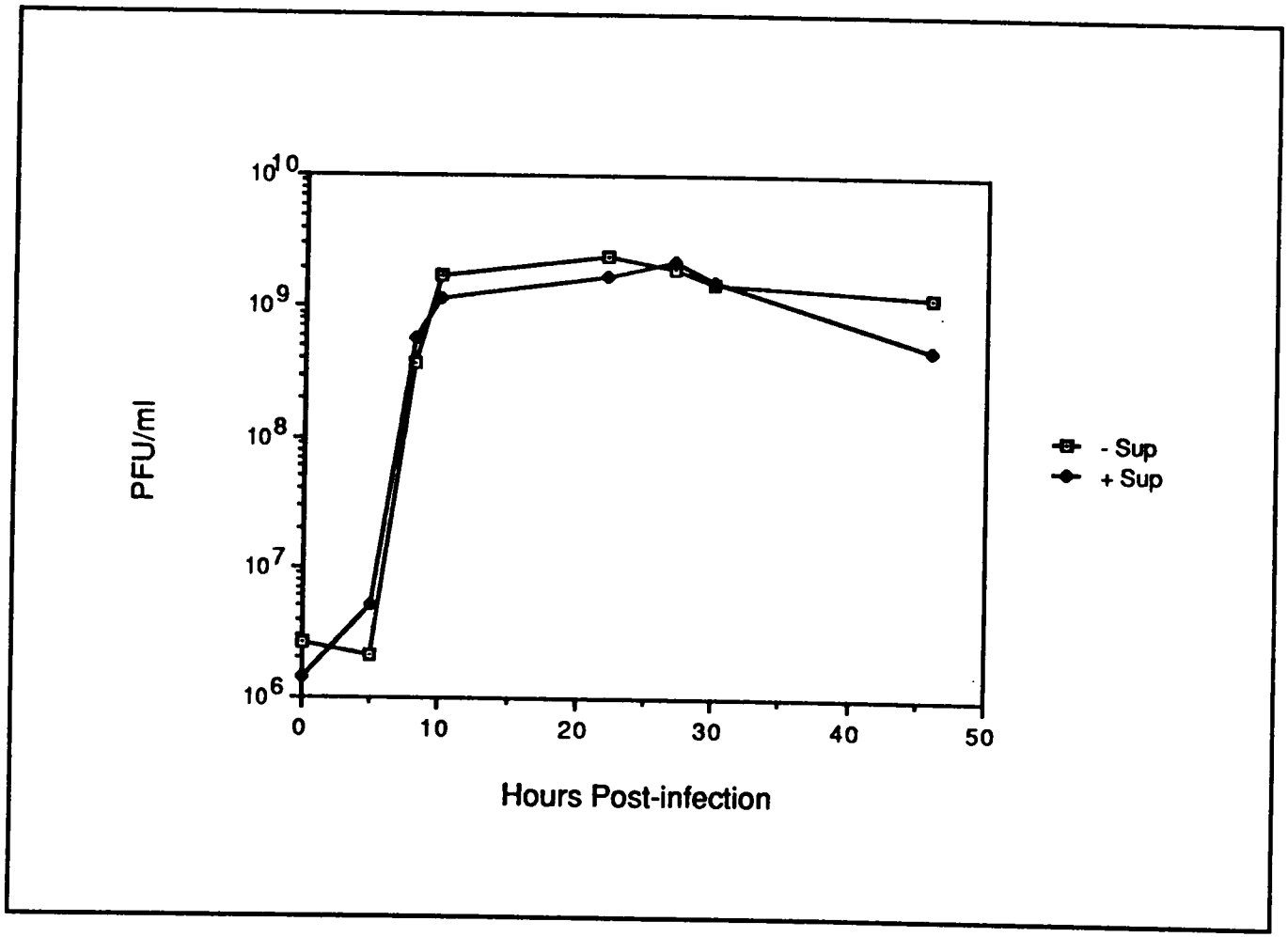


Figure 14. Viral yields from SFV-infected L929 cells cultured in the presence or absence of a 1:10 dilution of cell-free peritoneal wash.

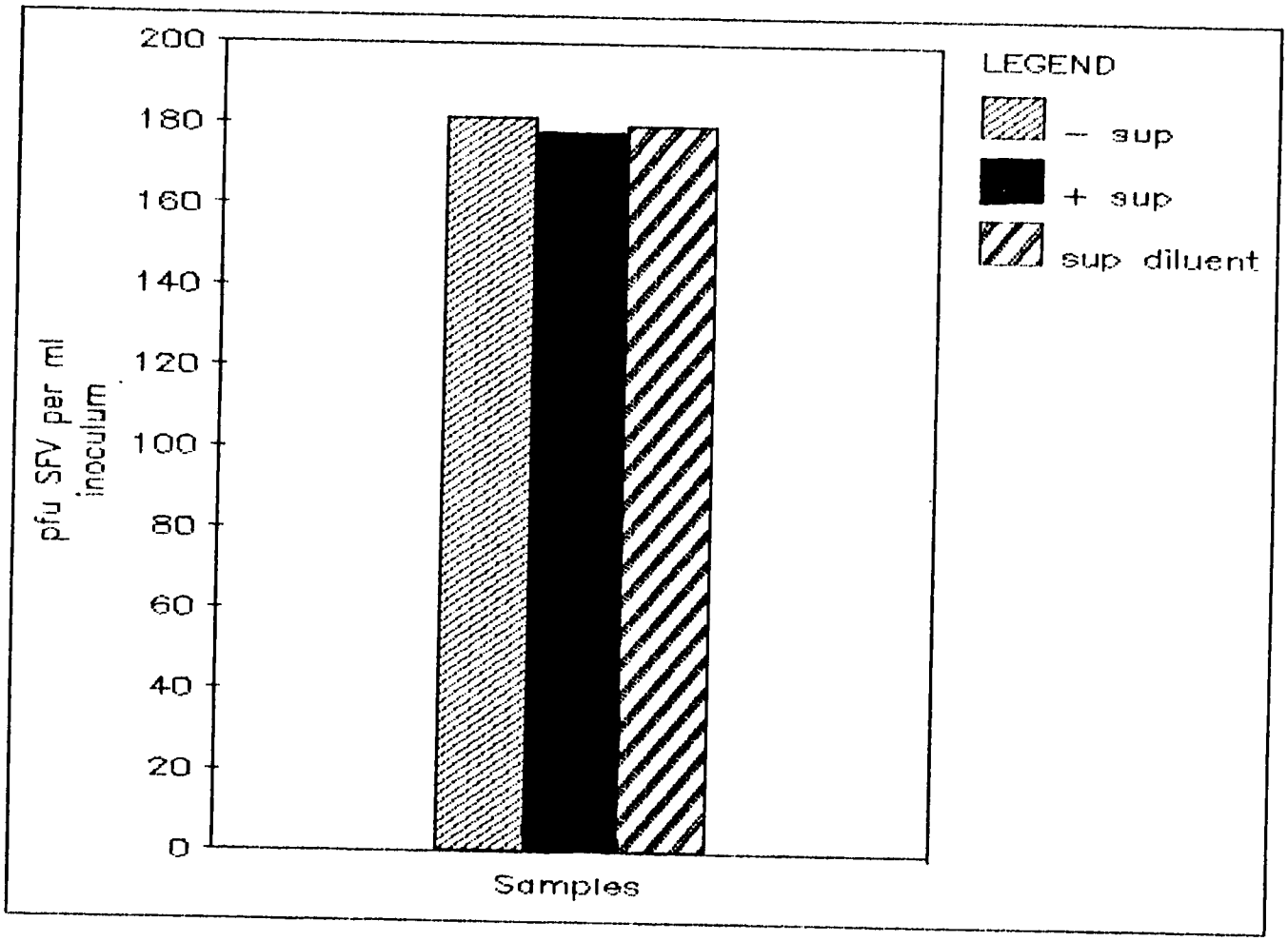


Figure 15. Number of plaques of SFV on L929 monolayers cultured with agar overlays containing a 1:5 dilution of fluid from a two hour culture of peritoneal cells.

presents the results. As can be seen, the presence of the supernatant fluid had no effect on plaque formation. Thus, this experiment suggests that peritoneal cells do not produce anti-viral factors constitutively, at least not at a high enough rate and concentration to account for the barrier-effects of the peritoneal cavity which limits viral spread. Another possibility, however, would be that peritoneal cells do not produce anti-viral factor(s) until exposed to the virus. The following experiment was designed to test this possibility.

Assay of the Peritoneal Cavity for Anti-Viral Factor(s) After SFV Infection

To test the possibility of induction of factors by virus, the peritoneal cavity was assayed for anti-viral properties after SFV-infection. Mice were infected, and the supernatant wash collected 24 hours later. As controls, L929 cells were treated for 24 hours with 100 International Reference Units (IRU) IFN α/β , supernatant wash from normal mice, UV-irradiated IFN α/β , and the already mentioned solutions which had been exposed to anti-IFN α/β for one hour before addition to the L929 cells. The wash from infected mice was UV-irradiated to inactivate the virus. It is important to note in this series of experiments that the supernatant wash was added undiluted to the L929 cells. The results are given in figure 16, and the following features

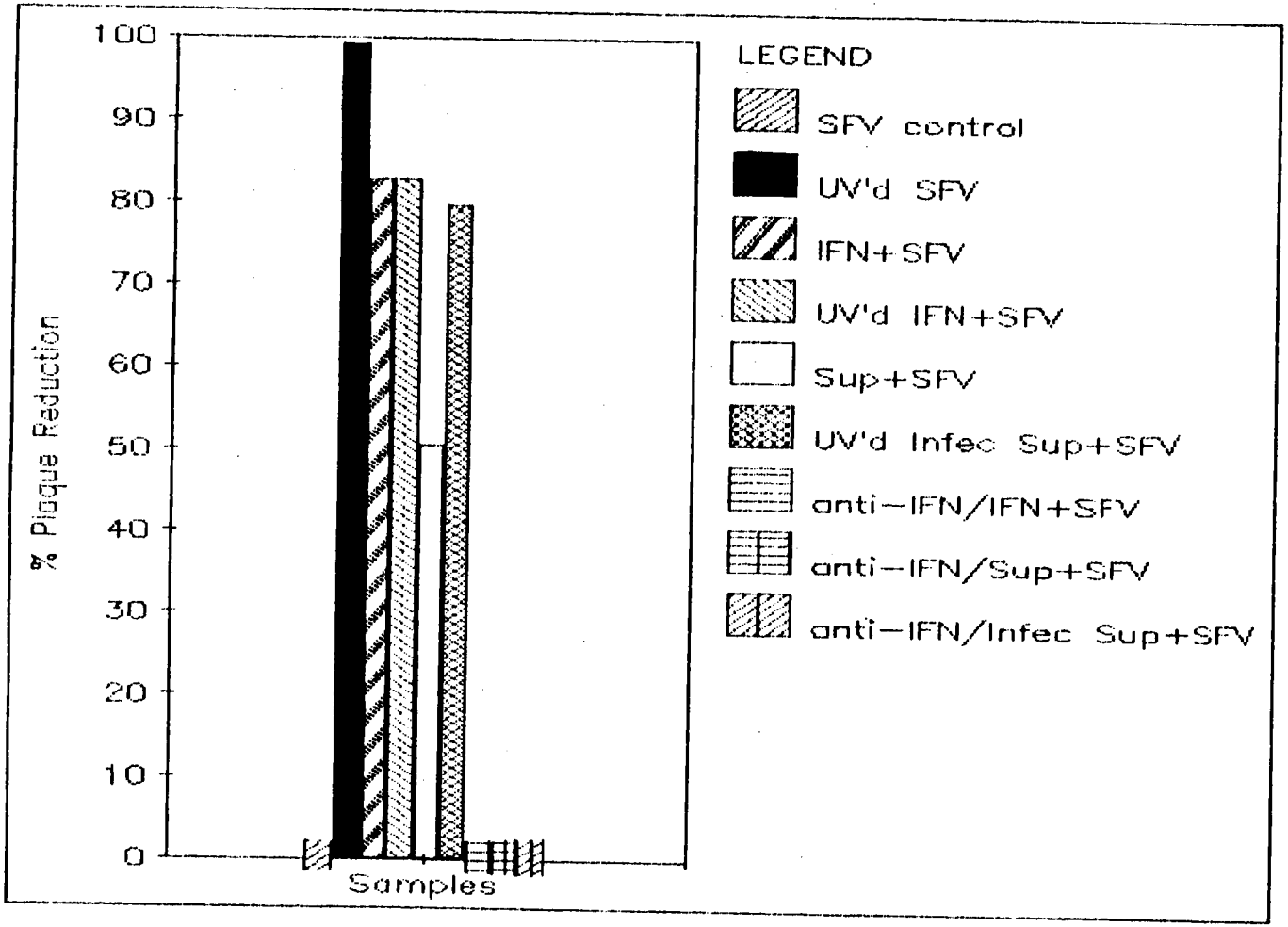


Figure 16. Percent plaque reduction of SFV on L929 monolayers pretreated for 24 hours with IFN α/β, normal cell-free peritoneal wash or UV-irradiated infected cell-free peritoneal wash.

are noted: First, this relatively high level of IFN caused plaque reduction by only 83% compared to untreated controls. Second, the normal undiluted supernatant wash caused a 50.7% plaque reduction. This is theoretically equal to one IRU or lower. In the earlier experiments, where a 25% concentration of supernatant wash was added for 18 hours in attempts to pre-treat L929 cells, no protection was observed. Owing to the low concentration of supernatant wash in that experiment, the protection of cells from SFV infection could not have been detected.

In the present experiment, undiluted supernatant wash from infected mice caused a plaque reduction which appeared to equal that caused by 100 IRU of IFN per ml of medium. Also, the protection of cells from SFV could be abolished with anti-IFN α/β serum. I conclude from this experiment that normal mice have a maximum of one IRU of IFN α/β in their peritoneal cavity, and that this level may increase marginally after SFV infection. However, although small amounts of IFN may be produced, it does not appear to protect L929 cells efficiently **in vitro** against SFV infection.

Effect of Anti-IFN α/β on the LD_{50} of SFV Administered IP

Since it was shown in the previous experiment that IFN α/β is produced during the course of an SFV infection of the peritoneal cavity, it was important to determine what effect the IFN has on the outcome of the infection. In other words, is IFN part of the barrier imposed on the virus in the peritoneal cavity? Mice were inoculated IP with anti-IFN α/β serum at a low dose, 1.25×10^3 Standard Neutralizing Units (SNU), and at a higher dose, 8.6×10^3 SNU. The low dose was calculated in the following way: If a mouse has 100 IRU of IFN α/β per ml in the peritoneal cavity after infection (aproximately 1 ml equivalent), one 25 gram mouse (25 ml equivalent) contains approximately 2.5×10^3 units of IFN α/β , assuming that IFN is equally distributed in the body. One half of that amount is 1.25×10^3, and 3.4 times that amount is 8.6×10^3 IRU. Note that the mice used were not 25 g, but of the same weights as descfribed previously. The values used for calculating the doses of serum are, however, purposely set high.

As presented in figure 17, mice given a "low" dose of anti-IFN α/β increases the susceptibility of those mice to SFV challenge (< 1 LD_{50}). A higher dose of anti-IFN α/β increases the susceptibility of the mice even more. Despite this increased susceptibility, anti-IFN α/β alone can not

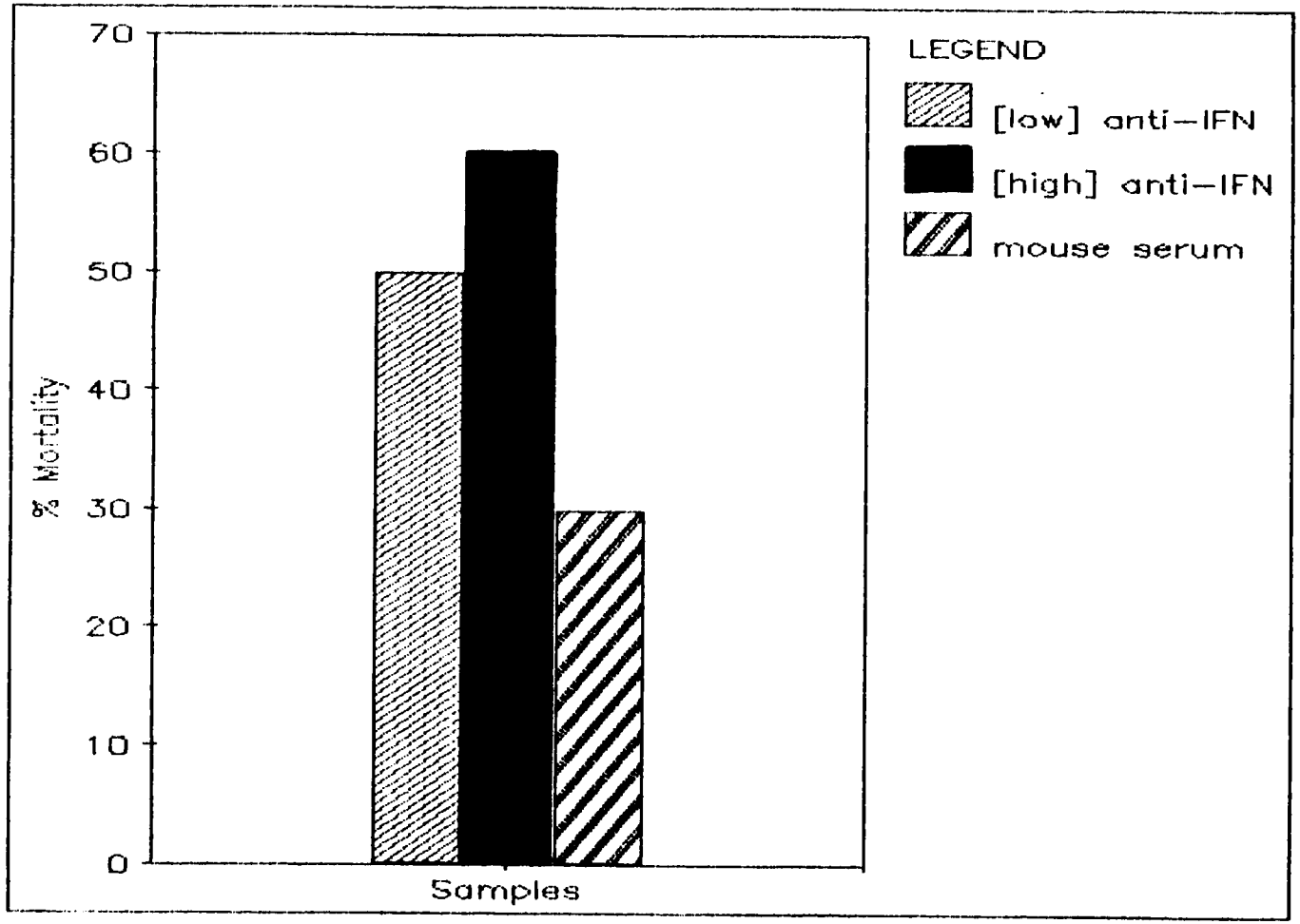


Figure 17. Mortality of mice to SFV given intraperitoneally after treatment with 1.25×10^3 SNU or 8.6×10^3 SNU of anti-IFN α/β. SFV = 0.8 LD_{50}

eliminate the barrier the peritoneal cavity imposes on the infecting virus. Therefore, the barrier cannot be IFN alone.

Effect of Indomethacin Treatment on the LD_{50} of SFV Administered IP

One question which arose during the course of this project was whether or not prostaglandins could be responsible for protecting the peritoneal cavity. The peritoneal cavity is a rich source of macrophages, which produce prostaglandins (12),(15),(8). It was therefore of interest to investigate the role of prostaglandins further. Indomethacin, which can inhibit prostaglandin synthesis (12),(15),(8), was injected IP in mice a day before, one hour before, and a day after viral challenge. If prostaglandins were involved in the early protective responses, this regimen should ensure its detection. As seen in figure 18, administration of Indomethacin had no effect on the viral infection. On the contrary, Indomethacin treatment actually protected mice somewhat when compared to control mice in which only the diluent of Indomethacin ($NaHCO_3$) was given. Therefore, prostaglandins could be ruled out as a protective barrier for the peritoneal cavity.

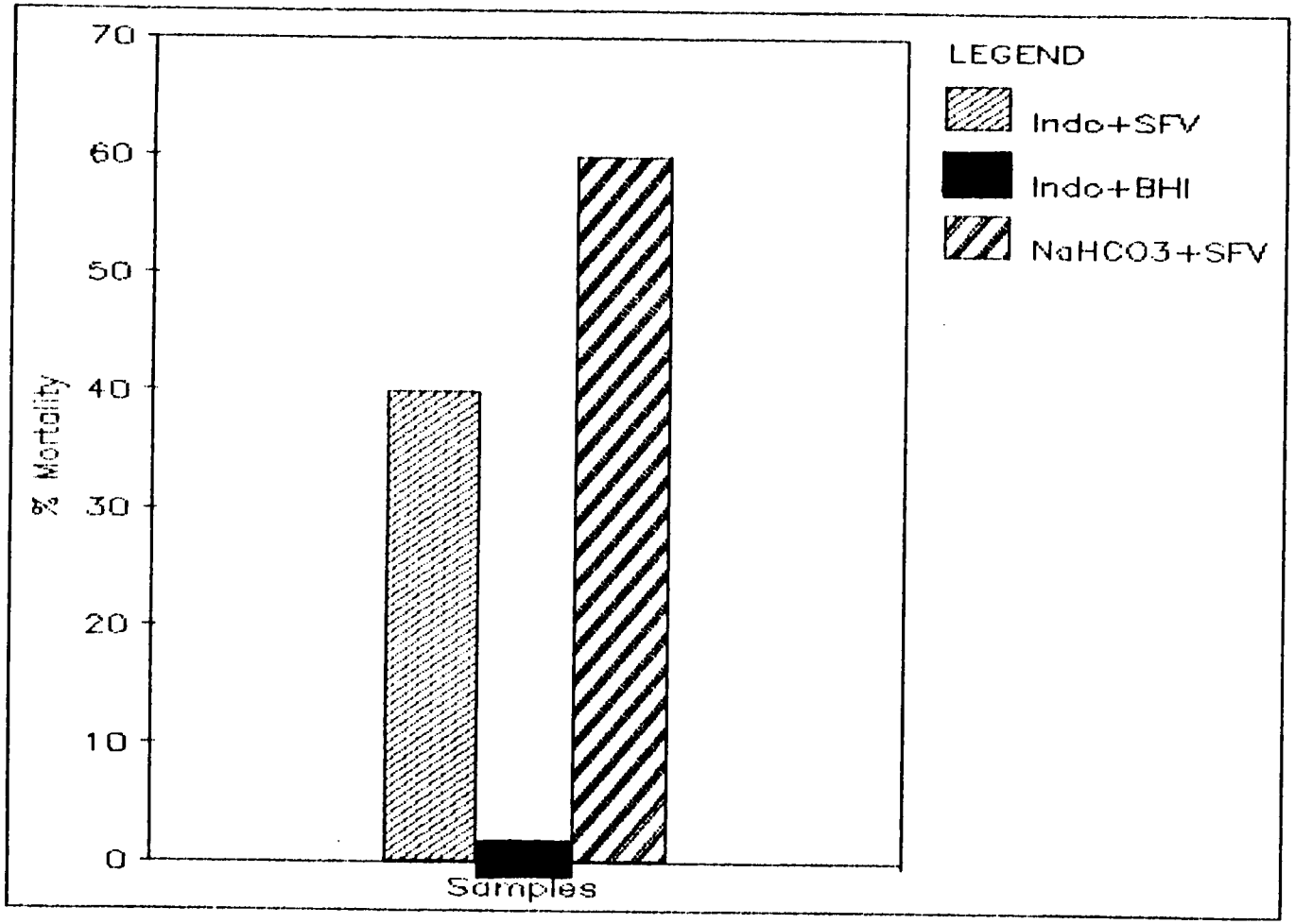


Figure 18. Mortality of mice to SFV given intraperitoneally after treatment with Indomethacin. Dose of SFV = 0.8 LD_{50}

Effect of LPS Treatment on the LD_{50} of SFV Administered IP

Owing to the fact that the C_3H/Ten mice had shown a delayed weight gain relative to age, it was suspected that these mice no longer were identical to the C_3H/Hej mice, the original parent. Furthermore, it is also known that C_3H/Hej mice are resistant to LPS due to a defect in the synthesis of lipoprotein lipase (8),(34),(66). If the C_3H/Ten mice were identical to C_3H/Hej mice, LPS treatment would have no effect. To test this, the C_3H/Ten mice were treated with LPS. At all doses of virus tested, LPS administration reduced the mortality of the mice (figure 19). This finding suggests that C_3H/Ten mice are not identical to C_3H/Hej mice, and that macrophages are involved in protecting the peritoneal cavity, since it is known that macrophages from sensitive animals produce cachectin (which suppresses lipoprotein Lipase) in response to LPS (35). Furthermore, it is also known that LPS causes increased phagocytosis of macrophages, and also causes them to be metabolically more active (8). Another interesting point is that LPS is known to cause production of IFN α/β (96), and that some IFN can be produced from macrophages in response to bacterial endotoxins (61). This would further support the findings presented in figure 17, which suggest that IFN α/β has some protective effects for the peritoneal cavity against SFV,

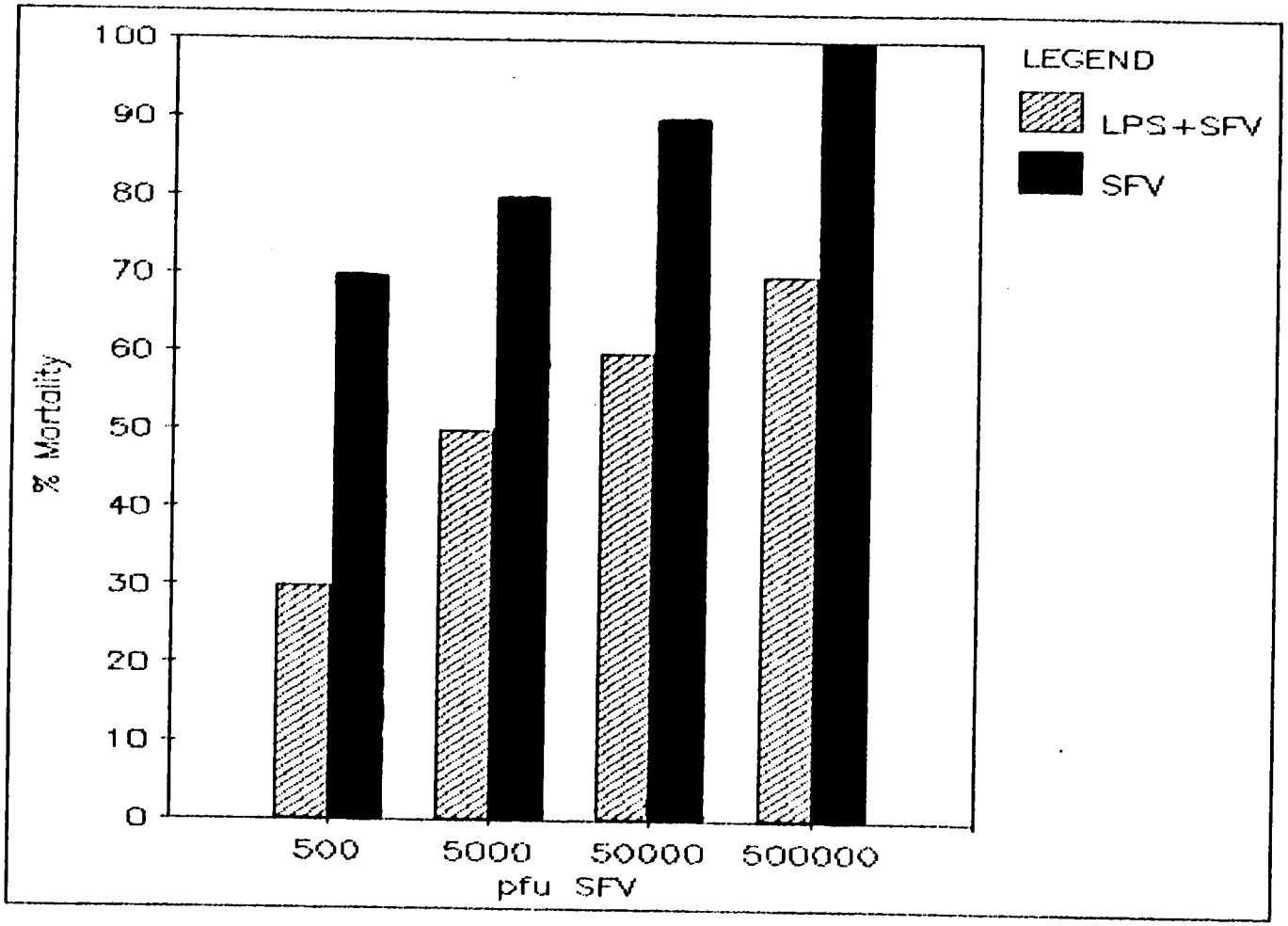


Figure 19. Mortality of mice to SFV given intraperitoneally after treatment with 25 μg of LPS.

and that macrophages may be involved. Consequently, the role of the macrophage had to be further investigated.

Effect of Protein A (Pansorbin) Treatment on the LD_{50} of SFV Administered IP

To rule out the role of T or B lymphocytes in the protection of the peritoneal cavity, an attempt to "activate" these cells before SFV IP infection was done. It is known that Protein A can cause induction in proliferation of T and B lymphocytes (70). Protein A was therefore chosen for this experiment. The Protein A was injected IP before viral challenge. The results in figure 20 represent three different viral concentrations. At low viral concentrations ($5x10^3$ pfu) virtually no protective effect of Protein A was noted. At higher concentrations ($5x10^4$ and $5x10^5$ pfu), a slight decrease in mortality was noted when Protein A treatment was given. At high levels of virus, artificial injection may be responsible for the decreased mortality from that expected (R. Moore, personal communication).

Effect of Thioglycollate Treatment on the LD_{50} of SFV Administered IP

Thioglycollate was used to attempt "activation" of the cells of the peritoneal cavity, particularly macrophages. As shown in figure 21, thioglycollate had absolutely no effect on the mortality of mice after SFV challenge, at

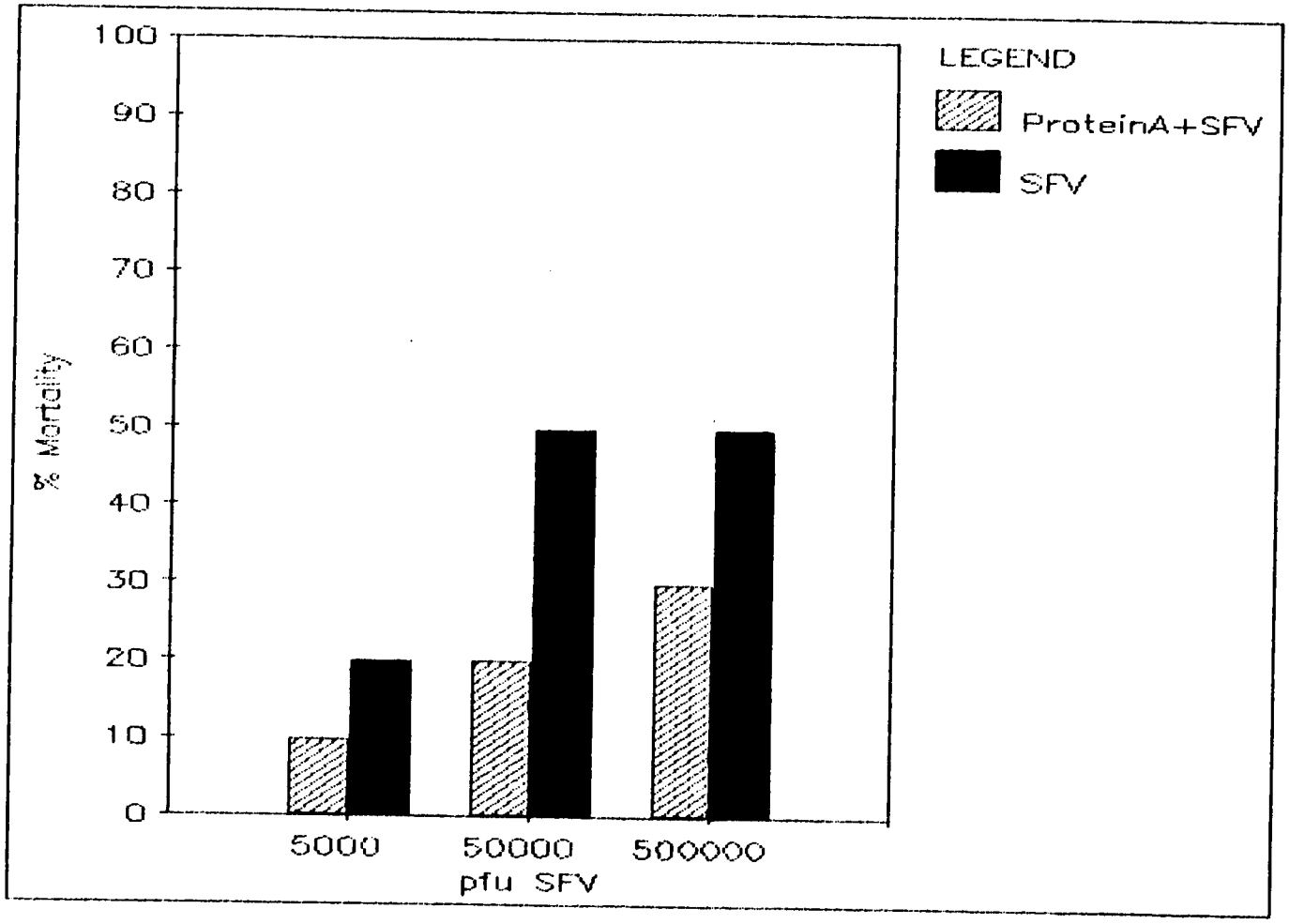


Figure 20. Mortality of mice to SFV given intraperitoneally after treatment with Protein A.

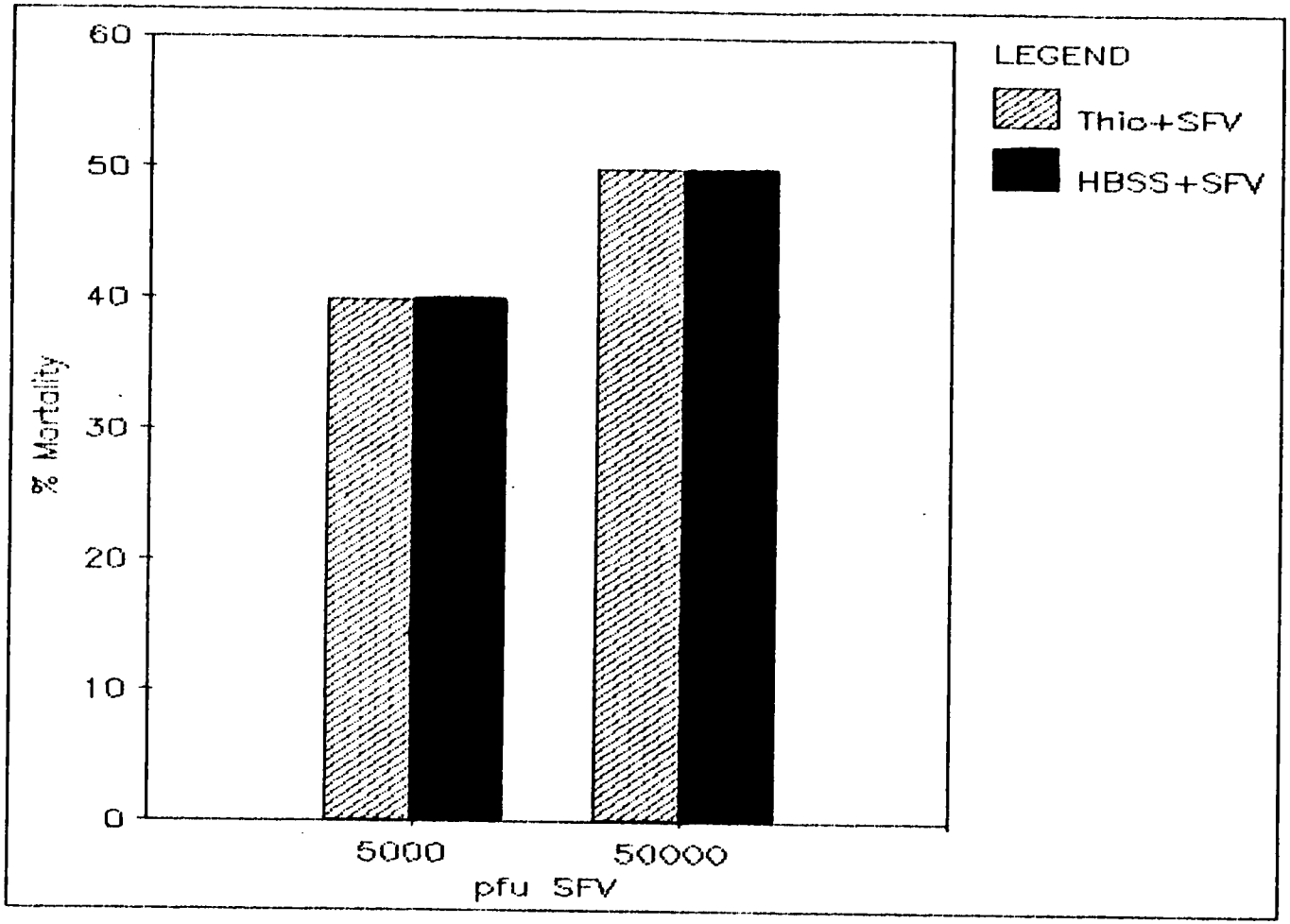


Figure 21. Mortality of mice to SFV given intraperitoneally after treatment with thioglycollate.

either of two viral concentrations tested ($5x10^3$ and $5x10^4$ pfu)

Effect of Silica Treatment on the LD_{50} of SFV Administered IP

Rather than attempting to activate cells of the peritoneal cavity, this experiment was designed to inactivate macrophages. Silica is known to damage or kill macrophages (3),(61), and was therefore chosen for this experiment. Silica was administered two days before virus. As presented in figure 22, only marginal effects on mortality of infected mice were noted. This may be because the silica was given two days before the virus, and an influx of undamaged cells from bone marrow could have replaced impaired ones. In fact, it has been shown that silica administered in large enough doses to cause death of macrophages, results in infiltration of immature monocytes and bone marrow stimulation (61).

Immunofluorescence of **In Vivo** Infected Macrophages

In order to evaluate the role of macrophages in the peritoneal cavity, an immunofluorescence experiment **in vivo** was done. Briefly, mice were inoculated IP with one LD_{50} SFV. Thirty minutes later, the peritoneal cells were harvested, and the adherent cell population incubated on glass coverslips for 18-20 hours. At that time, adherent

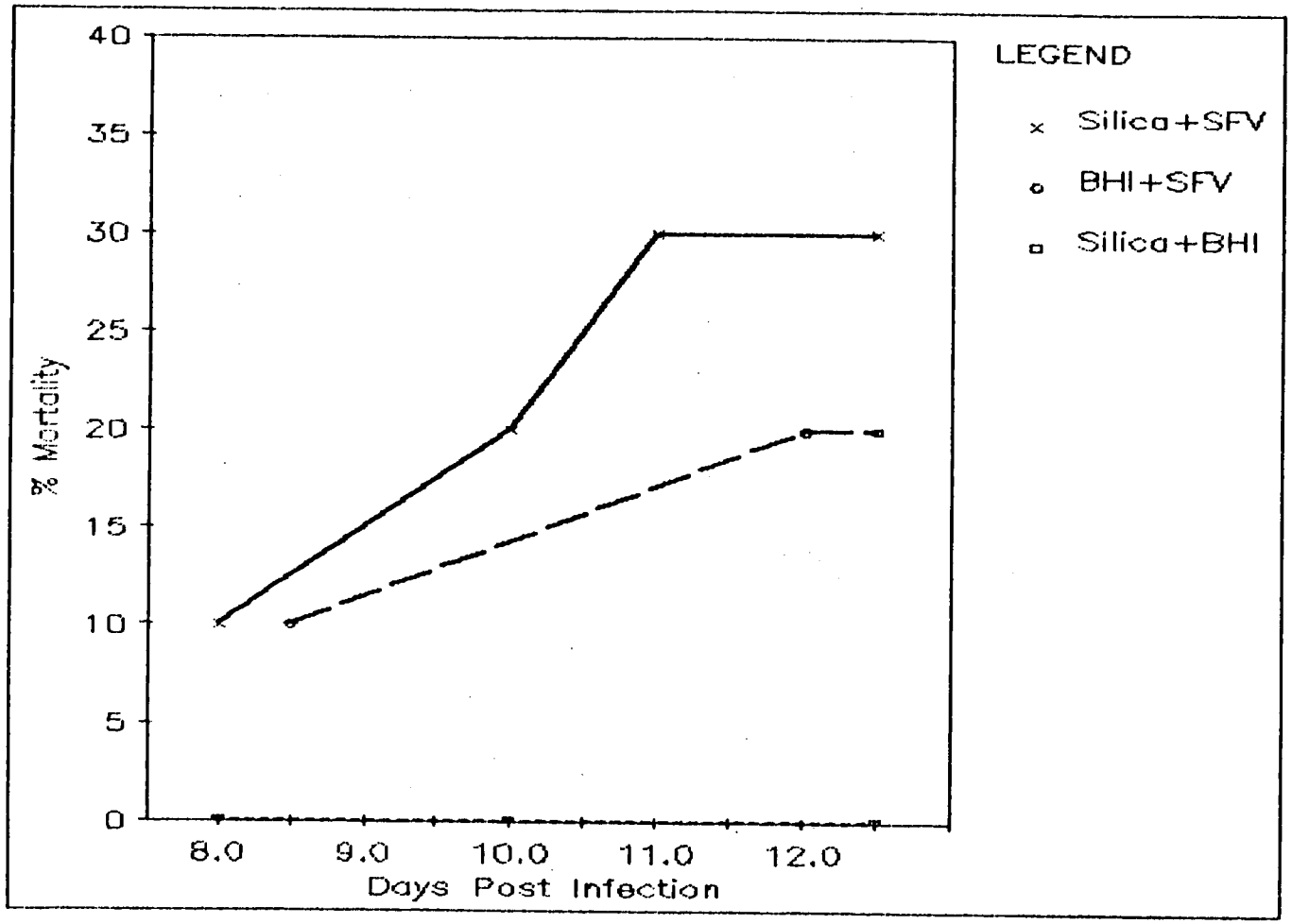


Figure 22. Mortality and day of death of mice to SFV given intraperitoneally after treatment with 300 μg silica. Dose of SFV = 0.8 LD_{50}

cells from normal controls as well as from infected mice, were either methanol fixed or left unfixed. Staining was accomplished by reacting the cells with rabbit hyper anti-SFV IgG, and then with FITC-conjugated goat anti-rabbit IgG. When viewed with a fluorescent microscope, very few cells were stained. The rough estimate would be approximately <2% stained. Of the stained cells, however, the fluorescent antibody could be seen as a thin rind on the outer edges of the cytoplasm, just on or beneath the plasma membrane. It is important to note this localization of viral antigens, since it is known that other cells infected with SFV contain viral antigens throughout the cytoplasm. It would therefore seem that even the cells which show viral antigens are highly resistant to infection. Figure 23a shows one example of an infected, fixed cell stained with rabbit hyper anti-SFV IgG as the first antibody. Figure 23b shows an infected, fixed cell stained with rabbit pre-immune serum, figure 23c a mock-infected, fixed cell stained with rabbit hyper anti-SFV IgG, and figure 23d a mock-infected, fixed cell stained with rabbit pre-immune serum as the primary antibody. Figure 24a-d shows the same cells as figure 23a-d, except that the cells are not fixed. Note that on both sets of figures no cpe can be seen. The convoluted membrane of macrophages appears to contribute to the high background since light-ripples are seen there on the unfixed cells. With respect to the small number of

Figure 23. Immunofluorescence of SFV-infected, methanol fixed peritoneal macrophages. a) Rabbit anti-SFV gamma globulin as first antibody. b) Normal rabbit serum as first antibody. c) Mock-infected macrophages with rabbit anti-SFV gamma globulin as first antibody. d) Mock-infected macrophages with normal rabbit serum as first antibody.

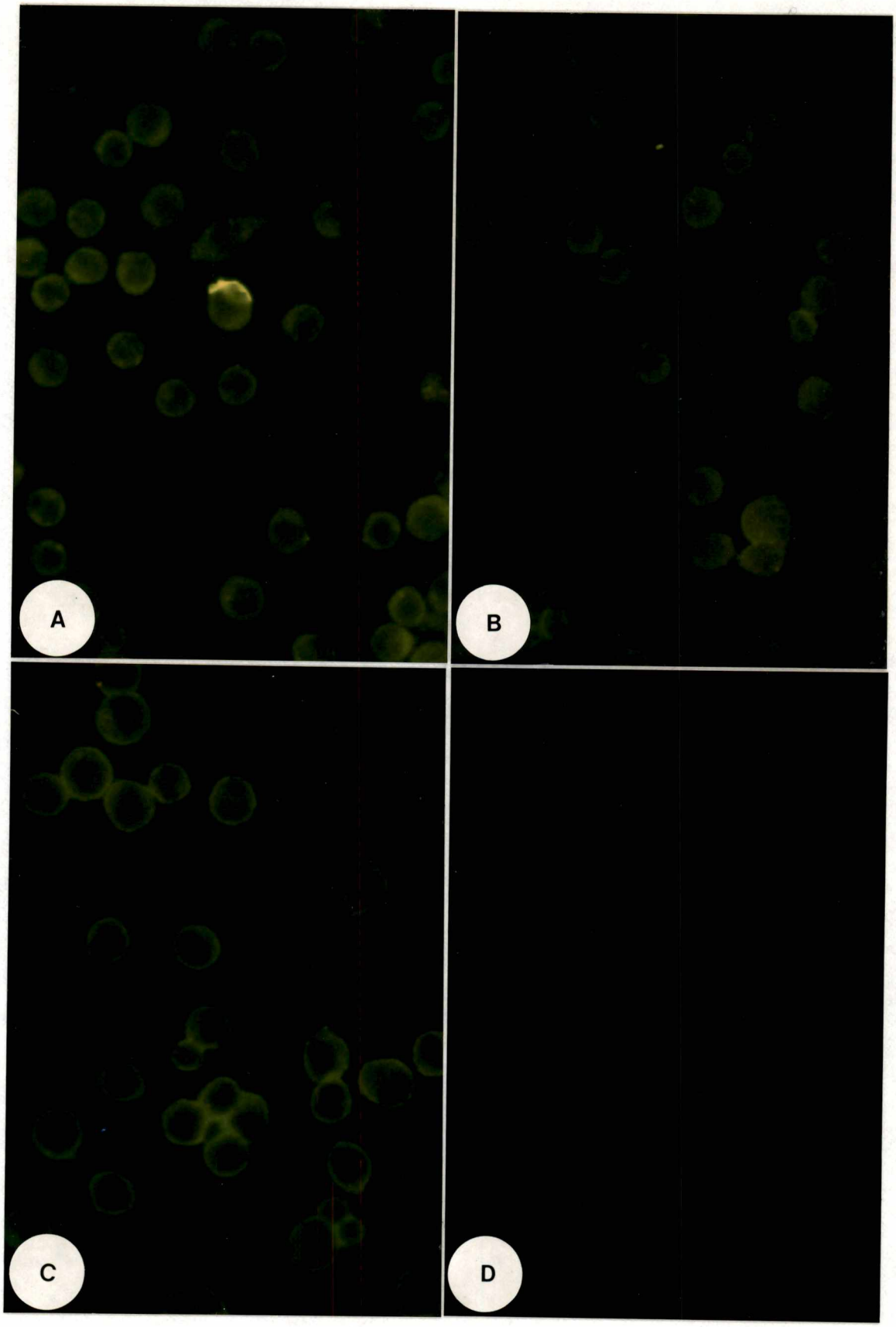
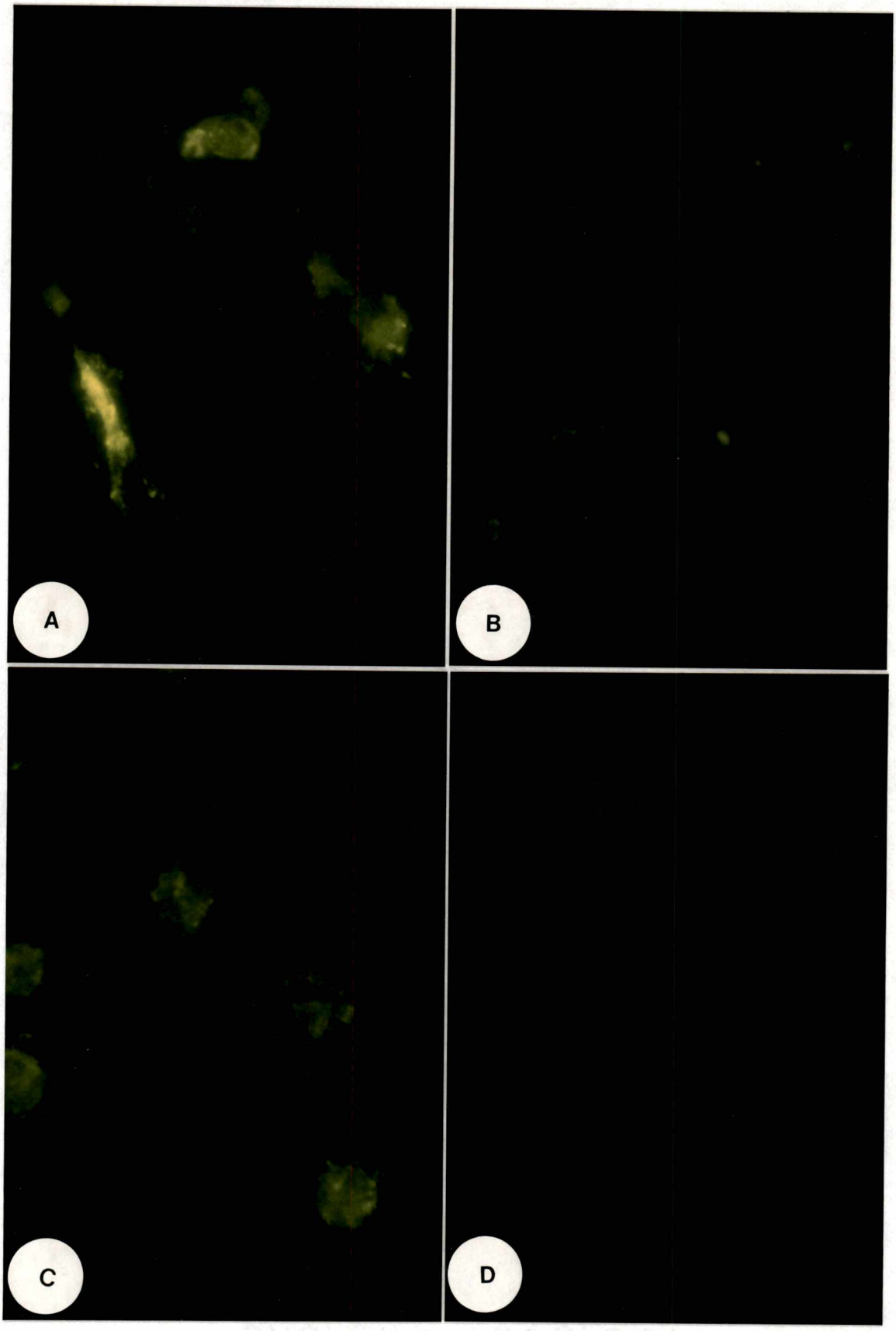

A
B
C
D

Figure 24. Immunofluorescence of SFV-infected, non-fixed peritoneal macrophages. a) Rabbit anti-SFV gamma globulin as first antibody. b) Normal rabbit serum as first antibody. c) Mock-infected macrophages with rabbit anti-SFV gamma globulin as first antibody. d) Mock-infected macrophages with normal rabbit serum as first antibody.


A
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cells stained positively, one can consider the following: $>10^6$ adherent cells can be obtained from one mouse and IP LD_{50} contains $4x10^3$ pfu of SFV. Thus, the **in vivo** MOI is approximately 0.002-0.004. On one coverslip, the number of cells are approximately $3x10^5$. At an MOI of 0.002, 600 cells would be stained, which is 0.2% of the total cells on the coverslip. In order to confirm the low percentage of stained cells, the experiment also had to be done **in vitro.**

Immunofluorescence of **In Vitro** Infected Macrophages

Since it was now clear that a small percentage of macrophages could take up or be infected with SFV at the low MOI of 0.002, it was important to determine if similar staining could be seen **in vitro**, and if at higher MOI more of the cells would be stained. Peritoneal cells were harvested from normal mice, and incubated for 18-20 hours onglass coverslips. The adherent cells were then infected at MOI of 0.002, 0.2 or 20, then fixed and stained as before. Upon viewing the cells under a UV-light microscope, it was found (a) that the macrophages could be infected or take up virus as was observed **in vivo**, (b) that the pattern of staining appeared exactly the same as cells infected **in vivo**, i.e., with a thin band of stain on the inner side of the plasma membrane, and (c) that the estimated number of cells stained remained the same at all three MOI. This latter observation was important since it would indicate

that only a limited small percentage of the macrophages could be "infected" or take up the virus, and those that did must be resistant too, to some degree, since viral antigens was not detected further beyond the cell membrane. This finding might indeed explain why the protective effects of the peritoneal cavity could be overcome with doses of SFV >one LD_{50}. At these MOI, virus might escape from the peritoneal cavity rather than being immobilized by these few susceptible macrophages.

To better understand whether the staining of macrophages observed with the immunofluorescence technique were due to active infection or simply engulfment of virus by the macrophages, another experiment was performed. Live virus was added to some cells and UV-inactivated virus was added to others. The UV-inactivation was accomplished by exposing SFV to a shortwave UV-light source for 2.5 minutes,which inactivated 99.4% of the virus. An immunofluorescence experiment was then performed as before. No difference in the number of cells stained with live as compared to UV-inactive virus was found. Therefore, it appears probable that macrophages can "engulf" inactivated virus; however, it does not rule out the possibility that live virus can "infect" macrophages, but if it does, the infection does not significantly proceed. In fact, experiments described below suggest some of the "engulfed" virus can be released as infectious virus (see discussion).

Immunofluorescence of Macrophages Infected **In Vitro** 0,1,2,3 and 4 Days PI

To determine how long the virus could stay in association with the macrophages, and to determine if the macrophages eventually show cpe, an additional immunofluorescence experiment **in vitro** was done. Peritoneal cells were harvested, fixed and stained as before. Coverslips were collected 30 minutes, one, two, three and four days post infection, and kept at -40^{o}C until all had been collected. These were then viewed with the UV-light microscope. Again, the same approximate number of cells were stained, with the same pattern of staining as seen before, on the samples harvested 30 minutes, one, two and three days PI. The coverslips from the 4:th day PI were questionable, in that the fluorescent staining could not be found as a bright green halo.

After viewing the stained cells, the coverslips were placed directly on a hemocytometer, and the number of cells counted. The results are shown in figure 25. From this experiment, the following conclusions can be drawn: (a) Macrophages remain stained in the same way for three days post infection as immediately after infection. Thereafter the fluorescence disappears, without notable cpe; (b) Cells surrounding the infected macrophages do not become infected several days after exposure to infected cells, showing

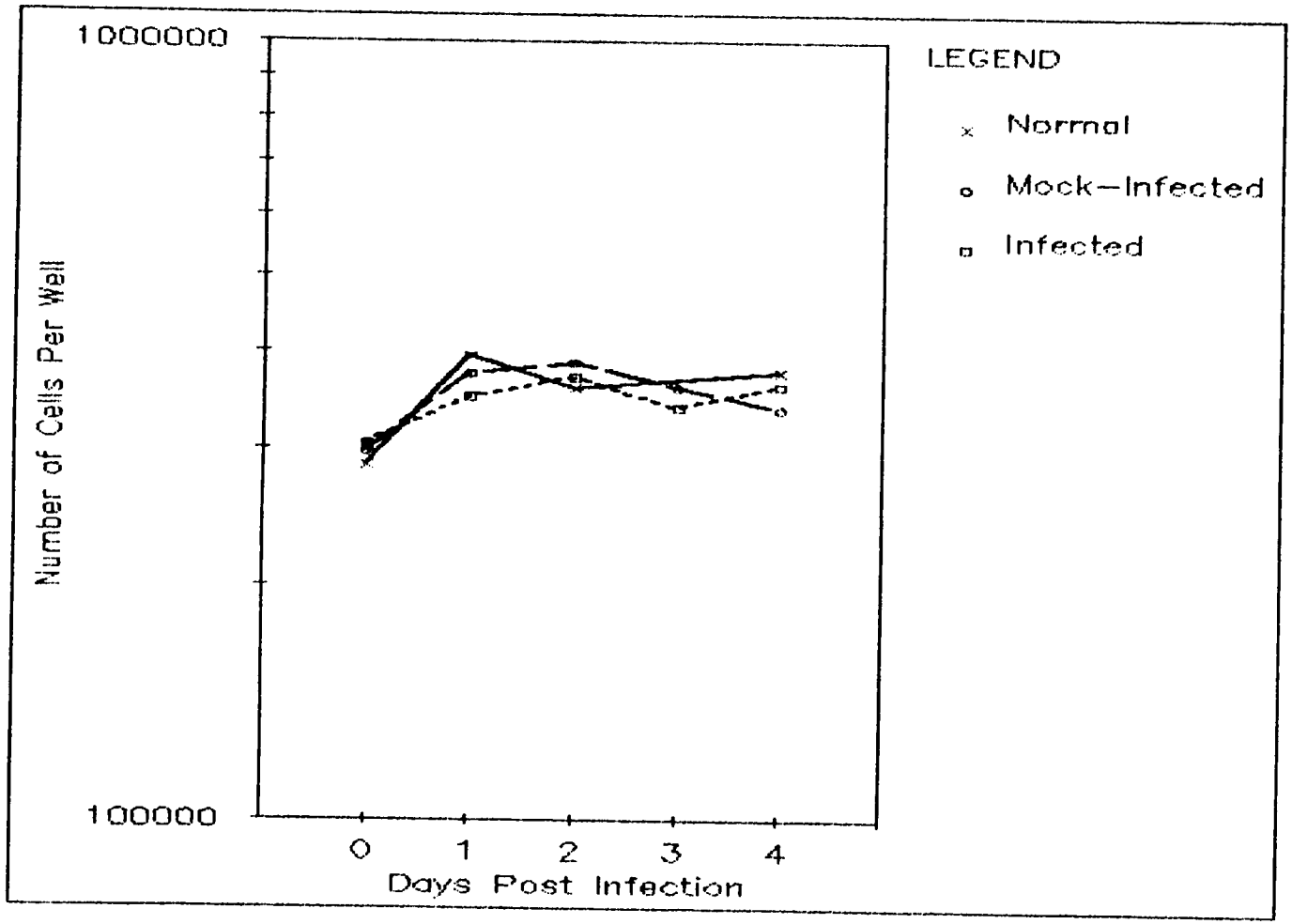


Figure 25. Number of macrophages at 0, 1, 2 and 4 days after SFV infection.

either that they are still resistant, and/or that no viral progeny are produced. The failure to detect antigens internally supports the last conclusion.

Assay of Infectious Virus From Macrophages Infected **In Vitro** for 0,1,3 and 4 Days

In order to determine if SFV was proliferating in infected macrophages, the following experiment was done: Briefly, peritoneal cells were harvested from normal mice, adhered to plastic dishes for 3.5 hours, and then infected at MOI equal to 0.002, 0.02, 0.2 or 20. Samples of the culture fluids were collected immediately after viral adsorption, and at one, three and four days PI, and stored at -70^{o}C until all samples were taken. The cells were likewise stored.

The samples were assayed for SFV on monolayers of CEF, and the viral yields calculated as pfu per macrophage for each timepoint. As seen in figure 26, the number of infectious SFV found per macrophage was very low at all times. As a reference, the viral yields from L929 cells infected at an MOI of 0.001 can be found in figure 14, page 74. Note that macrophages infected with the lowest MOI, i.e., 0.002, also show the lowest viral titers, and cells infected with higher MOI show higher viral titers, up to day three PI. However, it is important to note that the lowest values were at the limit of detection for this assay, and

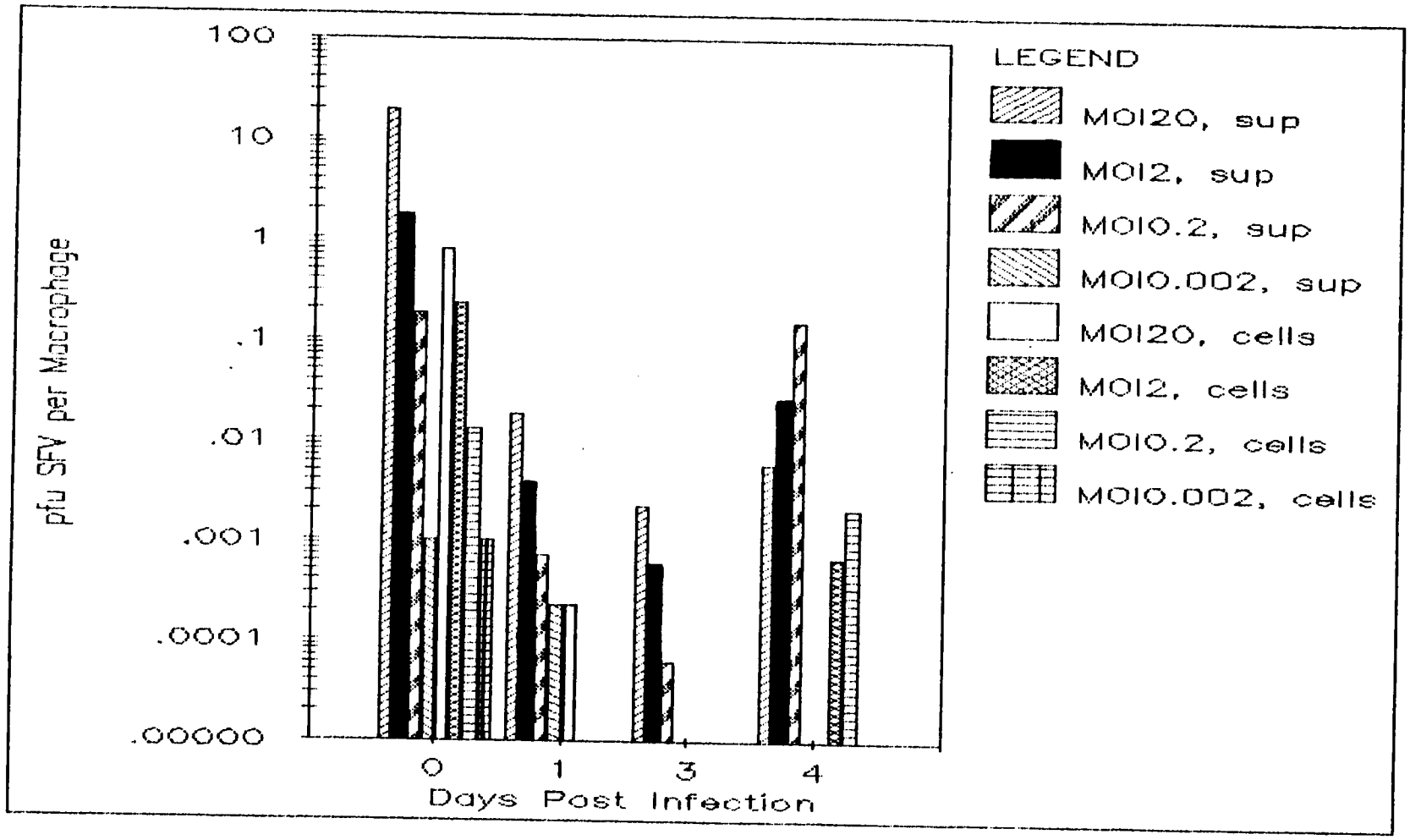


Figure 26. Yields of SFV from infected macrophages assayed from culture fluids or by infectious centers at 0, 1, 3 and 4 days PI.

that the highest titers were also very low, at best. Furthermore, there is a decrease in viral titers up to day three PI; however, on day four PI, viral titers increase. As previously presented, immunofluorescence of day four "infected" cells were negative for virus.

The infectious center assay using cells, showed similar results in that the pfu per cell was very low at all timepoints examined. Thus, it was difficult to obtain reporducible quantitative data. Part of the problem may be explained by the fact that these cells were physically scraped off the plastic plate. If <1% of the cells actually did contain virus, those few cells could have been destroyed or missed during the process of scraping or dilution.

These data support the concept developed further in the Discussion, that the virus that is detected represents residual virus after initial exposure to the inoculum, and/or virus that is slowly released. It is not virus that has replicated.

Neutral Red Uptake by **In Vitro** Infected Macrophages 0,1,2,3,4 and five Days PI

It was of interest to determine the viability of the infected macrophages over a period of five days PI. Briefly, the cells were harvested and infected **in vitro** at MOI 0.002, 2 and 20 as before. As an added control, L929 culture fluids were added at a 10% concentration to mock-

infected macrophages to provide growth factors, such as colony stimulating factor (CSF). At various times after infection, i.e., directly after viral adsorption, one day, two, three, four and five days PI, the cells were stained with the vital dye neutral red. In addition to mock-infected cells, empty wells were also stained in order to obtain a background color. This has been subtracted from the values shown (figure 27). After staining, the samples were frozen down at -70^{o}C until all had been collected. At that time, all plates were centrifuged, supernatants transferred to new plates, and read in an ELISA reader. As shown in figure 27, the cells infected at the three different MOI (i.e., 0.002, 2, 20), the mock-infected cells as well as the mock- infected cells with L929 culture supernatant, all showed similar significant degrees of dye uptake. Thus, it can be concluded that the cells are viable, and that the virus does not kill the cells even at the high MOI. The cells cultured with L929 cell supernatants may not show higher degrees of dye uptake because (a) not enough CSF was present, or (b) the other cells were equally active due to the processing or the artificial support alone i.e., the plastic.

IP Infection of Mice Following a Peritoneal Rinse

In this experiment, the objective was to infect mice IP immediately following a peritoneal rinse. It was

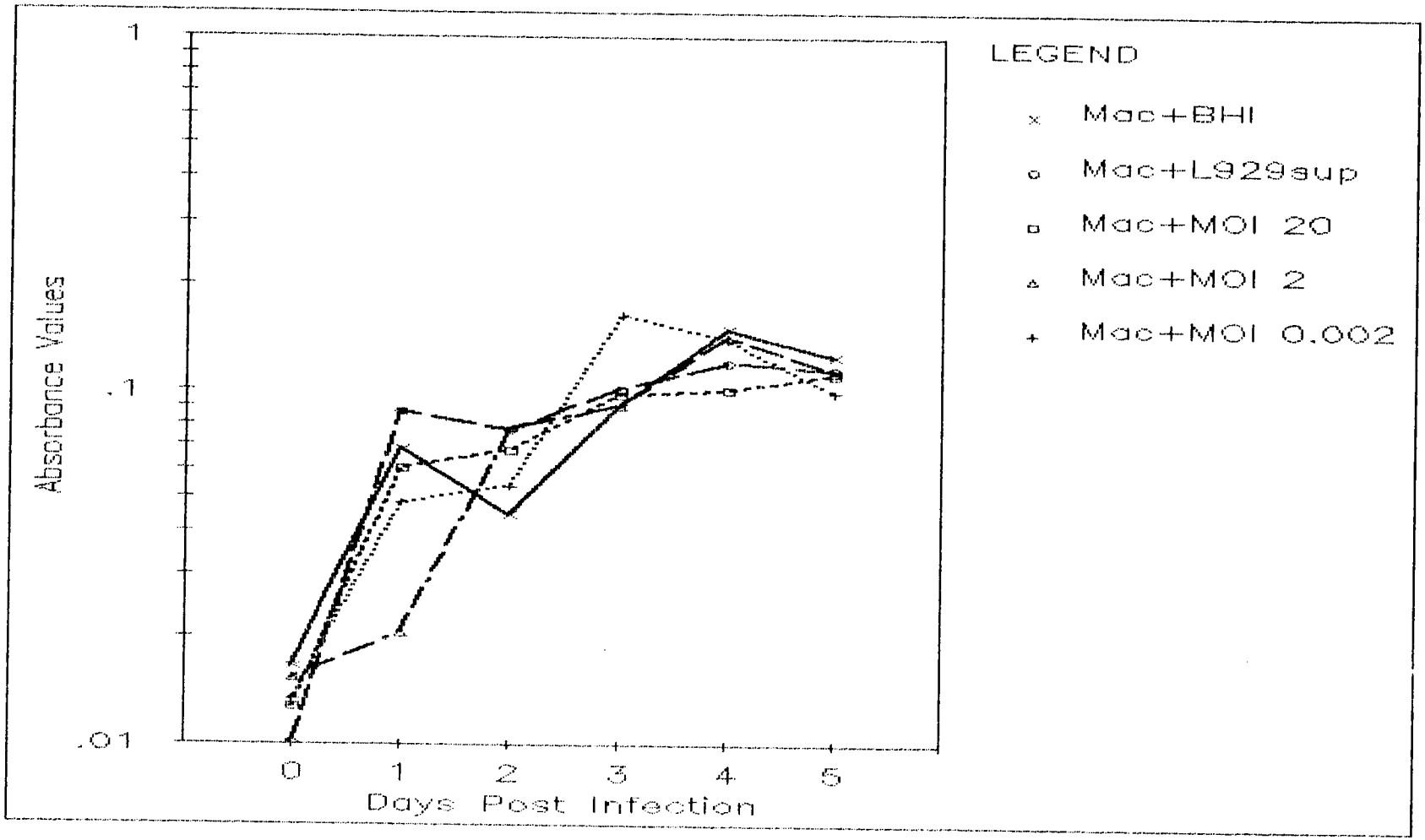


Figure 27. Measurement of neutral red dye released from macrophages that were stained 1, 2, 3 and 5 days PI.

hypothesized that if the macrophages could be removed from the peritoneal cavity, the barrier to viral spread would also be removed. If enough cells could be removed in this fashion, it was hypothesized that a lower dose of virus would be enough to cause encephalitis. The experiment did not, however, successfully demonstrate this (figure 28). Only a marginal difference could be seen in survival after SFV challenge between normal and rinsed mice.

FP Infection of Mice Following Resection of the Sciatic Nerve

In light of the fact that the FP LD_{50} of SFV is almost as low as the IC LD_{50}, it was of interest to investigate if this neurovirulent virus could possibly travel along peripheral nerves from the site of infection (i.e., the FP) to the brain. Therefore, 40 mice were anesthetized, and a 2-3 mm long section of one of their sciatic nerves was removed. The following day, half of the animals received 20 pfu SFV (10 LD_{50}) into the footpad of the leg with a cut nerve, while the other 20 mice received virus into the opposite footpad. Interestingly enough, 45% of the mice which had a cut nerve and injection of virus on the same leg, died. In the other group of mice, with the nerve cut on the side opposite to the viral injection, 75% died (figure 29). There was also a distinct difference in the times of death between the two groups. The group which had

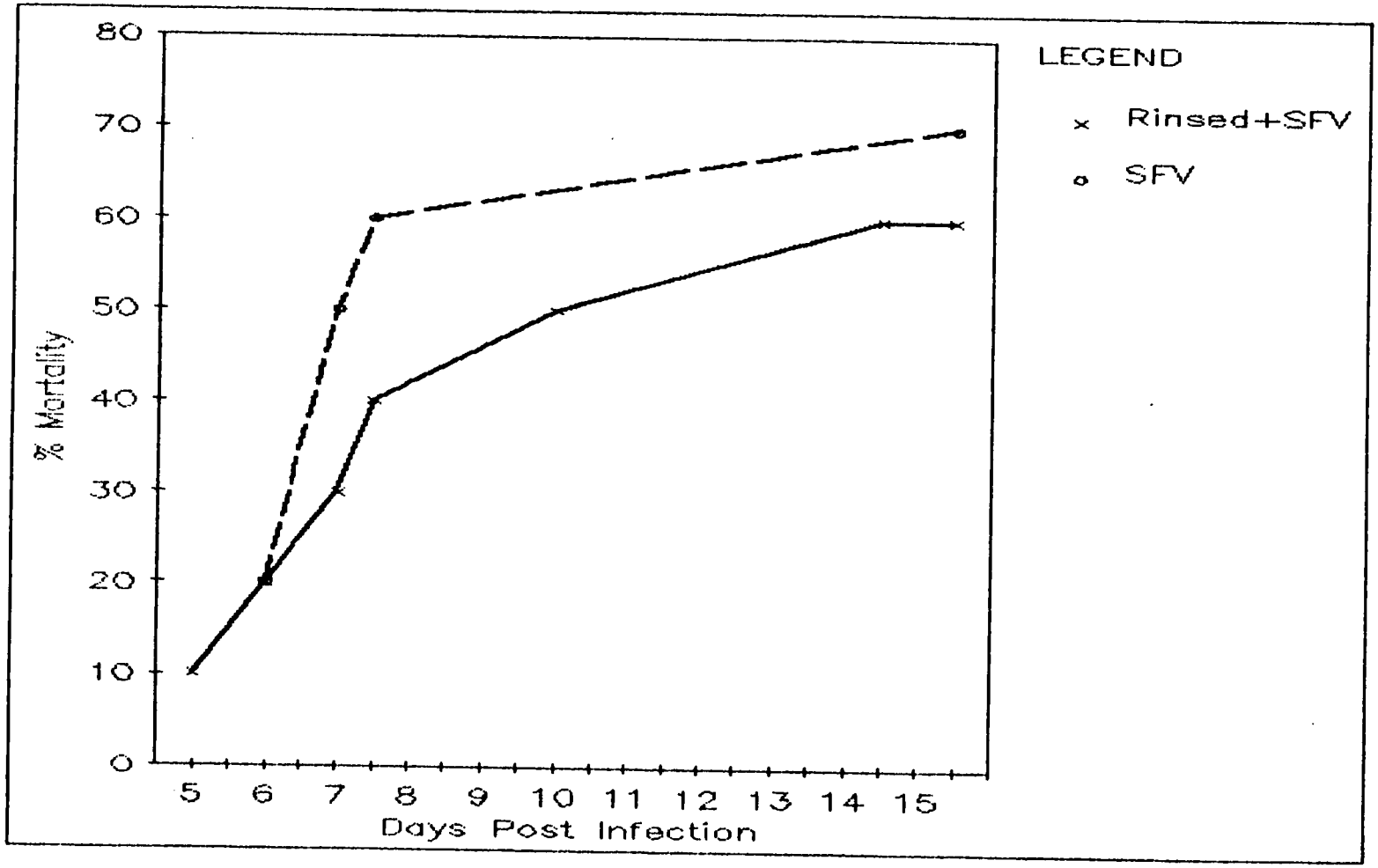


Figure 28. Mortality and day of death of mice given SFV intraperitoneally after peritoneal wash. Dose of SFV = 1 LD_{50}

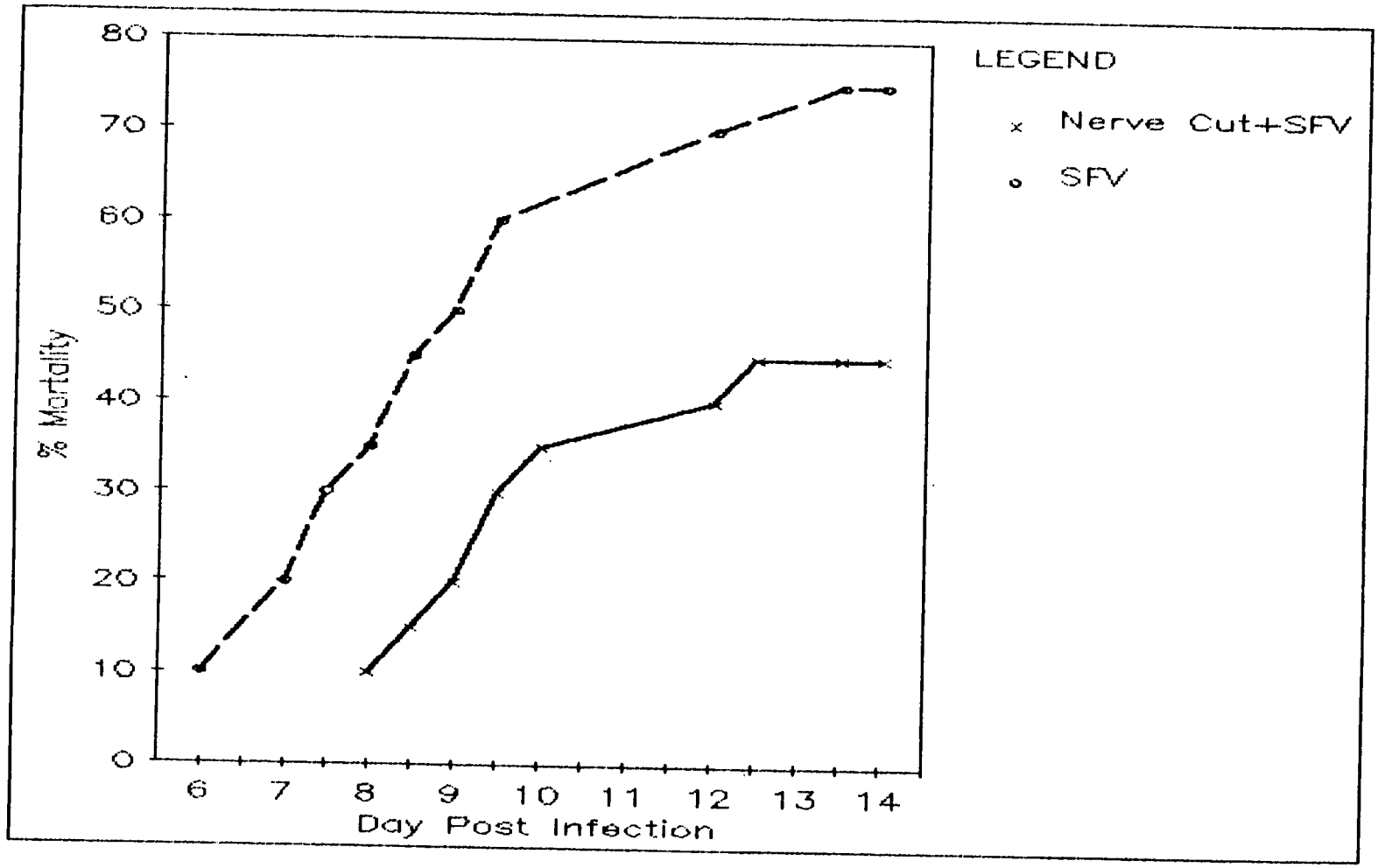


Figure 29. Mortality and day of death of mice given SFV in footpads after resection of the sciatic nerve. Dose of SFV = 10 LD_{50}

a cut nerve plus virus injected on the same side, began to die on day eight PI, whereas the control group began to die on day six PI. The average DOD was 9.67 and 8.77, respectively. Even though there is an increased survival of the mice in which the nerve was cut on the same side as virus was injected, all mice did not survive. One possibility could be that the virus injected into the FP is forced through the tissue by the pressure created by the inoculated volume. The virus might be forced through the tissues, and eventually forced into the blood stream. From the data presented in figure three, one could postulate that if only one pfu would enter the blood stream, it could cause death of the mouse. The results of this experiment would therefore argue for the possibility that SFV is capable of infecting the CNS via peripheral nerves.

SFV Virulence for CEF Versus L929 Cells

An interesting observation, which was not pursued, was that SFV appeared to be more virulent for some cells than others **in vitro**. To demonstrate this, a titration of the virus was performed on both CEF and L929 cells in parallel. The average size of the plaques on CEF were four mm in diameter two days PI. On L929 cells, the plaque size was only 1-2 mm after two days. Previous experiments have shown that the plaque size on L929 cells reach four mm in diameter

on day 4-5 of culture. Not only were the plaque sizes different in general, but on L929 cells two sizes of plaques could be distinguished. One, which was very small, and visible first on day three PI, and the "regular" larger sized plaque. Both plaque-sizes could be neutralized with hyper anti-SFV IgG. This suggests that there are two cell types present in the L929 cell line, one which is more susceptible to SFV than the other, or there is a mixture of the viruses giving the two plaque sizes. This phenomenon has been observed before, and was attributed to a high rate of mutation between large and small plaques on L929 cells (18). The virulence of SFV on CEF versus L929 cells could be seen as a difference in viral titers as well. On CEF, the titer was 2.7×10^9 pfu/ml, whereas on L929 cells, the titer was 3×10^7 pfu/ml, which is a 90-fold difference (table 1).

Table 1. Infectivity of SFV on CEF Versus L929 Cells

Cells	SFV titer (pfu/ml)	Plaque Diameter (mm) Day 2 PI	Day 4 PI	# Plaque Sizes (Day 3 PI)
CEF	2.7×10^{9}	4	7	1
L929	3.0×10^{7}	1-2	4	2

CHAPTER V

DISCUSSION

The work presented in this thesis was originally begun because of an interesting observation made while working with T cells from popliteal lymph nodes. It was found that the dose of virus required to kill 50% of C_3H/Ten mice was several thousand-fold higher for the IP route of infection than for the FP route. The IP LD_{50} was found to be $4x10^3$ pfu (fig. 1), whereas the FP LD_{50} was approximately two pfu (fig. 2). It was then hypothesized that the virus injected into the FP might possibly be infecting the CNS via peripheral nerves in the region of infection, i.e., the sciatic nerve. If this indeed were the case, one would have expected that perhaps other routes of infection, such as the IV route, would require a high LD_{50} since Pathek and Webb (1974) observed, by electron microscopy, the passage of SFV through the endothelium of cerebral capillaries after an IP injection (60), suggesting that SFV spreads to the blood after an IP infection. This hypothesis was thus tested, by performing an IV LD_{50} titration. Surprisingly, the LD_{50} was found to be <one pfu (fig. 3). Previous work in this laboratory has shown the IC LD_{50} to be approximately one pfu. It was evident that the peritoneal cavity provided some characteristics which made it refractory to SFV infection.

SFV was also injected via various routes into mice of an outbred strain, ICR. Even in these mice the IP LD_{50} was found to be high, actually higher than in C_3H/Ten mice, i.e., $5x10^3$ pfu (fig. 4). The IV and FP titrations were not definitive owing to a shortage of mice; however, the presumptive LD_{50} for both routes was considerably less than for the IP route (fig. 5 and 6), but higher than in C_3H/Ten mice. In a third strain of mice, the C_3H/Hen mice, the IP LD_{50} was again found to be high, approximately $5x10^3$ pfu (fig. 7). It could therefore be suggested that the strong resistance to IP infection with SFV, is not an unusual phemomenon, and not dependent on the strain of mouse.

As this project was proceeding, a problem arose: For unknown reasons, the IP LD_{50} for C_3H/Ten mice decreased. In fact, a new LD_{50} titration revealed the IP LD_{50} to be <40 pfu (fig. 8). When this was found, it was noted that the mice were quite small. A weight-versus-age relationship was plotted, and the results are displayed in figure 9. In contrast to the weight-versus-age curve of C_3H/HeJ mice provided by Jackson Laboratory, (the original source of C_3H/Ten) C_3H/Ten strain gain weight at a later time than the C_3H/Hej mice. For example, when our mice are seven weeks of age, they weigh 12.4 g, on the average, but the parent mice, C_3H/Hej, weigh 19 g at the same age. In all further experiments, the mice were weighed, and used only if between 17.5 and 19.0 g.

To further investigate the relationship between increasing age and increasing viral resistance, LD_{50} were determined for the IP (fig. 10) and FP (fig. 11) routes with young (6-8 weeks) and old (16-18 weeks) mice in parallel. It was found that older mice are more resistant to IP SFV infection than are younger mice. Similar findings were obtained when the FP infection route was used. It was concluded that older mice become increasingly resistant not only to IP SFV infections, but also to FP SFV infections.

When consistent responses to the viral infection were realized, the primary question could be addressed: Why was the peritoneal cavity so refractory to SFV infection? The first approach was an attempt to identify anti-viral factor(s) present in the peritoneal cavity.

It is known that virus inoculated into the peritoneal cavity can spread into the circulation within minutes (38),(64). From previous IV LD_{50} titrations, noted before, one pfu in the blood should be sufficient to cause encephalitis. Therefore, it was hypothesized that any anti-viral factor(s) in the peritoneal cavity, which would neutralize the virus or protect the cells, would do so rapidly. When L929 cells were pre-treated with the washings from the peritoneal cavity for 1.5 hours before SFV infection, or when the virus was pre-treated for 30 minutes before addition to normal L929 cells, no difference in plaque numbers could be found (fig. 12). However, if the

anti-viral factor(s) were constitutively present in the peritoneal cavity, it might be necessary for the target cells to be exposed to the presumed factor(s) for an extended period of time. Consequently, L929 cells were pre-treated with growth medium containing a 1:4 dilution of peritoneal cell-free washings for 18 hours prior to SFV infection. Again, no reduction in the number of plaques formed on the treated, as compared to control cells could be seen (fig. 13). In another attempt to detect anti-viral factor(s), L929 cells were infected with SFV, then cultured in medium with or without a 1:10 dilution of cell-free peritoneal rinse. As seen in figure 14, the viral yields from these cells, as measured in terms of plaque-formation on CEF, was not different in the test and control samples. Thus, any inhibitory factor(s) that might be present would have to be in very low concentrations, below the level of detection.

Next, an effort was made to identify anti-viral factor(s) that might be actively produced by peritoneal cells. In this effort, peritoneal cells were harvested and cultured **in vitro** for two hours. The cultures were then centrifuged to remove cells and debris, and added at a 20% concentration to agar overlays of infected L929 cells. As presented in figure 15, culture supernatant fluids did not lower the number of plaques (expressed as pfu SFV per ml inoculum). Therefore, it could be concluded that peritoneal

cells do not produce anti-viral factor(s) **in vitro**, and presumptively at least, not at a concentration or activity high enough to limit viral spread from the peritoneal cavity.

The experiments discussed thus far have considered the normal peritoneal cavity, as it would be before viral infection. Another possibility is that anti-viral factor(s) may be produced in response to the actual viral infection. This possibility was investigated. Briefly, cell-free peritoneal washings from normal and SFV-infected (24 hours PI) mice were obtained, and the washings from infected mice were then UV-irradiated to inactivate residual virus. L929 cells were exposed to the undiluted washings for 24 hours. As a positive control, some cells were pre-treated with medium containing 100 IRU/ml of IFN α/β. As additional controls, samples of the two washings were treated with anti-IFN α/β before being added to L929 cells. As was noted in figure 16, (a) the IFN α/β alone reduced the number of plaques by 83%, and this reduction could be abrogated by treatment of the IFN with anti-IFN α/β. (b) The undiluted washings from infected mice also caused a reduction in the number of plaques by almost the same amount as did IFN α/β. Interestingly, treatment of the infected washings with anti-IFN α/β abrogated the anti-viral effect indicating that the effect was caused by IFN. (c) Not only the washings of infected mice exhibited anti-viral properties, but also

those of normal mice, since pre-treatment of the cells with normal washings caused a reduction in plaque number by 50%. This inhibition could be abrogated by treatment of the washings with anti-IFN α/β serum. It was concluded that mice do have some IFN α/β normally present in the peritoneal cavity, and the amount increases after infection with SFV. Retrospectively, in the previous experiment in which L929 cells were treated with normal, cell-free peritoneal wash for 18 hours before SFV-challenge (fig. 13), the IFN activity would not have been expected to be detected, since a 25% concentration (i.e., a 1:4 dilution of the washing) was used.

Since it appears that IFN α/β is produced in response to an IP SFV infection, it was necessary to evaluate the role of the IFN in limiting viral spread from the peritoneal cavity. Mice were injected IP with anti-IFN α/β , and then challenged with SFV. As seen in figure 17, the anti-IFN α/β increased the mortality of the mice, in a dose-dependent fashion. It should be emphasized that the two doses used, i.e., 1.25×10^3 and 8.6×10^3 IRU, are relatively high. Also, this treatment did not result in 100% mortality of the mice, as would have been expected if IFN α/β were the only agent protecting the peritoneal cavity. In addition, IFN is not produced immediately after viral infection, but requires aproximately 16-18 hours. Thus, the virus should have time to escape from the peritoneal cavity before sufficient IFN

is produced. It could be concluded that IFN alone is not responsible for the resistance of the peritoneal cavity to SFV infection.

Regulatory factors produced by macrophages are prostaglandins, among others (8),(12),(15). It was of interest to investigate the possible role of prostaglandins in influencing viral spread from the peritoneal cavity. Mice were injected with Indomethacin, which is known to inhibit prostaglandin synthesis (8),(12),(15), before viral challenge. As presented in figure 18, the inhibition of prostaglandin synthesis did not increase the percentage number of mice that died, as might be expected if prostaglandins were involved in protection within the peritoneal cavity. In fact, the percent number of mice that died was reduced (see fig. 18). Based on these observations, one could speculate that prostaglandins contribute to the infectivity and dissemination of the virus, rather than being protective or beneficial.

The next attempt to identify resistant factors in the peritoneal cavity, was to examine the cell types which may be involved. Protein A is known to induce proliferation of T and B lymphocytes (70) and thus, these cell types are excluded as possible candidates.When mice were injected IP with Protein A prior to viral challenge, no great difference in survival of treated mice was seen, as compared to controls (fig. 20). For example, when $5x10^3$ pfu of SFV was

used as a challenge, there was a difference in mortality of only one mouse out of 10 between control and test groups.

A substance that can activate peritoneal macrophages from most strains of mice is LPS (8). However, C_3H/Hej mice are known to be resistant to LPS (8),(34), and the C_3H/Ten mice are supposed to be genetically identical to those mice. Since the C_3H/Ten mice had been shown to mature at a later time than the C_3H/Hej mice, I considered the possibility that the C_3H/Ten mice might be genetically altered so that they were responders to LPS. Therefore, LPS was tried, and as seen in figure 19, LPS did have an effect on C_3H/Ten mice. Treated mice survived SFV challenge better than control mice. For example, at a dose of 500 pfu SFV, a difference of four out of ten mice died between the test and control groups. These results suggest that the C_3H/Ten mice are no longer identical to C_3H/Hej mice, and more importantly, that macrophages may be involved in protection of the peritoneal cavity against SFV. In addition, LPS can induce macrophages to produce IFN (96). The results from this experiment would therefore support the findings presented in figure 16 and 17, which indicated that IFN α/β is increased in response to SFV and may serve to protect the peritoneal cavity to some extent.

Thus far, macrophages have not been discussed as being directly involved in IP SFV infection, so the following experiments were designed to show this. For example,

thioglycollate, which is known to activate macrophages, was administered IP to mice prior to SFV challenge. As presented in figure 21, the thioglycollate had no effect whatsoever. In a similar experiment, silica (which is known to damage or kill macrophages [3],[61]) was administered prior to SFV challenge and there was no significant effect on mouse survival. These results may be explained by the fact that silica-inactivated macrophages can be replaced by infiltration of immature,committed monocytes from the bone marrow (61).

To better evaluate the role of macrophages in protection from SFV, a new set of experiments was designed. First, it was of interest to find out if the macrophages were infected by the virus without allowing further spread, or if the virus was taken up by the macrophages. Firstly, an immunofluorescence experiment done on macrophages harvested from **in vivo** IP infected mice showed that very few cells contained viral antigens. Furthermore, the positively stained cells (<2%) displayed only a thin rind of green along the outer edges of the plasma membrane. These results indicate two things. (a) Most macrophages are resistant to SFV "infection" and (b) the cells which displayed viral antigens also must be highly resistant, since virus could not be seen anywhere in the cytoplasm except on or just below the plasma membrame.

In order to confirm the low percentage of positively staining cells, an **in vitro** experiment was done with macrophages "infected" at MOI 0.002, 0.2 and 20. Increasing doses of virus was used to see if more cells could be "infectable" at higher MOI. Some macrophages were also "infected" with UV-inactivated SFV, to see if inactivated virus could still result in the staining of cells. Fluorescent antibody staining in this case would show that an active infectious process is not necessary. For all macrophage groups tested, only a very low percentage contained viral antigens, displayed on the outer edges of the plasma membrane. These findings suggest that only a small subpopulation of peritoneal macrophages can presumably bind and possibly "endocytose" virus, since inactive as well as live virus were taken up by the macrophages; no replication occurs, since viral antigens were limited to the periphery of the "infected" cells. Interestingly, the fraction of cells stained never increased above that which was seen when one LD_{50} was used to infect the cells **in vivo**. One might hypothesize that above one LD_{50}, a significant number of viruses can no longer be prevented from spreading to the blood stream by the few cells capabale of "endocytosing" the virus which supports observationn made by others about rapid spread of viruses from the peritoneal cavity to the blood (38).

In another immunofluorescence experiment, the possibility was examined that "infection" of peritoneal macrophages takes longer than that of other cells. Macrophages were infected **in vitro**, and then cultured for 30 minutes, one, two, three or four days. Only a few cell were stained up to three days PI, and the viral antigens could be seen only on or just beneath the plasma membrane, as had already been seen in the previous experiments. However, the sample taken on day four PI, was different. No viral antigens were detected, and no cpe could be seen. The fate of the virus was unknown. It was unlikely that viral proliferation had occurred, since there was no increase in viral antigens with time, and no cells surrounding the initially "infected" cells contained viral antigens. Therefore, it could be concluded that macrophages initially resistant to SFV remained resistant after several days in culture in the presence of infected cells, and/or, that no viral progeny had been produced from the cells shown to contain viral antigens initially. The latter conclusion is supported again by the fact that even those few cells which contained viral antigens, never appeared to have the antigens in the cytoplasm.

The latter possibility was investigated in a virus yield assay. Supernatant fluids from "infected" macrophages up to four days in culture were collected. Viable virus could be found at all timepoints although at very low and

decreasing titers (i.e., 0.1-0.0001 pfu per cell). The titer of virus decreased through the third day, but on day four PI, there was an increase in virus titer to 0.1-0.01 pfu per cell. However, the titer on day four was much less than what was initially used to infect the cells (MOI of 20), strongly confirming that macrophages are resistant to SFV replication. To further verify this finding, the infectious center assay was used. The results showed more variability (probably because very few plaques were involved), but essentially confirmed the conclusion that SFV does not replicate in macrophages. The variability of the latter assay could be explained by the fact that cells were phyically scraped off the plastic support. If less than 2% of the cells adsorb virus, positive cells could be missed in the assay. Using supernatant fluids, however, the virus would be found even at low concentrations.

An interesting point can be made from the day four PI data. Earlier, it was shown by immunofluorescence that there were no viral antigens on day four cells. Thus, SFV must be adsorbed by <2% of the macrophages, held in them for several days, and then released without replication.

The macrophages used in these experiments were viable based on several criteria: Firstly, the number of cells increased throughout cultivation regardless of viral infection (fig. 25); Second, neutral red dye could be readily taken up by these cells, infected or mock-infected,

to the same extent as cells to which growth factor containing L929 culture fluids had been added.

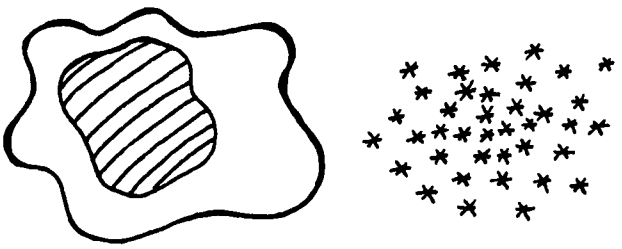
It was evident at this point that the macrophage limits the viral spread from the peritoneal cavity. In an additional effort to convincingly demonstrate this. Attempts were made to remove macrophages, and, thus, the protective barrier. Peritoneal cavities were rinsed with medium to remove the bulk of the cells, and SFV injected immediately. However, the proceedure did not remove protection. It is probable that sufficient numbers of macrophages remain after such washes.

The following schematic diagram (fig. 30) sums up the events we believe to occur in the peritoneal cavity after an SFV infection.

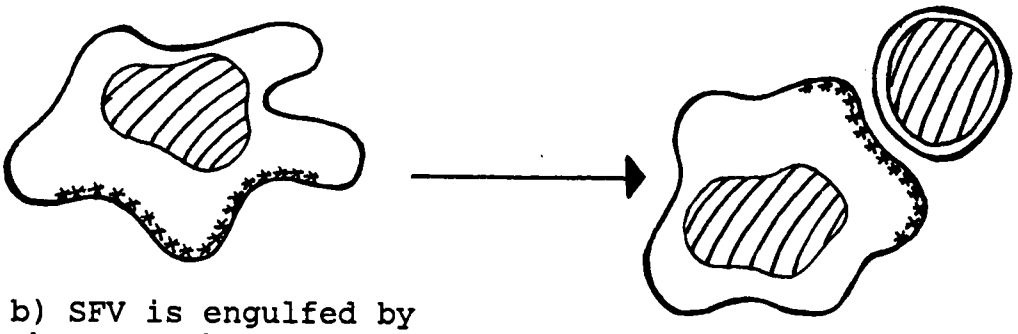
Since infectivity by the FP route was sensitive and has not been reported to our knowledge, we were interested to learn if SFV could spread from the site of infection via peripheral nerves even though hematogenous spread is well known. It would seem plausible that a neurotropic virus could infect nerves if deposited in the proper area (i.e., CNS directly and nerves in footpad). To test this, the cutting of the sciatic nerve was done followed by FP injection of SFV. The hypothesis was that a virus that spreads via the nerve to the CNS cannot infect the CNS if the nerve is severed. The results (fig. 29) showed that mice with cut nerves survived better than control mice (55%

versus 25% survival). Although the results are only suggestive, they support the conclusion that nerves may be one of the pathways of viral spread. This conclusion is further supported by a recent report (33). However, it is probable in my experiments that the pressure generated by the injected volume in the FP causes hematogenous dissemination of some of the virus. If just one pfu of SFV were to enter the blood stream, an IV LD_{50} would be achieved. Furthermore, since it is known that SFV can replicate to very high numbers in many cell-types, for example, fibroblasts, muscle tissue etc. (52),(64), it may replicate in cells in the area of the FP, such as the hindleg muscles, when the primary route for dissemination (i.e., the nerve), is severed, and thereafter enter the blood stream.

The average DOD for the mice was of interest. The test group (cut nerve + SFV on same side) had an average DOD of 9.67, whereas the control group had an average DOD of 8.77. The first deaths also occurred later in the test group (day 8.0 PI) compared to the control group (day 6.0 PI). If one assumes that the virus prefers to enter nerves, alternate routes to the CNS might be expected to take longer. Therefore, the data suggest that SFV can spread to the CNS from peripheral sites of infection, such as the FP, via peripheral nerves.



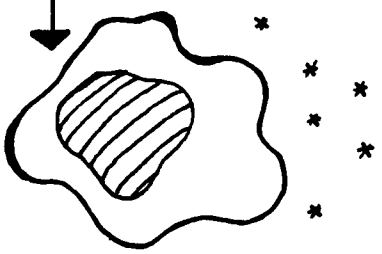
a) Macrophage is exposed to SFV.



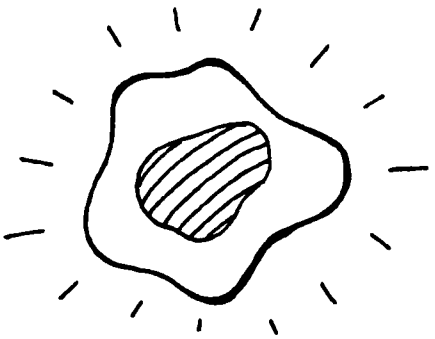
b) SFV is engulfed by the macrophage.

c) Presentation of viral antigens

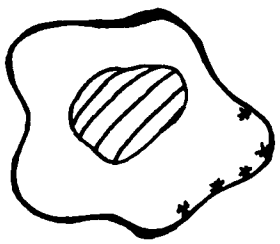
Ab induction begins



c) After several days, infectious SFV is inactivated and/or released, in fewer numbers.



c) Stimulation of IFN production.



d) The remaining SFV is again engulfed by a macrophage.

INHIBITION OF VIRUS REPLICATION

Figure 30. Sequence of events after IP SFV infection.

The work which has been presented in this thesis can be summarized in the following way: It was found that the peritoneal cavity is several thousand-fold more resistant to SFV infection than the FP, IV, or IC route. The resistance increases with age, and is a phemomenon that can be seen in several different strains of mice. LPS administration before viral challenge to increased survival of the mice, but thioglycollate treatment had no effect. Silica-treatment or peritoneal washings failed to remove the protective barrier of the peritoneal cavity, presumably by a) not killing or removing the bulk of the macrophages, or b) because replacement of the peritoneal cells is very rapid. IFN appears to be produced after SFV infection, but is not fully responsible for the peritoneal barrier. Prostaglandins, on the other hand, may participate in reactions harmful to the mice, and may add to the viral infectious process. Less than 2% of macrophages could be shown to bind and possibly endocytose viral antigens **in vivo** and **in vitro**, without allowing viral replication or cpe to occur; the viral antigens could be found only on or below the plasma membrane. The fraction of macrophages which appear to contain viral antigens could not be increased by increasing the dose of virus or by allowing the virus a longer time to "infect" the macrophages (several days). Live, or UV-inactivated virus appear to be equally taken up by macrophages. After four days of culture, however, viral

antigens could no longer be detected in cells by immunofluorescence, but could be found as live virus in the culture supernatants of the "infected" cells, at very low titers. Macrophages capable of "endocytosing" live virus and later releasing only a fraction of them without replication, are postulated as being responsible for the resistance of the peritoneal cavity to SFV.

In addition, SFV has herein been shown to use the sciatic nerve as one of the ways to be transported to the brain from the FP.

In the future, it would be interesting to investigate the reason(s) why SFV is unable, or prevented from, replicating in certain peritoneal macrophages, and yet remain live within these cells for extended periods of time. It would also be of interest to examine macrophage sub-populations of the peritoneal cavity, with the aim of characterizing those with distinct properties in virus infections (i.e., are virus positive cells Ia+, what fraction produces IFN?, etc.). Furthermore, if the precise mechanism by which SFV is prevented from replicating in macrophages could be understood, it may serve as a model in producing prophylactic treatments against SFV or related viruses which do cause serious diseases in man or animals.

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VITA

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