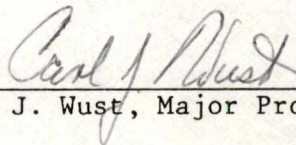


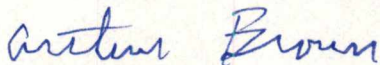
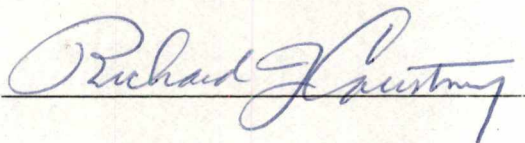
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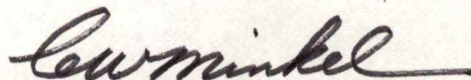


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PERSISTENT INFECTION OF THE PLURIPOTENT
HEMATOPOIETIC STEM CELL LINE, K-562,
WITH SEMLIKI FOREST VIRUS

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Denice D. King

June 1985

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ABSTRACT

A persistent alphavirus infection has been established in the pluripotent hematopoietic stem cell line, K-562, with Semliki Forest virus (SF) using a multiplicity of infection (MOI) of 1. The infected cell cultures have been maintained for over one year.

The SF infection of K-562 cells can be characterized by three stages: 1) an early stage lasting up to 72 hours post infection (pi); 2) an intermediate stage extending from 72 hours to approximately three weeks post infection; 3) a persistent infection beginning at three weeks pi and continuing at least 15 months pi. In the last stage, small cytological changes occurred throughout the observation period.

The early stage is characterized by the production of infectious virus in high titer (2.9×10^2 pfu/cell); however, there was only a subtle cytopathic effect (CPE) seen as cytoplasmic vacuolization of the cells. Viability of SF-infected cells approached 100% during this period with cell concentration and metabolism being comparable to mock-infected controls. All cells were judged to be infected as determined by indirect immunofluorescence assay and antibody dependent complement mediated cytolysis with antiviral serum. These observations are in contrast to the obvious lytic CPE seen with this virus in other mammalian cells (including human cells).

The intermediate stage (after 72 hours pi but before three weeks) was characterized by a viability that decreased but fluctuated from 75-95%. Cell concentration decreased and then stabilized at 2×10^5 cells/ml, and cell metabolism slowed to about half as measured by uptake of $^{51}\text{CrO}_4$, $[^3\text{H}]$ thymidine, and $[^3\text{H}]$ -amino acids. Production

of infectious virus declined until it was undetectable by 21 days pi. Cells in this stage of infection were fragile to fixation for immunofluorescence studies but showed positive ADCMC reactions with anti-viral sera.

The persistent stage (after three weeks pi) was unique in that no detectable infectious virus was found but cells showed similar characteristics in respect to growth and metabolism as in the intermediate stage. These cells were especially fragile in fixation procedure for immunofluorescence (IF). By examining the cell debris in IF, it was estimated that nearly all the cells showed fluorescence. ADCMC was lower than in the other stages (48% versus 57% in intermediate, and 70% in early stage) but substantial.

An additional characteristic of persistently infected cells was that they are killed by antiviral antibody in the absence of complement. Uninfected control cells are not killed. In addition, anti-SIN hyperimmune serum could also mediate the death of SF-infected K-562 cells although to a lesser extent than the homologous anti-SF serum, suggesting that both homologous and cross-reacting antigens were present on the cell surface. This killing is analogous to that reported previously with anti-K-562 serum and uninfected K-562 cells.

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LIST OF ABBREVIATIONS

ADCMC	Antibody-dependent complement mediated cytotoxicity
BCS	Bovine calf serum
CEF	Chicken embryo fibroblasts
CML	Chronic myelogenous leukemia
CPE	Cytopathic effect
CPM	Radioactive counts per minute
⁵¹ CR	Radioactive chromium fifty-one
DE	Defective interfering particles
EMEM	Eagle's Minimal Essential Medium
FBS	Fetal bovine serum
FCS	Fetal calf serum
IgG	Immunoglobulin G
MOI	Multiplicity of infection
NBS	Newborn Bovine serum
NK	Natural killer(s)
Pi	Post infection
pfu	Plaque forming unit(s)
Ph ¹	Philadelphia chromosome
RPMI	RPMI 1640 growth medium
SF	Semliki Forest virus
SIN	Sindbis virus

CHAPTER 1

INTRODUCTION

Semliki Forest virus (SF) has been isolated from a variety of insect and vertebrate hosts including mice, birds, and mosquitoes in Africa and from mosquitoes in the U.S.S.R. (7). While fever or inapparent infection are the usual result of SF infection in nature, the virus is highly cytopathic in a number of cell populations in vitro. Antibody reactive with SF has been detected in wild and domesticated animals and rodents in Africa, and neutralizing antibody has been found in humans in Africa, India, Southeast Asia, Malaya, Borneo and the Philippines (7). While the host-vector cycle of SF has as yet not been identified, the infection of vertebrates as well as mosquitoes suggests an enzootic cycle. It is believed that while transovarial transmission may be the mode of maintaining SF in environments where mosquitoes appear to be the major vector, chronic infection and lasting viremia in vertebrates may serve this purpose in other areas (7). A study of chronic infections such as those found in nature may elucidate this as a mechanism of virus persistence within a geographical area.

Persistent infection of some cell populations with SF in vitro has been reported. SF and Sindbis virus (SIN) readily produce persistent infections of mosquito cell lines (7,69, 70, 72); however, persistent infections of vertebrate cell lines is established only under unusual conditions such as at very high multiplicity of infection and/or with virus preparations rich in defective interfering (DI) particles (59,89).

The goal of the research presented herein was to study the infection of an interesting human cell line with SF. The infection was established and characterized in the hematopoietic leukemia stem cell line, K-562. Interestingly, this led to the establishment of a persistent SF infection of this cell line. Although the K-562 cell line has not been reported in virological studies, it was used in the present study owing to its human origin, and its world wide use.

CHAPTER II

SURVEY OF THE LITERATURE

Alphaviruses

Classification

The viruses of the family Togaviridae are icosahedral, enveloped, and contain a single non-segmented strand of infectious RNA. There are four groups, of which the alphaviruses and flaviviruses were previously classified as Group A and Group B arboviruses, respectively. These two groups are defined on the basis of antigenic relationships and on arthropod transmission vectors. The two other groups, rubivirus (Rubella) and pestivirus (eg. foot and mouth disease virus), consist of viruses that conform to togavirus morphology and molecular but are not transmitted by arthropods (78).

Two alphaviruses were used in this study. Semliki Forest Virus (SF), originally isolated in Uganda in 1944, and Sindbis Virus (SIN) isolated in Egypt in 1952 and designated Strain Ar339. The two viruses are morphologically similar and replicate in a similar manner but, although they are antigenically related in hemagglutination inhibition and complement-fixation assays, they belong to different sub-groups in that immunization with one does not cause the production of cross-neutralizing antibodies (78).

Virion Structure

The alphavirus nucleocapsid consists of a positive single stranded 42S RNA molecule ($M_v = 4.1-4.3 \times 10^6$) (43,47,82) and a single capsid protein.

The capsid protein ($M_w=2.97 \times 10^4$) (36,62,82) and the nucleic acid interact to form an icosahedral configuration of 180 units (82). This interaction of RNA and capsid protein makes it difficult to determine the triangulation number of the nucleocapsid but it is postulated to be 3 based on the molecular composition (56.6% protein, 42% RNA) (82). The nucleocapsid is surrounded by an envelope derived from the host cell lipid bilayer membrane. The envelope contains two (SIN) or three (SF) virus specific glycoproteins arranged on 180 spikes coincident with capsid protein molecules. The glycoproteins are E1 ($M_w=5 \times 10^4$), E2 ($M_w=5 \times 10^4$), and E3 ($M_w=1 \times 10^4$) and are present in equimolar concentration on each spike (19,82). The E3 of SIN is released into the medium (61). The molecular weight of the virion is approximately 4×10^7 (40,82).

Infection and Replication

The first step in SF infection of a cell is the binding of the virion to the cell surface. The receptor for the virion is apparently common to a variety of cells since the virus can infect many vertebrate and some non-vertebrate cell types (61). It had been proposed that antigens encoded by the major histocompatibility complex (MHC) serve as the virus receptors since the viral glycoproteins can bind to these cellular glycoproteins (32). However, cells that do not express MHC glycoproteins can be infected by SF (62).

After attachment of the virus to the cell surface, the virion is endocytosed and appears to migrate to endosomes where a low pH micro-environment allows fusion of the viral envelope to the endosomal lipid

membrane (34,59). After fusion, the nucleocapsid is released into the cytoplasm where replication occurs (82).

The mechanism for dissociation of the 42S RNA from the capsid protein is not known. However, when the 42S RNA is released from the nucleocapsid, it is available for immediate translation (46). The first four proteins translated are nonstructural and are synthesized as a polyprotein precursor that is cleaved proteolytically (Fig. 1) (46,82). After cleavage, one protein is an RNA-dependent RNA polymerase, which directs the production of a negative 42S strand of RNA from the positive-stranded virion RNA (47,82). Positive-stranded 42S and 26S RNAs are synthesized in turn (46,82). The 42S RNA will be packaged in developing capsids while the 26S RNA, representing the 3' one-third terminus of the virus genome, is translated into a polyprotein that is cleaved subsequently to give capsid protein and the three glycoproteins (Fig. 2) (46,82). The 26S RNA has one known initiation site for translation and the polyprotein contains, in sequence, capsid, E3, E2, and E1 (8,23,46,50,82). The first cleavage results in freeing the capsid protein, which is released into the cytoplasm where it is available to interact with the 42S RNA to form nucleocapsid (8,20,21,77). During translation, the ribosomes become associated with the rough endoplasmic reticulum (RER) and the remaining polyprotein is inserted into the membrane of the RER. This polyprotein is cleaved into two products: designated PE2 in SIN and NVP62 in SF, and E1 (77).

Glycosylation begins immediately after translation (80, 82). The oligosaccharide $(\text{Glc})_3(\text{Man})_9(\text{GlcNAc})_2$ is transferred by host enzymes to the peptide chain by way of a dolichol pyrophosphate

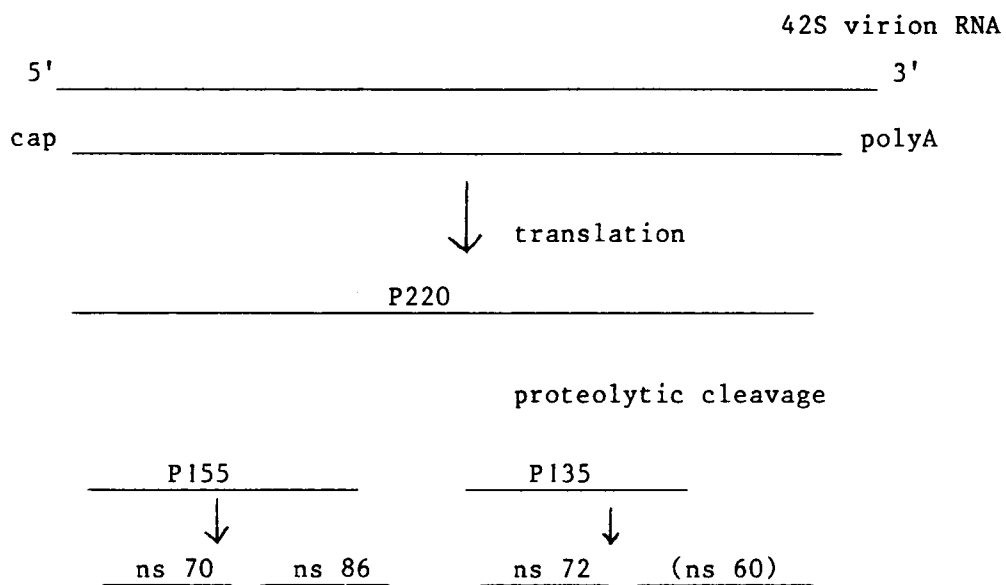


Figure 1. Synthesis and Processing of SFV Non-structural Proteins

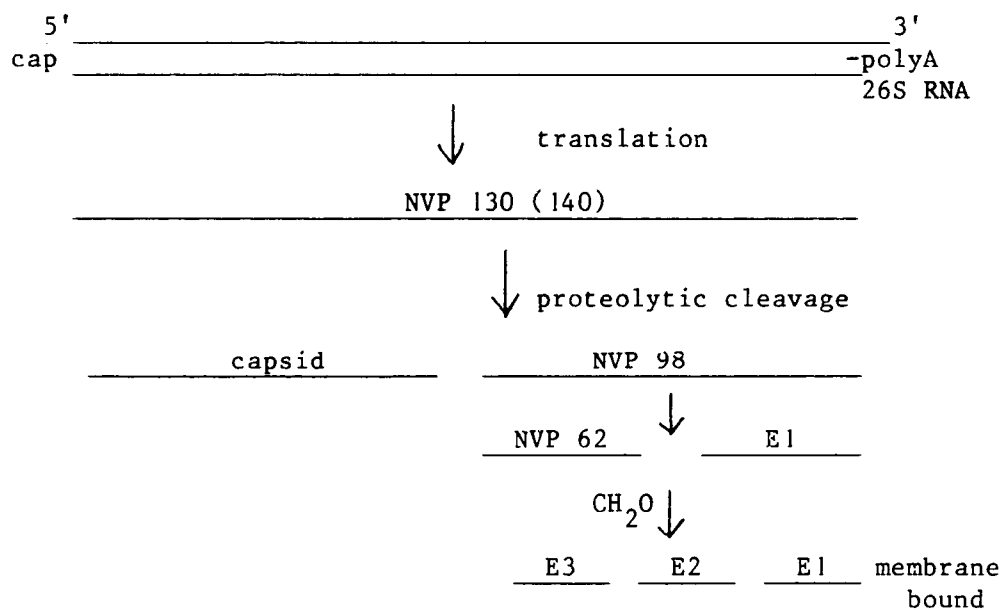


Figure 2. Synthesis and Processing of SFV Structural Proteins

intermediate and is joined to the polypeptide by way of -N-glycosidic linkages between N-acetyl-glucosamine and asparagine (44,82,84). The glycoproteins then migrate to the Golgi apparatus where they undergo further processing including fatty acid acylation, trimming, and addition of terminal sugars (44,79,82). While there is strong evidence that NVP62 appears intact on the infected cell membrane and that cleavage occurs after insertion (3,42,100), it has been reported that NVP62 is cleaved into E2 and E3 either in the Golgi apparatus or in transport from the Golgi to the plasma membrane (44). External antiglycoprotein antibody treatment of infected cells prevents cleavage of NVP62. This could be misleading in that antibody can be endocytosed and thus acting intracellularly to block cleavage. Evidence supporting the latter mechanism is provided by monensin treatment of SF infected chicken embryo fibroblast cells (CEF). Monensin, the Na^+K^+ ionophore, inhibits glycoprotein and secretory protein transport within the Golgi stacks. Monensin treatment of CEFs prevents the transport of the viral glycoproteins to the cell membrane but does not prevent cleavage of NVP62 to E2 and E3 (44). It has been shown that this cleavage occurs approximately 5 minutes after the peptide becomes resistant to endoacetylglucosaminidase H. This would place the protein in the Golgi or in transport to the membrane at the time of cleavage (29). The glycoproteins then insert into the plasma membrane. The enzymes involved in the proteolytic cleavages of SF proteins are not well characterized. It is believed that at least one virally encoded protease is active. Whether one

viral protease, more than one, or a mixture of viral and cellular proteases are involved is not known (82).

The nucleocapsid associates with the glycoproteins in the plasma membrane where the capsid protein interacts with the cytoplasmic domain of the E2 glycoprotein, which is associated with the E1 and E3 glycoproteins, giving a ratio of 1:1:1 of capsid and glycoproteins. When the nucleocapsid is surrounded by the glycoprotein containing membrane, it evaginates from the cell as virion (82).

K-562 Cell Line

The human leukemic pluripotent stem cell line, K-562, was established from the pleural effusion of a patient in the blastic crisis stage of chronic myelogenous leukemia (CML). Since its establishment in 1970, the cell line has been serially passaged at weekly intervals and aliquots of various passages have been frozen in liquid nitrogen. Sublines have been established from these frozen stocks (52).

Unlike other CML lines, all K-562 sublines have retained the chromosomal marker associated with CML, the Philadelphia chromosome (Ph^1) (52,54). This marker consists of a reciprocal translocation of chromosomes 22 and 9, $t(22; 9)(q11; p.23)$ (52,54,58). Other chromosomal markers and a number of translocations are present in sublines. Sublines established from early passages exhibit a hypodiploid modal chromosome number, while those established from later passages as well as the parent cell line are hypotriploid (52,58).

K-562 cells are highly undifferentiated and are of variable size (average 14-17 μm in diameter, ranging from 7-55 μm). Most cells are round or oval with basophilic cytoplasm that is agranular, with few

vacuoles. While nuclei are pleomorphic, the predominant form is round with a slight indentation and there are one to four nucleoli. A second commonly observed cell type has pale blue cytoplasm and a large nucleus with two to six nucleoli (52,58). Mean cell generation time, which was 50 hours in early passages, decreased and stabilized at 10-12 hours after passage 85. K-562 cells may be cloned with high efficiency in soft agar (52,58). Cloned cultures always contain a mixture of the two cell types. Cells tested in short term culture (passaged at one week intervals), exhibit no myeloperoxidase or alkaline phosphatase activity, produce no lysozyme and do not phagocytose inert particles, bacteria or sensitized erythrocytes (52,58). The cells were negative when tested for Epstein Barr virus genome and nuclear associated antigens, Friend murine leukemia virus, Mason-Pfizer monkey virus, leukemogenic monkey virus, and herpes virus-like particles as well as reverse transcriptase (54). Cells show no interferon production when stimulated with Newcastle's Disease virus (NDV) and tested for plaque reduction of NDV or vesicular stomatitis virus on Vero cells (52,53,54). K-562 cells do not express human leukocyte antigens (HLA) or Ia- like cellular antigens, do not form E rosettes, or produce immunoglobulin (53,54).

Recent discoveries in the study of cellular protooncogenes include the mapping of C-abl protooncogene in the region of chromosome 9 that is translocated to chromosome 22 in the Ph¹ chromosome (54,55). This shift puts C-abl in the same locus as the C λ light chain (10). Both the C-abl and C λ genes are amplified in K-562 cells. However, there is at this time no evidence to support amplification of C-abl

due to the association with C λ as is seen in the Burkitt's lymphoma 22;8 translocation that puts C-myc adjacent to the C λ switch gene (16). A unique 8.0-kb RNA transcript of C-abl has been detected in K-562 cells and cells from CML patients (81). Two protein species (190 Kd and 240 Kd) which react to two antisera raised against C-abl and have tyrosine protein kinase activity have been isolated in K-562 cells. Studies are currently under way to examine the protein kinase activity of these two proteins and the role of C-abl in normal hematopoietic cell differentiation and development of CML (4,18)

CML is thought to be a clonal tumor which develops from a pluripotent hematopoietic stem cell. Patients in the blastic crisis stage present with cell populations of either lymphoid or myeloid lineage (17). The K-562 line is thought to be a cancerous model for this pluripotent stem cell because long term cultured cells (10-11 days) show markers and functional characteristics of both lymphoid and myeloid lineages (38,55,58).

When K-562 cells are kept in culture for 10-11 days (reaching stationary phase on day 9), in the absence of exogenous differentiation inducers, they begin to spontaneously differentiate into early precursors of the monocytic, granulocytic, erythrocytic and megakaryocytic series. This is shown by production of fetal hemoglobin, granulocyte and monocyte specific enzymes, platelet glycoproteins and the presence of granules. Preliminary differentiation of K-562 cells can also be achieved using chemical inducers. No terminal differentiation of K-562 cells has been attained and any differentiation of cells is reversed when cells are passaged into fresh medium (55,58).

Recent advances in hybridoma technology has made possible the comprehensive examination of K-562 cell surface markers using monoclonal antibodies. These studies indicate that K-562 cells, without the addition of chemical inducers, possess markers of all five major bone marrow cell lineages and thus should be capable of partial differentiation along all five of these lines. Markers present include:

1. precursor hematopoietic cell antigens found on bone marrow stem and precursor cells;
2. transferrin receptor and glycophorin A which are erythrocyte-associated antigens;
3. those reactive with the monoclonal antibodies My-1 and PMN 29 which are specific for myelocytes and neutrophils respectively;
4. those reactive with 6 3D3 monoclonal antibody which is specific for monocytes;
5. platelet glycoprotein I specific for megakaryocytes; and
6. those reactive with OKT-17, HNK1, and BA-1, specific for T lymphocytes, natural killer cells, and B lymphocytes respectively (38,55,58).

Monoclonal antibodies raised against K-562 cells were shown to react with antigens shared by normal hematopoietic progenitors, leukemia cells, and transformed lymphocytes. Myeloid and erythroid colony formation was inhibited when normal bone marrow cells were treated with this anti-K-562 monoclonal and complement (99). A monoclonal antibody raised against normal bone marrow (E-10) reacts with only 3% of normal bone marrow cells but reacts strongly with K-562 cells (76) and is capable of causing antibody-induced killing of K-562 cells in the absence of complement or effector cells (36).

Xenogeneic anti-K-562 sera have been used to immunologically characterize K-562 cells. The antiserum, made in goats and also from

a monkey and in rabbits, is usually absorbed with normal human bone marrow cells and/or peripheral blood leukocytes to remove antibody not specific for K-562 or leukemia associated antigens. Absorbed sera exhibit cytotoxicity specific for leukemia by complement-mediated (ADCMC) or cell mediated (ADCC) mechanisms (56,58). Antibody alone, whole antiserum, gamma globulin, or Fab fragment, is cytotoxic for K-562 cells in vitro in the absence of complement or effector cells and in vivo in decomplemented mice (56,58, 98).

A leukemia specific antigen (nonglycosylated protein mw=80,000 d) has been found on K-562 cells and, when isolated, inhibits the specific ADCMC of goat anti-K-562 serum (9). Other studies showed that rosette formation of sensitized sheep erythrocytes complexing with the K-562 Fc receptor was inhibited by absorbed goat anti-K-562 serum. While this might suggest that the specific antigen is the K-562 Fc receptor, which is antigenically distinct from that of mature cells, the finding that goat anti-K-562 also reacts with non-Fc receptor bearing leukemia cells suggests that the antibody reacts with a specific leukemia antigen that might be closely associated with the Fc receptor (66).

K-562 cells are susceptible to cytolysis mediated by human natural killer cells (NK). Since this finding in 1976, K-562 cells have been used world-wide as a target cell in studies of NK cell-mediated cytolysis (41). Since NK cells are suspected to have a role in regulation of hematopoiesis (24,28, 48, 93,94,95), the likelihood is that the K-562 cells treated with chemical inducers of differentiation are less susceptible to NK cytolysis than are untreated K-562 controls (31).

Persistent Alphavirus Infection

Persistent viral infection in vivo is associated with some animal viruses. Viruses such as those of the family Herpetoviridae are widespread in human populations and are notorious for their proclivity in establishing latent infections that undergo recrudescence when the viral infection is apparent and sometimes fatal (13). Viruses that can persist have been implicated in contributing to a malignant state (eg. HSV-2 linked to cervical carcinoma, EBV in Burkitt's lymphoma) (15). Recent studies of long term debilitating diseases in vertebrates, including man, suggest that viruses or virus-like particles may be the causative agents (14). Persistent infection of insects and vertebrates has been proposed for the maintenance of a virus in an ecosystem(7). Thus, it is important that models for laboratory study of persistent viral infection continue to be developed.

There are numerous reports of persistent infections in vitro by RNA viruses which are normally considered highly cytolytic (2,25, 26,45, 67,90). One or more of the following criteria often must be met for persistent infection to be established: 1. the virus inoculum must be at a high multiplicity of infection to give autointerference of virus replication; 2. the virus inoculum must be rich in defective interfering particles; 3. the virus inoculated must be a temperature sensitive mutant; 4. interferon must be present in cell cultures (39, 60, 91). During the establishment of persistent infection, cells commonly undergo a burst of virus production followed by periods of crisis where up to 95% of the cells are lysed by the virus. Persistently infected cell cultures, established from the surviving cells

after periods of cytolysis, usually exhibit cellular morphology and growth rates similar to the uninfected parent cell (67, 91). Further, many cultures continue to produce low levels of infectious virus. Virus produced in a persistent infection has been noted as undergoing mutations for unknown reasons, such as becoming temperature sensitive (2,25,35,45). Persistently infected cells usually become resistant to superinfection by wild type virus (25).

Persistent infection of mammalian cells with SF (L-929 mouse cells) or SIN (BHK-21 hamster cells) has been established using one or more of the above-mentioned criteria. SF can persistently infect L-929 cells if interferon is present in the culture or if the virus inoculum is rich in defective interfering particles. The persistently infected cells undergo periods of virus induced cytolysis during which up to 95% of the cultured cells lyse. While DI particles are essential for establishment of persistent infection, it is the virus induced interferon that appears to be necessary for maintenance of the persistent state, as well as possible mutations that may occur. Infectious virus is produced during persistent infection but it is estimated that only 0.4-4.5% of the total cells produce virus and only 1-20 % of the total cells are positive for viral antigens when tested in the fluorescent antibody technique (60).

Persistently infected cultures treated with antiviral antibody do not undergo cell crises and death but may be cured of the infection. This observance indicates that extracellular virus is necessary for maintenance of the persistent infection state. This requirement for extracellular virus along with the high percentage of cells negative

for viral antigens suggests that an apparent persistent infection is a balance of a few infected cells mixed with a high percentage of cells that are totally uninfected. The high percent of uninfected cells is presumably the consequence of protection by interferon (60).

Persistent SF infection in vivo has been reported when mice are inoculated with either DI particles or are passively immunized against SF (1).

Persistent infection of SIN in BHK cells was established using a viral inoculum enriched for DI particles. As with SF infected L-929 cells, SIN infected BHK cells undergo an initial crisis period. Unlike infected L-929 cells, the SIN infected cells did not present additional crisis periods, interferon did not seem to be necessary for maintenance of the infection, and most cells contained viral RNA and thus were considered to be infected. Cells cloned from the original infected culture lost all characteristics of infected cells (91).

While persistent alphavirus infection of mammalian cells is possible only under specific conditions, such an infection (SF,SIN) of mosquito cells is easily achieved. Cultures of Aedes albopictus cells infected at an MOI of 5-10 undergo no CPE. Peak virus yields of 10^8 - 10^9 pfu/ml are reached during the first week post infection and gradually decrease and plateau at 10^5 - 10^6 pfu/ml by week five post infection throughout the life of the culture. The virus mutates to a small plaque temperature sensitive mutant. Persistently infected cells produce DI particle RNA, and are resistant to superinfection by homologous virus (70,71,73,86). Extracellular virus appears to be necessary for maintenance of the persistent infection (73). The

existence of a low molecular weight anti-viral factor produced by persistently infected mosquito cells has been reported. This material is capable of decreasing virus yield when added to infected mosquito cell cultures during the time of peak virus production. The factor is both virus and cell specific (74).

CHAPTER III

MATERIALS AND METHODS

Cell Cultures

K-562

The K-562 cell line was established in 1970 from the pleural effusion of a patient in the blastic crisis stage of chronic myelogenous leukemia. Cells of various weekly passages were provided by Dr. Carmen B. Lozzio and subcultured before use.

Cells were grown in EM-15A supplemented with 10% newborn bovine serum (NBS; Gibco, Grand Island, NY) and subcultured in RPMI 1640 medium supplemented with 10 to 15% bovine calf serum (BCS; KC Biologicals, Kansas City, MO), NBS, or fetal bovine serum (FBS, Gibco) and containing Gentamycin (5 mcg/ml; Gibco). After initial infection, SF-infected cells were subcultured at one week and then at tri-weekly intervals in RPMI 1640 medium supplemented with 15% FBS, Gentamycin (5 mcg/ml, Gibco), Penicillin/Streptomycin (100 units/ml, Gibco), and Fungizone (1 mcg/ml, Gibco).

MRC-5

The MRC-5 cell strain was established in 1966 from the normal lung tissue of a 14-week old human male fetus by J. P. Jacobs. It is a human diploid fibroblast-like cell strain. Cells will undergo 42 to 46 doublings before decline. The cells are used in senescence studies, are susceptible to infection by a number of human viruses, and are used in the production of virus vaccines. These cells were provided by Dr. R.J. Courtney and were routinely grown in EMEM supplemented with 5% FBS (Gibco).

CEF

Chicken embryo fibroblasts (GEF) were prepared from 10 day old embryos as primary cell cultures. The cells will undergo 5-10 doublings before decline. They are used for propagation of virus stocks and for plaque assays.

L-929

The L-929 cell line was established in 1948 by K. K. Sanford, W. R. Earle, and G. D. Likely. It is a clone of a mouse connective tissue cell line designated Strain L established in 1940 by Earle. The original strain was derived from normal subcutaneous areolar and adipose tissue of a 100 day-old C3H/AN mouse. Clone 929 was selected from the ninety-fifth subculture of the parent strain. These cells are widely used in virological as well as other studies.

These cells were provided by Dr. Arthur Brown and were routinely grown in McCoy's medium (Gibco) supplemented with 5% FBC (Gibco) and Gentamycin (5mcg/ml, Gibco).

Preparation of Virus Stock

SIN Ar339 and SF viruses were provided by Dr. Arthur Brown. The stocks were grown in CEF roller bottle cultures (Corning, Corning, NY), and infected at a MOI of 0.01. Virus particles were harvested at 24 hours post-infection by removal of culture medium. Cellular debris was removed by centrifugation at 1700 x g for 15 minutes at 4°C, and stored at -70°C. Virus titer was obtained by plaque assay on CEF monolayers.

Infection of K-562 Cells with SF

K-562 cells from a particular age of culture and concentration were washed once with RPMI 1640 tissue culture medium (Gibco) containing 2% BCS (KC Biologicals), NBS (Gibco), or FBS (Gibco) and suspended in 1 ml of the same medium. SF at an MOI of 0.33, 1, or 3, or medium for mock infection, was added to separate cell suspensions. The suspensions were then incubated with occasional rocking at 37°C in 5% CO₂ for 60 minutes to allow adsorption of the virus. After incubation, the cells were washed 3 times in medium and suspended to a concentration of $1-2 \times 10^5$ cells/ml in RPMI 1640 supplemented with 15% of the same serum used in the initial wash medium, Fungizone (1 mcg/ml, Gibco), Penicillin/Streptomycin (100 units/ml, Gibco), and Gentamycin (5 mcg/ml, Gibco). The suspensions were placed in tissue culture flasks (Gibco, Nunc) and incubated at 37°C in 5% CO₂.

Preparation of Chicken Embryo Fibroblasts (CEF)

Virus-free fertilized chicken eggs (Truslow Farms, Maryland) were incubated at 38°C for 10 days. The eggs were then sprayed with 70% ethanol, cleaned with egg wash (iodine, ETOH), opened, and the embryos removed. The embryos were decapitated, eviscerated, and washed 5 times in Saline A solution (0.8% NaCl, 0.1% dextrose, 0.04% KCl, 4 ml phenol red in 1000 ml dH₂O) before being forced through a 10 ml syringe into a 200 ml solution of 0.025% trypsin-Saline A. The suspension was incubated with constant stirring at 37°C for 45 minutes. After incubation, the cell suspension was filtered through sterile gauze to remove tissue clumps and the filtrate was collected in a 50 ml centrifuge tube

(Corning) containing 2 ml NBS to inhibit trypsinization. Cells were centrifuged at 800 x g for 15 minutes and the supernatant fluid was discarded. The cell pellets were suspended by gentle vortexing in Medium-199 with 5% NBS (Gibco), Gentamycin (5 mcg/ml, Gibco), Penicillin/Streptomycin (100 units/ml, Gibco), and Fungizone (1 mcg/ml, Gibco). The cell suspensions were then pooled, the number of cells determined and diluted as necessary to seed 6-well tissue culture plates (Lindbro, Flow Laboratories, McLean, VA) for plaque assay or tissue culture flasks (Nunc) for future use. These primary CEF cultures were viable through three passages.

SF Plaque Formation on CEF Cells

Chicken embryo fibroblasts (CEF) were prepared as described and seeded into 6-well tissue culture plates (Lindbro). Confluent monolayers were washed once in HBSS (Gibco) and 0.1 ml of virus sample or 0.1 ml HBSS (mock-infected cell control) was added to each well. The plates were incubated with occasional rocking at 37°C in 5% CO₂ for 60 minutes to allow adsorption of the virus. After incubation, the fluid was removed by aspiration and the monolayers were overlaid with 3 ml of M-199 in 0.9% agar (Gibco). The plates were then incubated at 37°C in 5% CO₂ for 48 hours. After incubation, an additional overlay of M-199 in 0.9% agar containing neutral red stain (1:300, 5 ml/100 ml) was made to each plate. After time was allowed for the neutral red to stain living cells (6-24 hours), plaques were counted.

Infectious Center Determination

Chicken embryo fibroblasts were prepared as described and seeded into 6-well tissue culture plates (Lindbro). SF-infected K-562 cells or uninfected control cells were washed one time, suspended and diluted in HBSS (Gibco). CEF monolayers were washed once in HBSS and 0.1 ml of cell suspension added to each well. The plates were incubated with occasional rocking at 37°C in 5% CO₂ for 60 minutes to allow adsorption. After incubation, the monolayers were carefully overlaid with 3 ml of M-199 in 0.9% agar (Gibco). The plates were incubated, stained, and plaques counted as described.

Staining of K-562 Cells

Cells were washed once in RPMI 1640 (Gibco) and suspended in fresh medium from which 1×10^5 cells were extruded onto microscope slides by centrifugation in a Cytospin II (Shandon Southern Instruments, Sewickly, PA) cytocentrifuge (500 x g, 5 minutes). The slides were stained with a Diff-Quik® hematological staining kit (American Scientific Products, Stone Mountain, GA). Stained cells were examined microscopically and photographed as appropriate.

Labeling of K-562 Cells With Chromium-51

K-562 cells (4×10^6 cells) were washed once in RPMI 1640 (Gibco) or EMEM (KC Biologicals) containing 5% FBS (Gibco). After centrifugation at 800 x g, the cells were suspended in 0.5-1.0 ml medium and 400 µCi of Na₂⁵¹CrO₄ (ICN, Irvine, CA; NEN, Boston, MA; Amersham, Arlington Heights, VA) were added. The mixture was incubated on a

rocking platform at 37°C for 45 minutes. After incubation, the cells were washed five times in RPMI 1640 and suspended to the desired concentration.

A ⁵¹Cr Release Assay of K-562 Cells After Virus Infection

Cells from a particular age of culture were labeled with $\text{CrO}_4^{=}$ as described and suspended to 1×10^5 cells/ml in EMEM-FBS. The cell suspension (3 ml) was infected with SIN Ar339 strain, or SF, at an MOI of 1, 5, or 10, or were mock-infected with medium. The infected cells were incubated at 37°C for 60 minutes on a rocking platform to allow adsorption of the virus. After incubation, the cells were washed three times with EMEM and suspended to 1×10^4 cells/ml for transfer to, and culture in, plastic 25 cm² tissue culture flasks (Corning, Corning, NY). The medium used for culture contained 90% fresh EMEM (containing 2% FBS), and 10% conditioned medium collected as the supernatant fluid of the K-562 culture used. The cell suspensions were then incubated at 37°C in 5% CO₂. At intervals of time, triplicates of 200 µl of supernatant fluid from each suspension were removed and placed in 12 x 75 mm borosilicate culture tubes (Fisher, Norcross, GA) for determination of total radioactivity which was done in a Beckman Gamma 4000 Spectrometer. The tubes were then centrifuged at 700 x g and 100 µl of the supernatant fluid were evaluated for radioactivity. Percent ⁵¹Cr release was calculated as follows:

$$\frac{\text{supernatant fluid cpm} \times 2}{\text{total cpm}} \times 100 = \% \text{ } ^{51}\text{Cr release}$$

Preparation of Rabbit Anti-alphavirus Serum

Purified SIN or purified SF (250 μ g, approximately 10^{10} pfu) were emulsified in complete Freund's adjuvant (CFA, Gibco) and given subcutaneously. A second injection (250 μ g of virus in CFA) was given one week later. Subsequent injections consisted of 250 μ g of purified virus without adjuvant and were given intravenously. Rabbits were bled, the serum obtained and either heat-inactivated or fractionated and stored at -40°C . The antiserum was considered normal immune if the injections were given over a period of less than six months, and were considered hyperimmune if the injections were continued over a period of greater than one year. The hyperimmune serum contained homologous virus neutralizing titers of 10^5 (assayed by plaque reduction on CEF monolayers) but no heterologous virus neutralization activity (1:5). Hyperimmune sera were shown to be cross-reactive by ADCMC of infected CEF and L-929 cells.

Preparation of Goat Anti-K-562 Serum

Goat preimmune and anti-K-562 sera were provided by Dr. Carmen B. Lozzio. The first of four weekly injections was made with 5×10^6 cells/kg of body weight in complete Freund's adjuvant (CFA, Sigma, St. Louis, MO). The second and third injections contained cells emulsified with incomplete Freund's adjuvant (IFA, Sigma). The fourth weekly dose of cells was given intravenously. The Goats were bled after 10 days, serum obtained from clotted blood and heat-inactivated. The serum was then absorbed five times with human buffy coat (Plasma Alliance, Knoxville, TN) and/or bone marrow (10^7 cells/ml serum).

Gamma globulin fraction was obtained by $(\text{NH}_4)_2\text{SO}_4$ fractionation (dropwise addition of 33% v/v saturated $(\text{NH}_4)_2\text{SO}_4$).

Immune Enhancement

Antiviral Antisera

SF or SIN were incubated with homologous antiserum (2 2-fold dilutions of antiserum past neutralization endpoint) on ice for one hour. The antibody-virus mixtures were used to infect K-562 cells as described. Cells were assayed for ^{51}Cr release due to CPE as described.

Anticellular Antiserum

K-562 cells were washed once in RPMI 1640 containing 5% NBS (Gibco) and suspended in goat anti-K-562 serum (1:50 dilution). The antibody-cell mixture was incubated at room temperature for one hour after which the mixture was centrifuged at 800 x g. The supernatant fluid was removed and the cells were washed three times in RPMI. The cells were then infected with SF or SIN as described and assayed for ^{51}Cr release due to CPE as described.

Antibody Dependent Complement Mediated Cytotoxicity Assay

^{51}Cr Release Assay

SF infected K-562 cells, or uninfected K-562 cells, were labeled with ^{51}Cr as described. Rabbit anti-SF hyperimmune serum, rabbit pre-immune serum, goat anti-K-562 serum or goat preimmune gamma globulin were heat-inactivated at 56°C for 30 minutes. The rabbit sera were absorbed with uninfected K-562 cells (1×10^6 cells/ml serum) for 1

hour at 37°C as described. Serial two-fold dilutions of sera were made in RPMI 1640 containing 5% NBS (Gibco) in 96-well round bottom microtiter plates (Dynatech Labs, Inc., Alexandria, VA) to give a final volume of 50 µl serum/well. K-562 cells (50 µl, 10⁴ cells) and 50 µl rabbit low-tox-human complement (final dilution 1:15) (Accurate Chemical and Scientific, Westberry, NY) were added to each well. Complement control wells contained 50 µl medium; and total releaseable count wells contained 50 µl cells and 100 µl Triton X-100 (Sigma) (added immediately prior to harvest). These control sets were made for each population tested. The plates were incubated at 37°C in 5% CO₂ for 90 minutes. After incubation, the plates were centrifuged at 700 x g for 7 minutes and 75 µl of supernatant fluid from each well was harvested into a 12 x 75 mm borosilicate glass culture tube (Fisher Scientific) for determination of radioactivity in a LKB RIA/Gamma spectrometer.

Trypan Blue Exclusion Assay

The trypan blue (Gibco) exclusion ADCMC assay is as described above except K-562 cells were not labeled with ⁵¹Cr and no total releaseable count wells were made. The percent specific killing was determined microscopically by determining the percent cells in test wells that were stained with trypan blue (dead cells) compared with the percent in control wells.

Indirect Immunofluorescence Technique

Cells were extruded onto microscope slides as described in the staining procedure. Those cells to be evaluated for internal immuno-

fluorescence were fixed in methanol and then washed three times in 0.5M PBS, pH 7.5. The primary antibody (20 μ l, 1:20 dilution) was added to the cell area and the slides were incubated in a humid chamber at 37°C in 5% CO₂ for 60 minutes. After incubation, the slides were washed three times in PBS to remove unreacted primary antibody. FITC-conjugated goat anti-rabbit IgG serum (20 μ l, 1:20 solution; Cappel Laboratories, Division of Cooper Scientific, Malvern, PA) was added to the cell area and the slides were incubated as before. Finally, the slides were washed three times in PBS to remove unreacted FITC-antibody and allowed to air dry. A 1:1 preparation of glycerol-PBS was used to mount coverslips before microscopic examination.

Isolation of Human Peripheral Blood Lymphocytes

Heparinized human peripheral blood was diluted 1:2 in RPMI 1640 and carefully layered onto one-half volume ficoll (Histopaque 1077, Sigma) in a 15 ml centrifuge tube (Corning). The tubes were centrifuged at 400 x g for 35 minutes and the mononuclear cell-containing band was harvested and washed three times (300 x g, 10 minutes) to remove ficoll and platelets. The cell suspension was placed in tissue culture flasks (for 30 minutes) to remove adherent monocytes/macrophages and the non-adherent cell population was harvested, counted, and suspended to the desired concentration for use in natural killer cell cytotoxicity assays.

Natural Killer Cell Cytolysis Assay

K-562 cells were labeled with ^{51}Cr as described and 100 μl of suspended K-562 target cells at desired concentration were placed in wells of 96-well, round bottom microtiter plates (Dynatech). Human lymphocyte effector cells (100 μl) were added to the target cells at effector:target ratios of 50:1, 25:1 and 12.5:1 with triplicate wells being seeded for each effector:target ratio, for spontaneous ^{51}Cr releaseable counts (100 μl K-562 cells + 100 μl medium), and for total releaseable counts (100 μl K-562 cells + 100 μl 3% Triton X-100). The cell mixtures were incubated at 37°C in 5% CO_2 for four hours. After incubation, the plates were centrifuged at 700 x g for 7 minutes. A sample of supernatant fluid (75 μl) was taken and placed in a 12 x 75 mm borosilicate culture tube (Fisher) for determination of radioactivity in a LKB RIA/Gamma gamma spectrometer.

Mixed [^3H]-Amino Acid Incorporation

K-562 cells were washed once in medium, suspended in amino acid-free medium and allowed to incubate at 37°C in 5% CO_2 for 30 minutes. The suspension was centrifuged, supernatant fluid discarded and the cells suspended in fresh amino acid-free medium to a concentration of 1×10^6 cells/ml. Mixed [^3H]-amino acids (Amersham; 50 μCi ; containing: L-[4,5- ^3H]Leucine >100 Ci/mmol; L-[4,5- ^3H] Lysine 75-100 Ci/mmol; L-[2,3,4,5,6- ^3H] Phenylalanine >100 Ci/mmol; L-[2,3,4,5- ^3H] Proline 80-110 Ci/mmol; L-[2,3,5,6- ^3H] Tyrosine 70-100 Ci/mmol) were added per 10^6 cells and 1 ml of the cell suspension was seeded in triplicate wells of 24-well tissue culture plates (Costar, Cambridge, MA). The

cultures were incubated for four hours at 37°C in 5% CO₂. After incubation 100 µl samples of each well were harvested and placed on paper disks (Whatman 23 mm, Fisher) as described.

[³H] Thymidine Incorporation

K-562 cells were washed once in medium and suspended to a concentration of 1×10^6 cells/ml. [³H] thymidine (Amersham, 100 µCi, 18 Ci/mmol) was added per 10^6 cells and 1 ml of the cell suspension was seeded in triplicate wells of 24-well tissue culture plates (Costar). The cultures were incubated for 18 hours at 37°C in 5% CO₂. After incubation, 100 µl samples of each well were harvested and placed on paper disks (Whatman 23 mm, Fisher) as described.

Trichloroacetic Acid Precipitation of Macromolecules

Samples of cell suspensions labeled as described with either [³H] thymidine (TdR) or mixed [³H]-amino acids were placed on paper disks (Whatman 23 mm, Fisher) and allowed to absorb. [³H]TdR-labeled samples were then immersed in ice cold 10% trichloroacetic acid (TCA, Fisher) for 30 minutes to precipitate cellular macromolecules into the filter disk. The disks were then transferred sequentially to each of the following for 5 minutes: 10% TCA, 5% TCA, 50% ETOH:50% ether, and finally 100% ether.

[³H]-amino acid labeled samples were immersed in ice cold 10% TCA for 30 minutes to precipitate cellular macromolecules and these disks were transferred sequentially in 5% TCA for 10 minutes, 5% TCA held in a boiling water bath for 20 minutes, 5% TCA for 5 minutes,

50% ETOH:50% ether for 5 minutes, and finally 100% ether for 5 minutes. Disks were allowed to dry under a heat lamp and then placed in scintillation counter vials (Wheaton, Fisher) with 4 ml Redi-sol scintillation fluid (Beckman, Irvine, CA). Radioactivity was determined in a Beckman beta spectrometer.

Effect of Rabbit Anti-SF Hyperimmune Serum on SF-infected K-562 Cells

Normal rabbit serum and rabbit anti-SF hyperimmune serum were heat-inactivated at 56°C for 30 minutes and each serum was absorbed with 1×10^6 uninfected K-562 cells (per ml of serum) at 37°C for 1 hour. After incubation, the serum samples were centrifuged at 800 x g for 10 minutes to remove the cells. SF-infected K-562 cells, 19 days post infection, or non-infected K-562 cells were washed once in RPMI 1640 and suspended to 2×10^5 cells/ml in RPMI 1640 containing 15% NBS (Gibco). Each cell population was seeded into 25 cm² tissue culture flasks (Corning) and rabbit hyperimmune, or normal preimmune serum was added to a flask of each cell population in a final dilution of 1:50. One ml samples were removed at five hour intervals up to 51 hours after serum addition and then daily through nine days after serum addition. Viability was determined by trypan blue exclusion and was expressed as percent number of viable to total cells.

Effect of Anti-viral Sera on SF-infected K-562 Cells

Normal preimmune serum, rabbit anti-SF hyperimmune serum (whole serum or gamma globulin fraction), rabbit anti-SF normal immune serum, rabbit anti-SIN hyperimmune serum, and rabbit anti-

SIN normal immune serum were prepared as described for rabbit anti-SF hyperimmune serum. SF-infected K-562 cells, 8 months post infection, or noninfected K-562 cells, were washed once in RPMI 1640 containing 10% FBS (Gibco). Each cell population was seeded into 25 cm² tissue culture flasks (Corning) and a different serum sample (in a final dilution of 1:50) was added to an individual flask of each cell population. One ml samples were removed at 24 hour intervals starting at two days after serum addition through nine days after serum addition. Viability was determined by trypan blue dye exclusion and was expressed as the percent number of viable to total cells.

CHAPTER IV

RESULTS

Virus Infection of K-562 Cells

Previous attempts in this laboratory to infect K-562 cells with SF had shown that this virus produced no apparent cytopathic effect on K-562 cells within 24 hours post infection as measured by ^{51}Cr release (Judith Wolcott, personal communication). Therefore, it was of interest to ascertain the characteristics of the virus infection in this cell line. In the present study, K-562 cells were tested for susceptibility to infection by Semliki Forest virus (SF). A number of parameters were examined to determine whether or not SF could produce an active infection in this cell type.

One criterion for determining SF infection of cell culture is the development of cytopathic effect. This virus is considered to be highly cytolytic for mammalian cell cultures producing cytopathic effect in virtually 100% of most cell types by 24 hours post infection. However, when virus was added to K-562 cells, no apparent cytopathic effect was observed upon microscopic examination of unstained cells, although after two days, the cell growth rate decreased dramatically. An examination of the growth curve for SF-infected cells (Fig. 3) indicates that infected cells proliferate normally for two days and then reaches a steady state in cell concentration, which is evident through six days. When the cells are passaged to new medium at six days pi, the infected cell culture was maintained at the initial level of inoculation, that is, approximately 2×10^5 cells/ml.

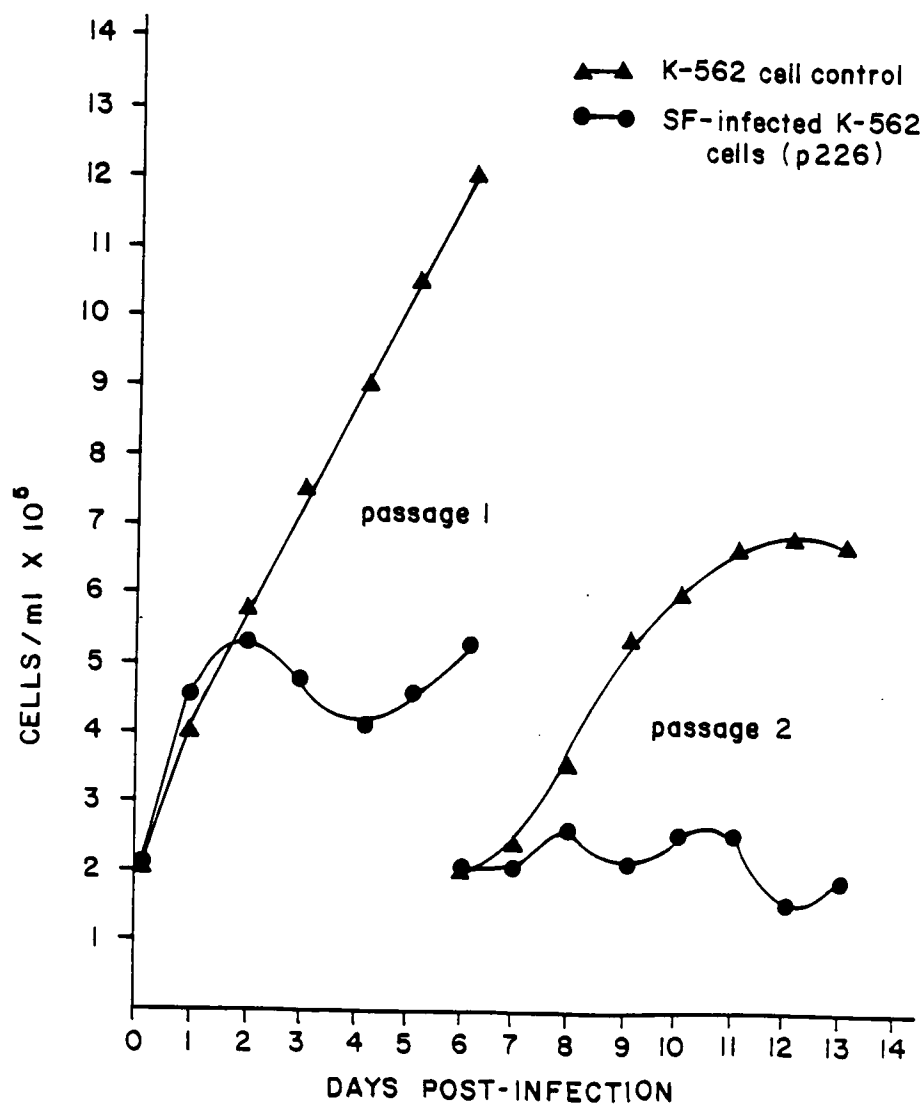


Figure 3. Growth of SF-infected K-562 Cells

K-562 cells were infected with SF (●-●) (MOI 1 pfu/cell) or mock infected (▲-▲). Cells were counted daily from day one to day thirteen post infection. Cultures were passaged to new medium (diluted to 2×10^5 cells/ml) on day six post infection.

Viability of SF-infected K-562 Cells

The decline in growth of K-562 cells infected with SF suggested that cells were being killed by the infection. Cell viability was observed each day (Fig. 4) and it was noted that there was only a slight drop in viability (80-95% of mock infected cultures). This indicated that SF might indeed be capable of infecting K-562 cells but not in the usual cytolytic manner. The SF infection more closely resembled that of mosquito cells in which no CPE is observed.

Infectious Virus Production in K-562 Cells

Since SF-infected K-562 cells do not exhibit any typical cytopathic effect, a very low level of virus infection might be expected. Therefore, the supernatant fluids of the cultures were tested for the presence of infectious virions. Samples were taken each day for 14 days pi and assayed for plaque forming units on CEF monolayers. Infectious virus was produced by the infected cells reaching a peak of 2.9×10^2 pfu/cells at 48 hours pi (comparable to CEF virus production of 2×10^2 pfu/cell at 24 hours pi) and falling to 0.8 pfu/cell at 14 days post infection (Fig.5). To show that virus was being produced and was not residual from the inoculum, an inoculating dose of SF was added to culture medium without cells. The titer decreased at a rate of 7×10^2 pfu/ml/day so that no virus was detected by day six. Again, this paralleled SF-infection in mosquito cells as did the decrease in virus production beginning at day three. In infected mosquito cells, virus production declines to a level of 10^5 - 10^6 pfu/ml by week five post infection. However, K-562 virus titers dropped steadily

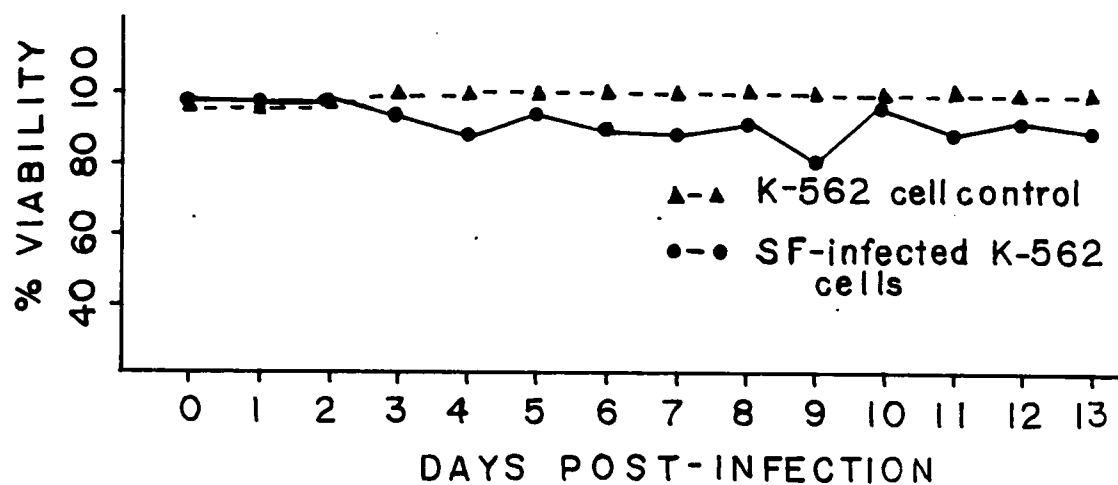


Figure 4. Viability of SF-infected K-562 Cells

K-562 cells were infected with SF (●-●) (MOI 1 pfu/cell) or mock infected (▲-▲), and viability determined daily on days one to thirteen post infection by trypan blue dye exclusion (% viable total cells).

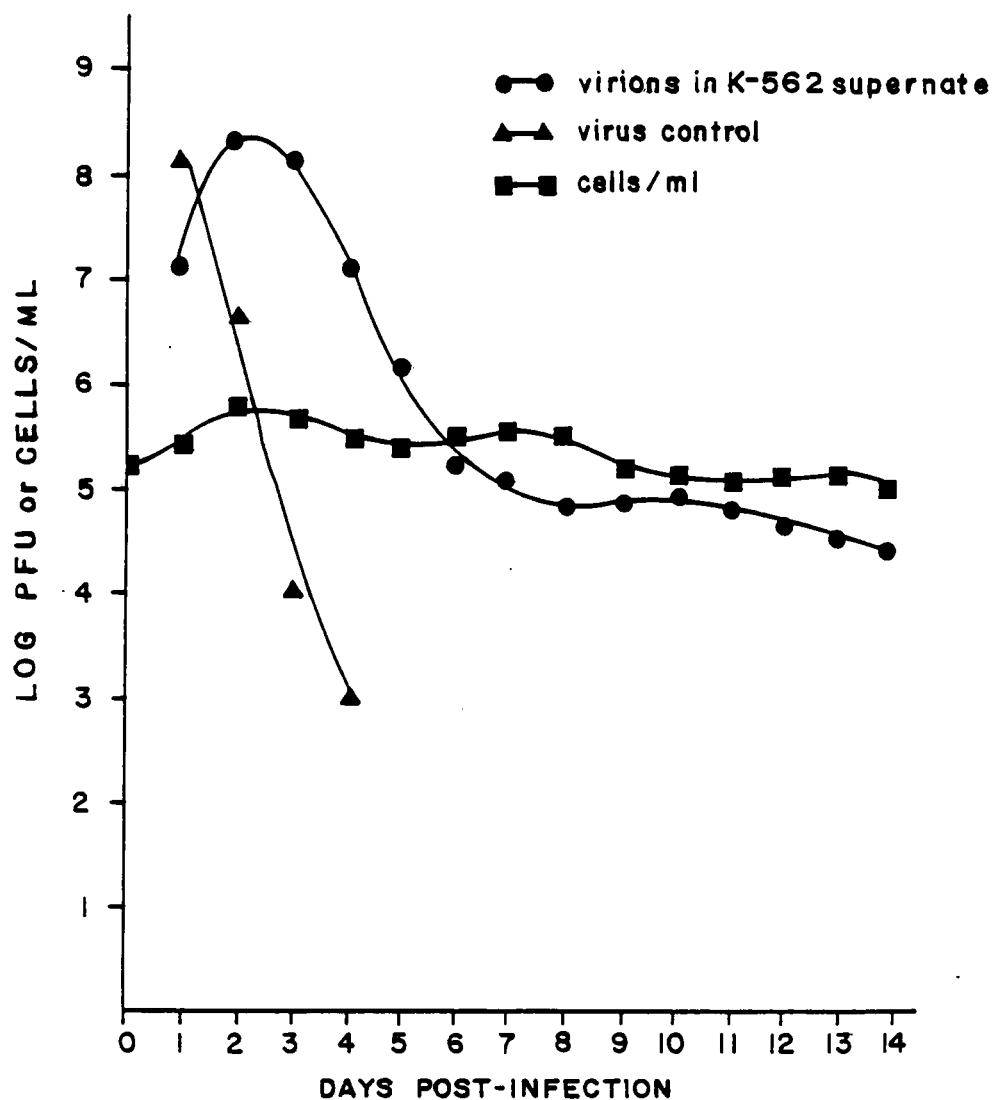


Figure 5. Infectious Virus Production in SF-infected K-562 Cells

K-562 cells were infected with SF (■-■) (MOI 1 pfu/cell). Culture fluid was removed daily on days one to fourteen post infection, centrifuged to remove cells, and assayed for infectious virus on CEF monolayers (●-●). Virus control, stock virus in cell free culture medium, indicates decline in virus titer (▲-▲).

through two to three weeks, eventually reaching a level of no detectable infectious virus in the cultures.

Microscopic Examination of Stained K-562 Cells

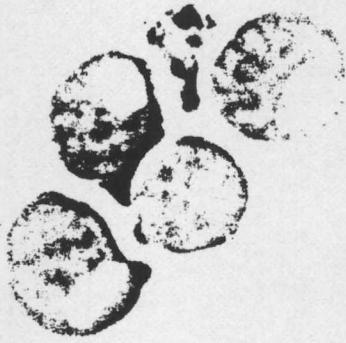
The production of infectious virus by SF-infected K-562 cells indicated that morphological changes might be present in the cells. SF-infected or mock infected K-562 cells were stained with a hematological stain and morphological changes noted. SF-infected K-562 cell cytoplasm contained numerous small vacuoles while few vacuoles were present in mock infected control cells (Fig. 6). This again correlates with the finding that small cytoplasmic vacuoles are formed in SF-infected mosquito cells, the vacuoles serve as centers of virus replication (27). No such correlation has been found in K-562 cells.

Determination of % SF-infected K-562 Cells

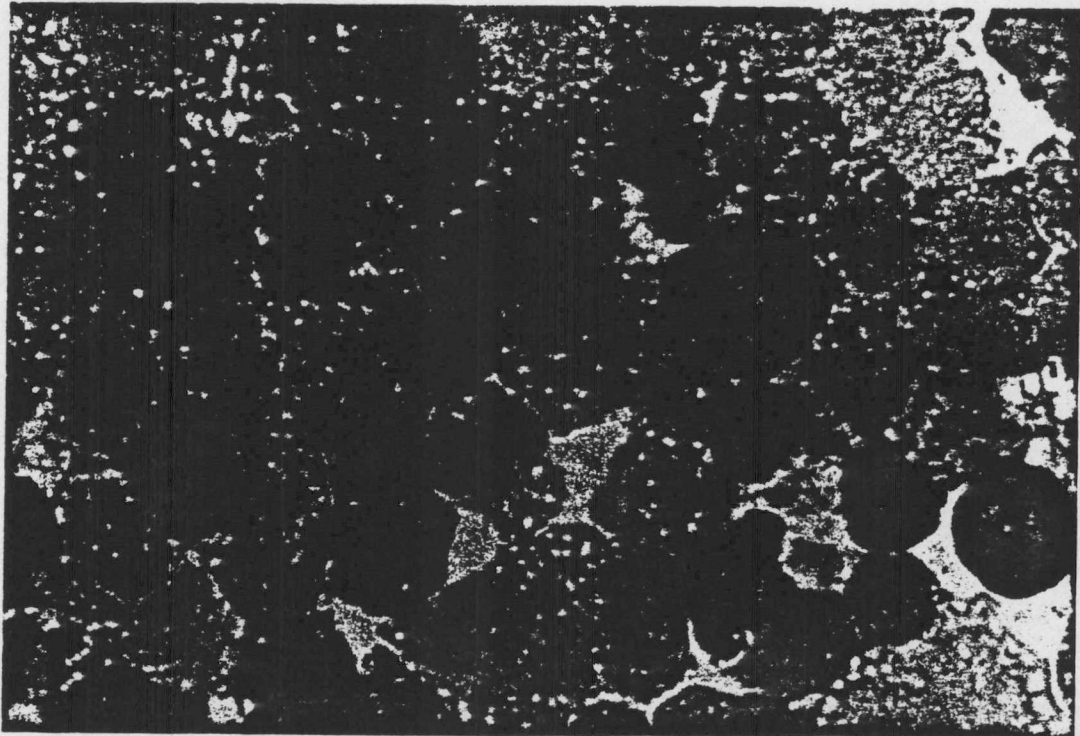
K-562 cultures contain a heterogenous population of blast-like cells with two distinct cell types predominating. Thus, it was of interest to determine the percent of K-562 cells in infected cultures which were actually infected with virus. An indirect immunofluorescence assay of cells at 24 hours post infection was performed to determine the percent of cells expressing viral antigens. Anti-SF hyperimmune serum reacted specifically with 100% of K-562 cells in infected culture (Fig. 7).

Figure 6. Morphology of SF-infected K-562 Cells

K-562 cells were infected with SF (a) (MOI 1 pfu/cell) or mock infected (b), and examined for morphological differences after 24 hours post infection. Cells (1×10^5) were washed, extruded onto microscopic slides with a cytofuge, stained with Diff-Quik hematological stain and examined at 100 X magnification.



Uninfected K-562 Cells



SF-infected K-562 Cells

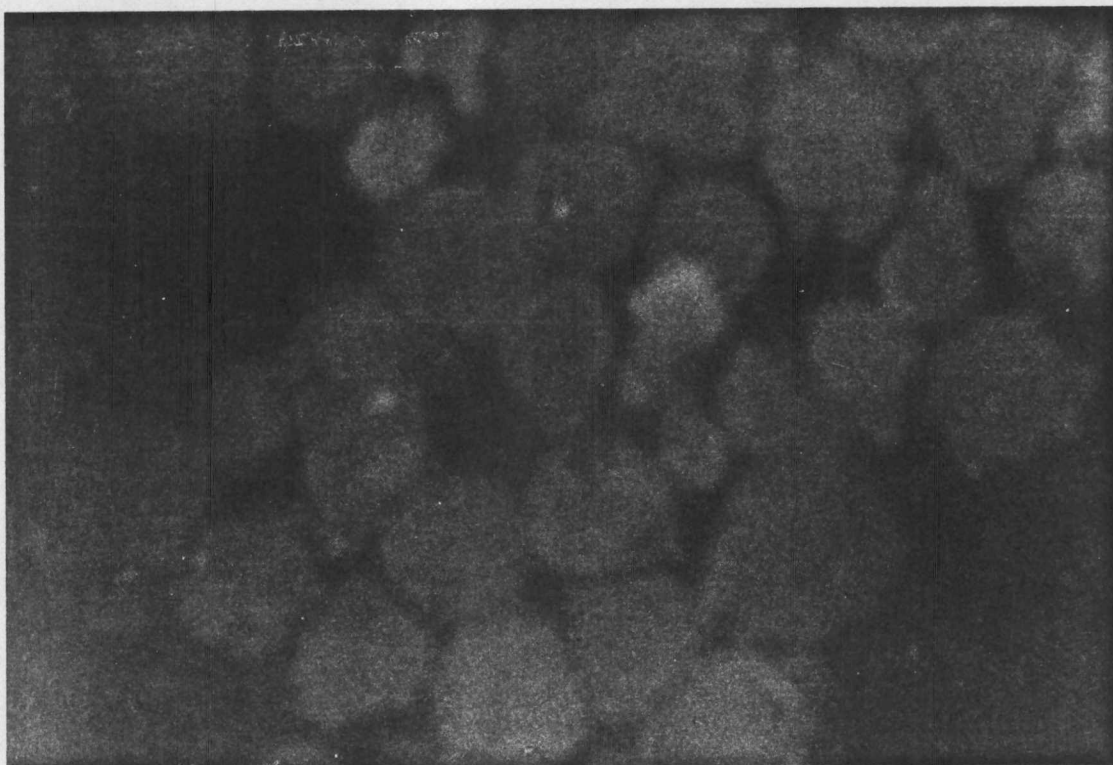


Figure 7. Immunofluorescence of Viral Antigens in SF-infected Cells.

K-562 cells were infected with SF (MOI 1 pfu/cell) and examined for presence of viral antigens at 24 hours post infection by indirect immunofluorescence assay using rabbit anti-SF serum for 60 minutes, followed by FITC conjugated anti-rabbit IgG (40 X magnification).

Release of ^{51}Cr from Infected Cells

Although no cytopathic effect of SF-infection on K-562 cells was evident even though high levels of virus (2.9×10^2 pfu/cell peak) were produced, cell damage was further assessed by noting the release of ^{51}Cr from labeled infected cells. Representative results (Fig. 8) show that no significant release of ^{51}Cr occurred in cells infected at three levels of MOI (1, 5, and 10) compared to that released spontaneously by uninfected cell controls. This suggests that while cell morphology was shown to be affected by virus infection, the cytoskeleton was apparently not significantly damaged after 48 hours pi.

Virus Infection of MRC-5 Cells

MRC-5 cells, a human diploid cell strain, were infected with SF to determine whether or not the lack of cytopathic effect was due to the human origin of K-562 cells. MRC-5 cells were infected with SF or SIN as described. At 48 hours pi, cell damage was assessed microscopically and approximately 90% of cells were determined to exhibit cytopathic effect (data not shown). This, along with reports of SF infection of the human Victor lymphoblastoma and HeLa cell lines(63) indicate that the unusual reaction of K-562 cells to SF infection is not dependent on the species of cell origin.

Antibody Augmentation of Infectivity

Antibody augmentation of togavirus infection has been reported in vivo with Dengue Fever virus (30). This immune enhancement effect is seen when virus reacts with sub-neutralizing concentrations of anti-

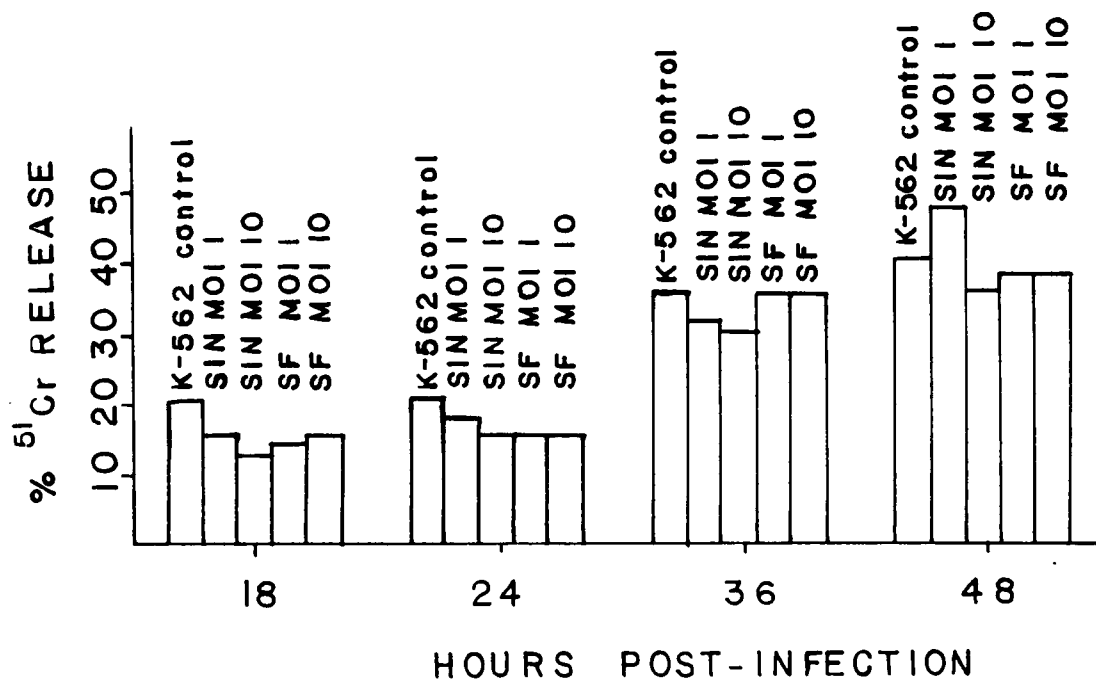


Figure 8. % ^{51}Cr Release From Infected K-562 Cells

K-562 cells were labeled with $^{51}\text{CrO}_4$, infected with SF or SIN (MOI 1, 5, or 10 pfu/cell) or mock infected and assayed for ^{51}Cr release at 18, 24, 36 and 48 hours post infection.

viral antibody. It was conjectured that anti-viral antibody or anti-cellular antibody might exhibit a similar effect on virus infection of K-562 cells and allow the establishment of a cytolytic infection. Sub-neutralizing concentration (2 2-fold dilutions beyond the neutralization titer) of anti-SF hyperimmune serum was mixed with the virus 30 minutes before addition to K-562 cells. No immune enhancement of infection was observed (Table I).

Similarly, anti-K-562 serum was added to cultures 30 minutes before virus to test whether the infection would be enhanced. No immune enhancement of infection was found (Table II).

Long Term Culture of Infected K-562 Cells

Since SF-infected K-562 cells remained viable for two passages as noted above, the cells were passaged at three week intervals and were maintained for over one year growing at the low steady rate noted for the first two passages. The presence of infectious virus was assayed at four and eight months pi and none was detected (Table III).

Although no direct detection of infectious virus could be made, it was considered as possible that if the cells were persistently infected, the virus could be rescued by cocultivation of the cells with susceptible cells. SF-infected K-562 cells at four and eight months pi were washed, added to CEF monolayers and overlaid with agar. No plaques developed on the CEF monolayers (Table IV), suggesting that the virus could not be rescued or that the K-562 cells were cured, although they had not regained their usual growth characteristics.

Table I. % Cytotoxicity of SIN- or SF-infected K-562 Cells by
Anti-SIN or -SF Serum

Cells	MOI	Ab Added	Average % Cytotoxicity	
			24 hr pi	48 hr pi
control	none	none	10.9	23.2
SIN-infected K-562	1	SIN	9.2	21.0
SIN-infected K-562	5	SIN	10.0	24.6
SIN-infected K-562	10	SIN	9.5	29.2
SF-infected K-562	1	SF	4.1	19.4
SF-infected K-562	5	SF	6.8	25.2
SF-infected K-562	10	SF	7.4	23.6

K-562 cells were labeled and infected with SIN or SF which had been pretreated with homologous antiserum for one hour (MOI 1, 5 or 10 pfu/cell) or mock infected. % cytotoxicity was determined at 24 and 48 hours post infection.

Table II. % Cytotoxicity of SIN- or SF-infected K-562 Cells by Goat Anti-K-562 Serum

	MOI	Anti-K-562 Serum Added	Average % Cytotoxicity at 24 hours pi
control	none	-	7.2
SIN-infected K-562	1	+	7.9
SIN-infected K-562	5	+	7.7
SIN-infected K-562	10	+	12.8
SF-infected K-562	1	+	4.9
SF-infected K-562	5	+	6.7
SF-infected K-562	10	+	8.5

K-562 cells were labeled with $^{51}\text{CrO}_4$, treated with goat anti-K-562 serum for one hour, and infected with SF (MOI 1, 5 or 10 pfu/cell) or mock infected. % cytotoxicity was determined at 24 hours post infection.

Table III. Infectious Virus Yield on CEF Using Culture Fluid
From SF-infected K-562 Cells

Cell-free Culture Fluid		pfu/ml
SF-infected K-562 cells	4 months post infection	0
SF-infected K-562 cells	8 months post infection	0
SF-infected K-562 cells	9 months post infection	0
Virus control ^a		1.4×10^9

^aStock virus was added to CEF monolayers as positive control.

K-562 cells were infected with SF (MOI 1 pfu/cell) and culture fluid tested for infectious virus production on CEF monolayers (at 4, 8 and 9 months post infection).

Table IV. Infectious Centers on CEF Using Long Term SF-infected
K-562 Cells

Cell Population	Cell Concentration	Infectious Centers
SF-infected K-562 cells 4 months pi	6×10^4 cells/0.1 ml	0
SF-infected K-562 cells 8 months pi	1.7×10^4 cells/0.1 ml	0
Virus Control ^a		1.4×10^9 pfu/ml

^aAs in Table III.

K-562 cells were infected with SF (MOI 1 pfu/cell) and assayed for infectious centers on CEF monolayers at 4 and 8 months post infection.

Viral Antigens in Long Term Cultures

SF-infected K-562 cells were examined for viral antigens on the cell membrane. Even though infectious virus could not be recovered, it was concluded that cells still were infected since the growth rate continued to be depressed. The possible presence of viral antigens on the surface of the cells was examined by the ADCMC assay. Cells infected for 72 hours, two and four months, as well as uninfected cells were used as targets. All of the post SF-infection target cell populations showed the same susceptibility to cytolysis by anti-SF hyperimmune serum plus complement in terms of antibody titer, that is, the antibody dilution that caused 50% lysis (Fig. 9). However, there was a significant difference in the level of cytolysis since cells infected for 72 hours showed a maximum of 68% cytolysis, while cells infected for two months gave 56% and Cells infected four months gave 47%. There was no cytolysis of uninfected control cells or with infected cells by preimmune serum.

These results suggest that the cells are persistently infected with the virus, express viral antigens on the cell surface but cannot produce infectious virions.

K-562 Antigens in Cells Persistently Infected

K-562 cells can be used to produce specific anti-K-562 sera in rabbits, monkeys and goats. After production the antisera can be absorbed with normal human peripheral blood leukocytes and normal bone marrow cells leaving antibodies that react with several leukemia cell lines. When the absorbed antisera are absorbed with a leukemia

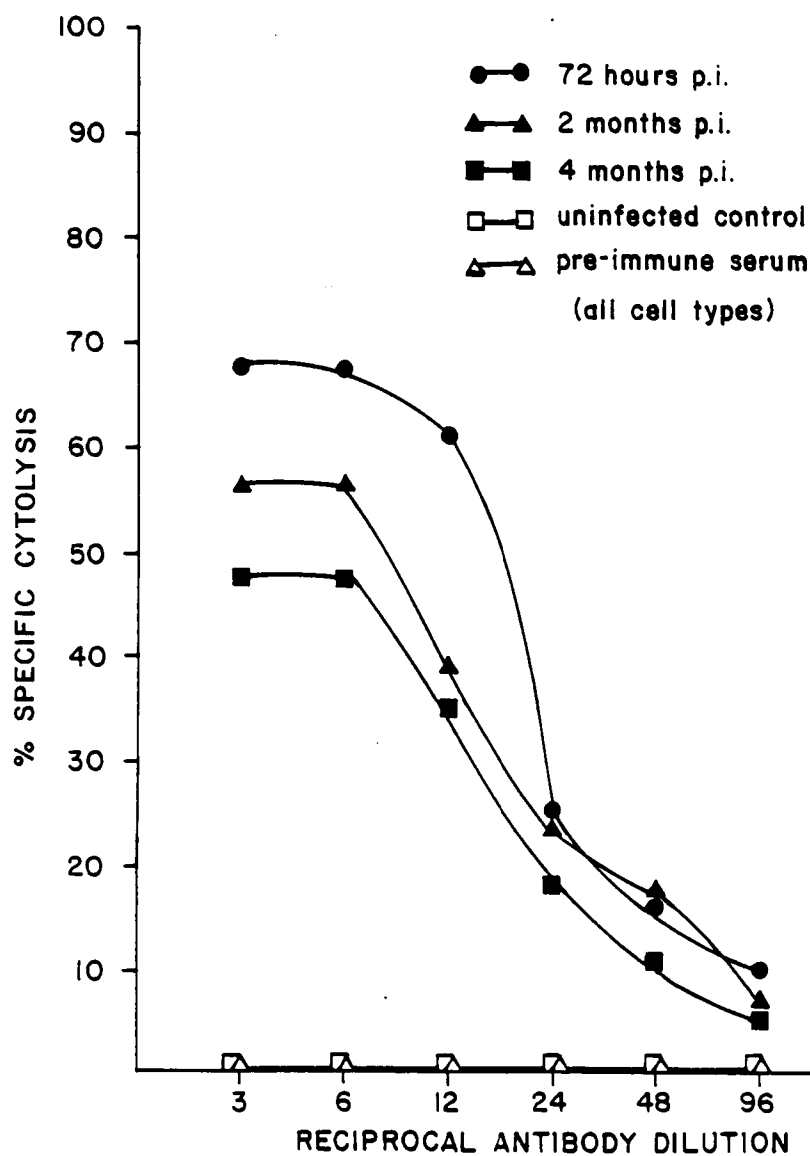


Figure 9. Antibody Dependent Complement Mediated Cytotoxicity of SF-infected K-562 Cells by Rabbit Anti-SF Serum.

K-562 cells were infected with SF (MOI 1 pfu/cell) and examined for viral antigens on the cell surface at 72 hours, 2 months and 4 months post infection by ADCMC assay using rabbit anti-SF serum. % cytolysis was determined by trypan blue dye exclusion.

cell line, antibodies remain that are specific for K-562 cells. It was considered possible that the infection would modulate the expression of the K-562 antigens since virus proteins are inserted into the cell membrane and mature virus buds from it.

The results of the ADCMC (Fig. 10) indicate no significant difference between infected and noninfected cells when measured by the maximum extent of cytotoxicity and the 50% endpoint.

Natural Killer Cell Susceptibility of Infected K-562 Cells

K-562 cells have been used extensively in numerous laboratories around the world as target cells to measure human natural killer (NK) cell activity. Susceptibility to NK activity depends on the recognition of still undefined antigens on the target cell by the NK cell. As another parameter for the effect of virus infection on the expression of cellular antigens by the K-562 cells, infected and noninfected cells were subjected to NK cells. The results (Fig. 11) indicate that there was no significant difference in the susceptibility of infected cells compared to uninfected cell controls; therefore, the NK target antigen(s) continue to be expressed at apparent normal levels during the persistent infection.

Effect of Persistent Infection on Metabolism of K-562 Cells

The reports of persistent infections by alphaviruses in other cell types stress that the infected cells are indistinguishable metabolically from cell controls (61,91). As seen by the growth curves of SF-infected K-562 cells, this does not hold true for this infection.

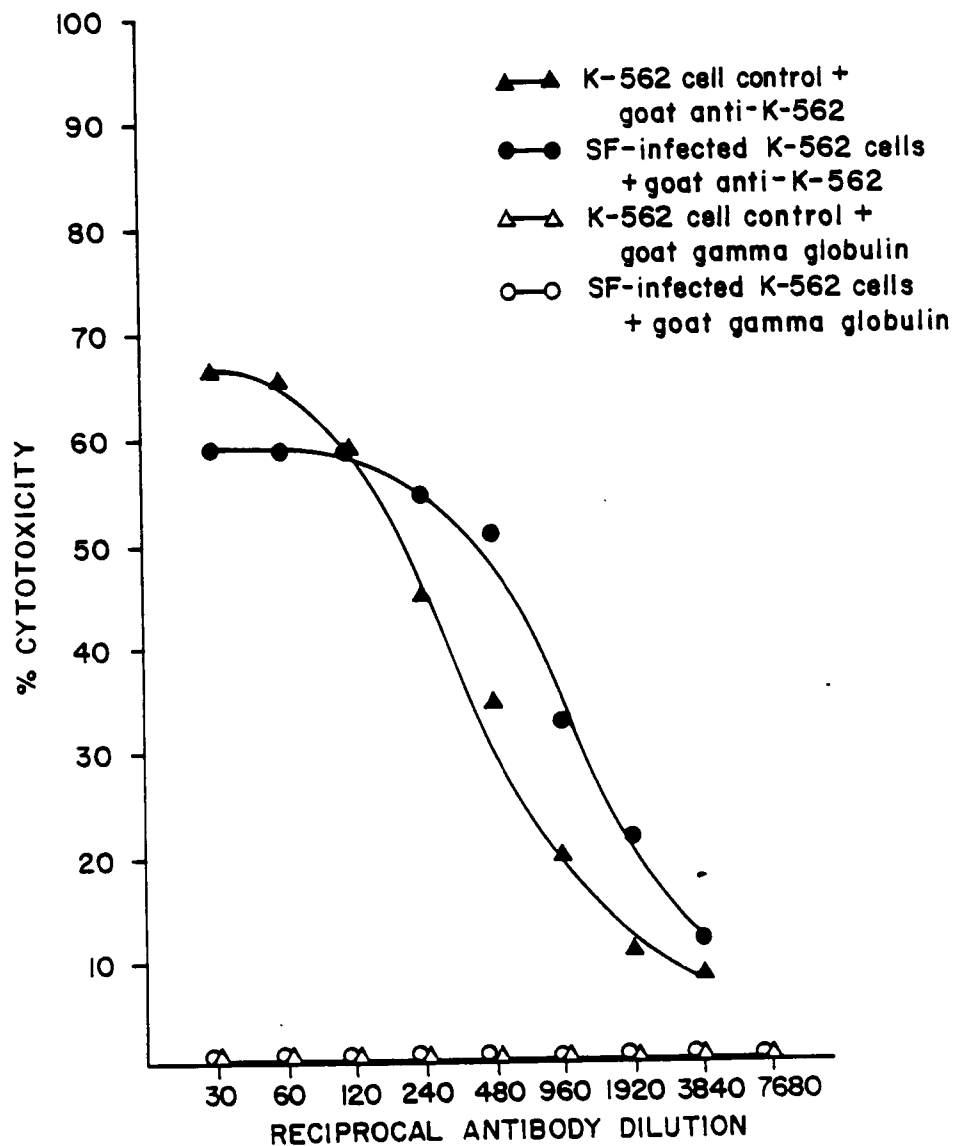


Figure 10. Antibody Dependent Complement Mediated Cytotoxicity of SF-infected K-562 Cells by Goat Anti-K-562 Serum.

K-562 cells were infected with SF (●-●) (MOI 1 pfu/cell) or mock infected (▲-▲) and examined for reactivity with goat anti-K-562 serum or with goat preimmune gamma globulin (SF-infected ●-●, cell control ▲-▲) by ADCMC. % cytolysis was determined by trypan blue dye exclusion.

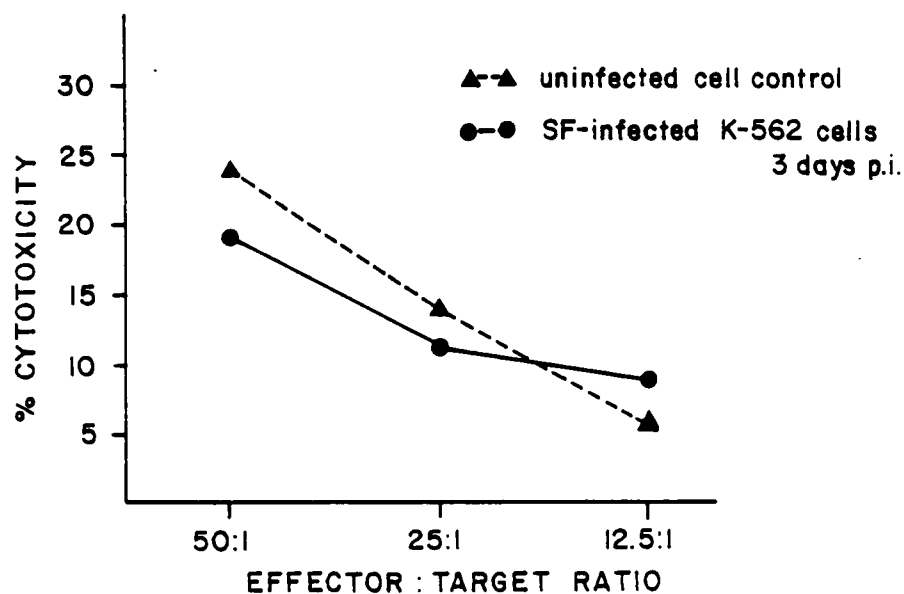


Figure 11. Lysis of SF-infected K-562 Cells by Natural Killer Cells

K-562 cells were infected with SF (●-●) (MOI 1 pfu/cell) or mock infected (▲-▲). At 3 days post infection cells were labeled with $^{51}\text{CrO}_4$ and assayed for susceptibility to human natural killer cell cytotoxicity. Human mononuclear peripheral blood lymphocytes were added to K-562 cells at effector to target ratios of 50:1, 25:1 and 12.5:1 and inoculated for 4 hours. % cytotoxicity was measured as test ^{51}Cr release as compared to total releaseable counts.

Since cell growth was markedly diminished, it was of interest to examine whether or not the cells were actively metabolizing. To examine this question three metabolic parameters were assessed: 1. uptake of $^{51}\text{CrO}_4^-$; 2. uptake and incorporation of radioactively labeled amino acids into protein; and 3. uptake and incorporation of radioactively labeled thymidine into DNA.

Uptake of $^{51}\text{CrO}_4^-$

The uptake of $^{51}\text{CrO}_4^-$ ion is a metabolic event in which Cr^{+6} is transported into the cell and converted to Cr^{+4} . The latter associates with organic anions, such as citrate and members of the tricarboxylic acid cycle, and remains internalized unless the cell is damaged and the Cr^{+4} ion is released. As Cr^{+4} ion it cannot be taken up by living cells.

SF-infected K-562 cells were labeled with $^{51}\text{CrO}_4^-$ at various times post infection and the level of labeling efficiency compared to uninfected control cells. The results (Fig. 12) show that ^{51}Cr is taken up at significantly reduced levels in infected cells indicating that the cells are metabolically active but not at the level of uninfected control cells.

Incorporation of Amino Acids

A mixture of [^3H]-amino acids was added to infected and non-infected cell cultures and the incorporation of label into hot trichloroacetic acid insoluble (100°C , 30 minutes) material measured. Hot TCA insoluble material is well known to indicate that the amino acids have been precipitated into the paper disks. The results indicate

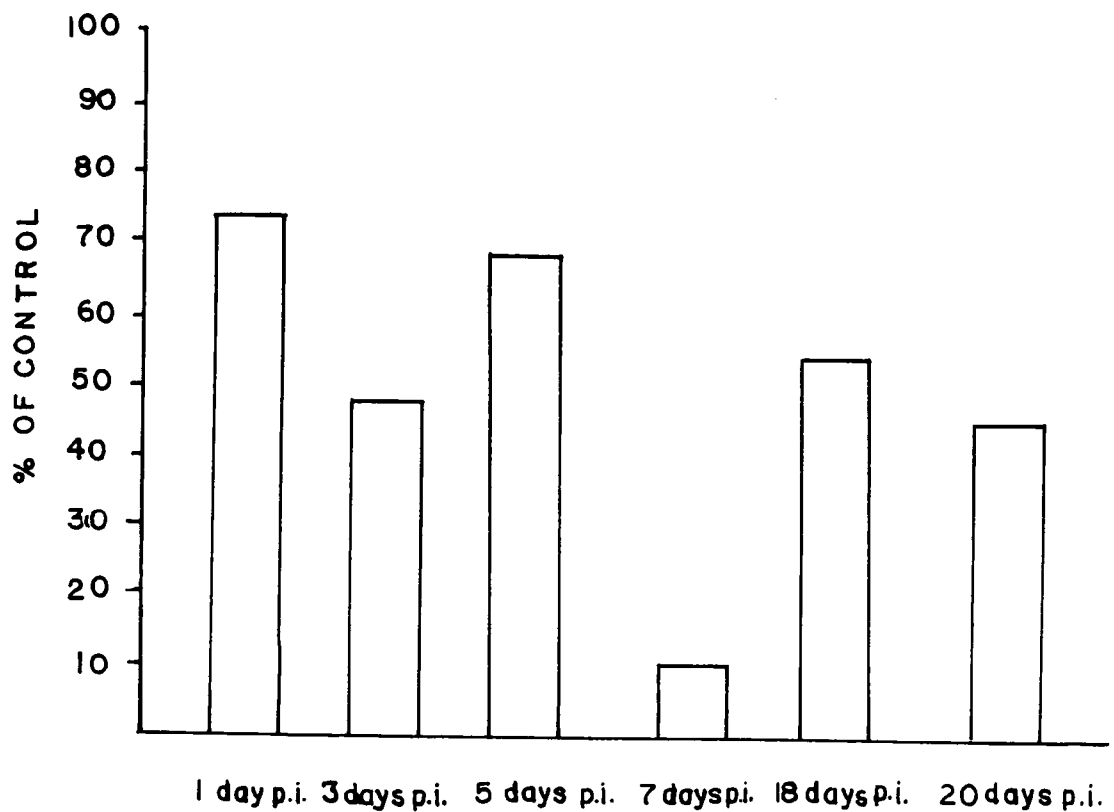


Figure 12. $^{51}\text{CrO}_4$ Incorporation by SF-infected K-562 Cells

K-562 cells were infected with SF (MOI 1 pfu/cell), labeled with $^{51}\text{CrO}_4$ at various days post infection and examined for % uptake of $^{51}\text{CrO}_4$ as compared to mock infected control cells.

that the labeled amino acids have been incorporated into protein of infected cells but at reduced levels(23-42%) compared to control cells (Fig. 13).

Incorporation of Thymidine

[³H] thymidine was added to infected and noninfected K-562 cells and the incorporation into cold TCA insoluble material was measured. The results (Fig. 14) indicate that DNA synthesis is reduced to about 40% of control levels in infected cells and this reduction did not vary from 13 days to 4.5 months post infection.

Antibody-Mediated Cytolysis of Infected K-562 Cells

It has been reported that K-562 cells are susceptible to antibody-mediated cytolysis in the absence of complement or effector cells (56,98). The mechanism was proposed to be interference with normal metabolic activity, which leads to cell death and lysis (98). It was of interest to test the effect of heat-inactivated rabbit anti-SF serum on the persistently infected K-562 cells.

The results (Fig.15) show that the immune serum had no effect on the infected cells until 16 hours at which time the cells began to die, with less than 20% viability at 48 hours and 7.5% viability at 216 hours. Preimmune serum had little effect on infected cells and hyperimmune serum had no effect on noninfected cells.

To further characterize this phenomenon, infected K-562 cell cultures received the following sera: preimmune, hyperimmune anti-SF, gamma globulin of the hyperimmune (to eliminate any possible residual endogenous complement), normal immune anti-SF (immunization 2-4

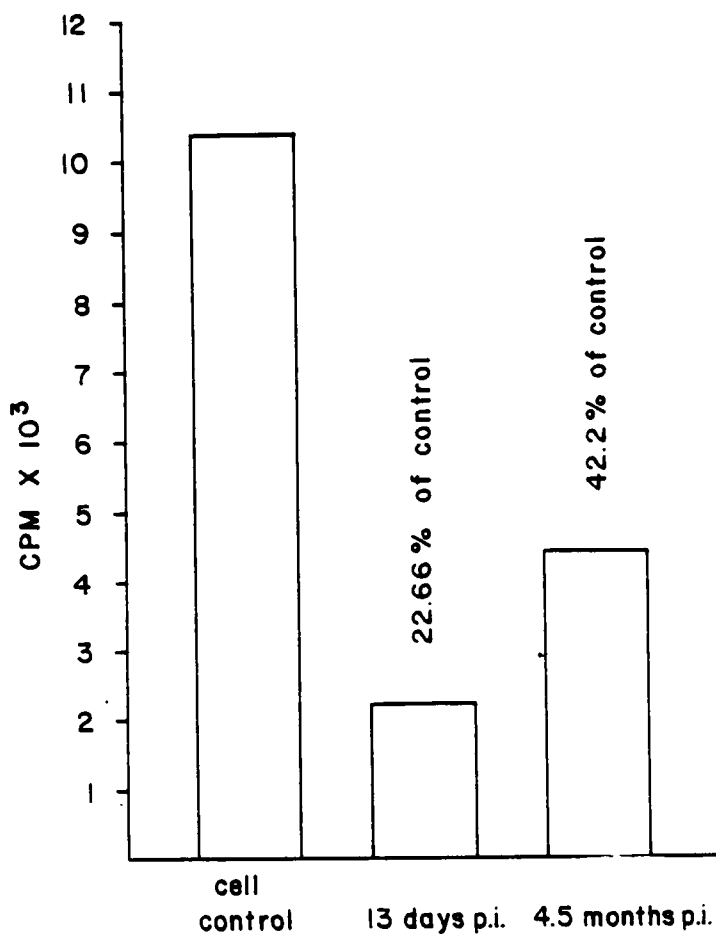


Figure 13. [³H]-mixed Amino Acid incorporation by SF-infected K-562 Cells

K-562 cells were infected with SF (MOI 1 pfu/cell) or mock infected and labeled with [³H]-mixed amino acids (100 μ Ci/10⁶ cells) at 13 days and 4.5 months post infection. Cells were placed on filter paper disks and proteins precipitated with hot TCA.

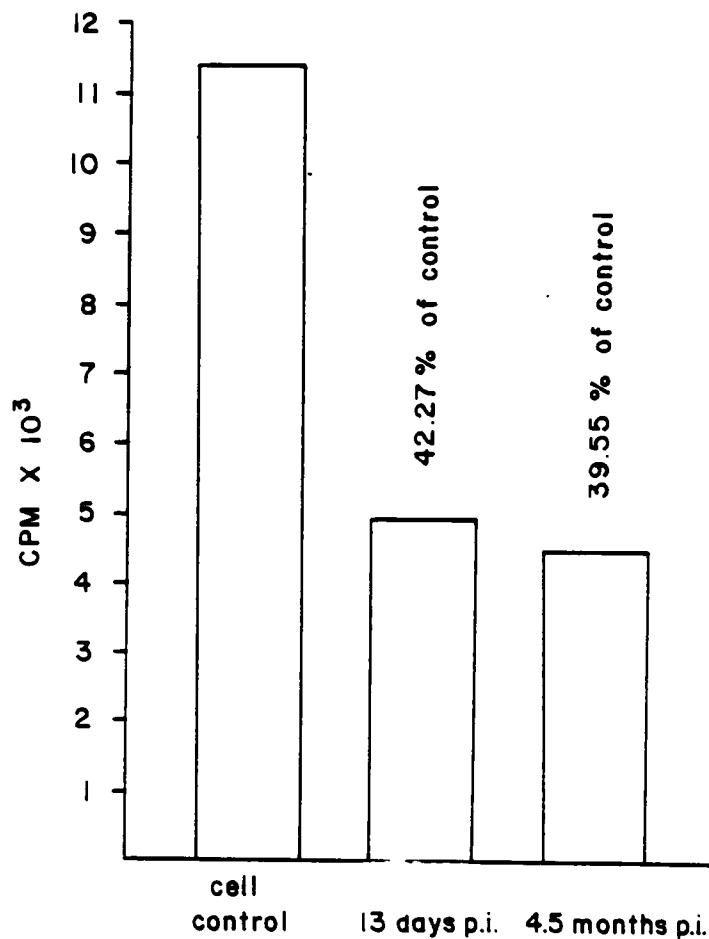


Figure 14. [³H] Thymidine Incorporation by SF-infected K-562 Cells

K-562 cells were infected with SF (MOI 1 pfu/cell) or mock infected and labeled with [³H] thymidine (100 μ Ci/10⁶ cells) at 13 days and 4.5 months post infection. Cells were placed on filter paper disks and DNA precipitated with ice cold TCA.

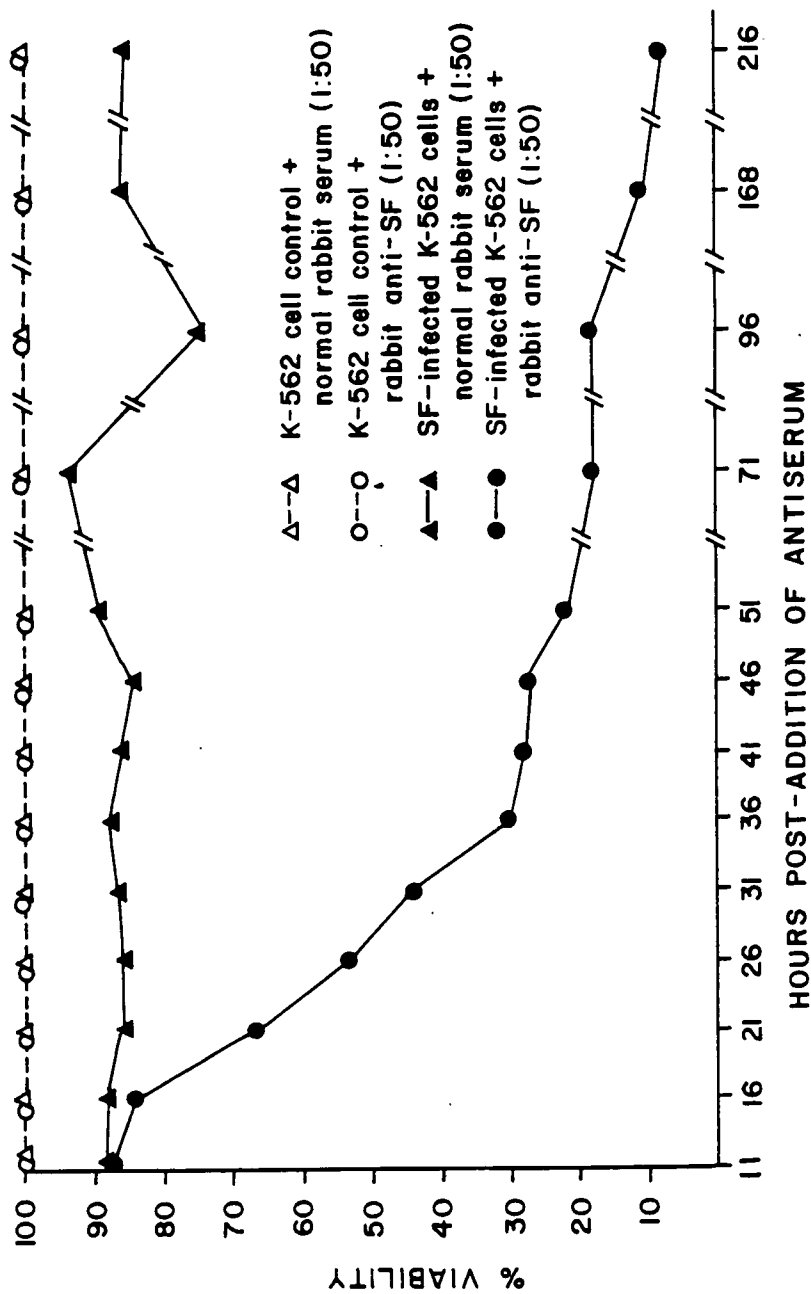


Figure 15. % Residual Viability of SF-infected K-562 Cells with Rabbit Anti-SF Serum

K-562 cells were infected with SF (MOI 1 pfu/cell) or mock infected and treated with rabbit preimmune serum (SF-infected $\Delta-\Delta$, cell control $\Delta-\Delta$) or rabbit anti-SF serum, (SF-infected $\bullet-\bullet$, cell control $\bullet-\bullet$) at 19 days post infection, and cell viability determined by trypan blue dye exclusion.

months), hyperimmune anti-SIN (cross-reactive antibody), and normal immune anti-SIN. Fractionated and whole anti-SF sera mediated cytolysis to the same extent (Fig. 16V, 16C), and anti-SIN (Fig. 16E) serum mediated cytolysis but at a lower level than the homologous. Pre-immune and normal immune sera had no effect on cell viability (Fig. 16A, 16D, 16F).

These results are of interest since only hyperimmune sera have been shown to give cross ADCMC reactions and the target antigens appear to be a unique E1 glycoprotein determinant and the capsid protein exposed on the infected cell surface (82,96,97).

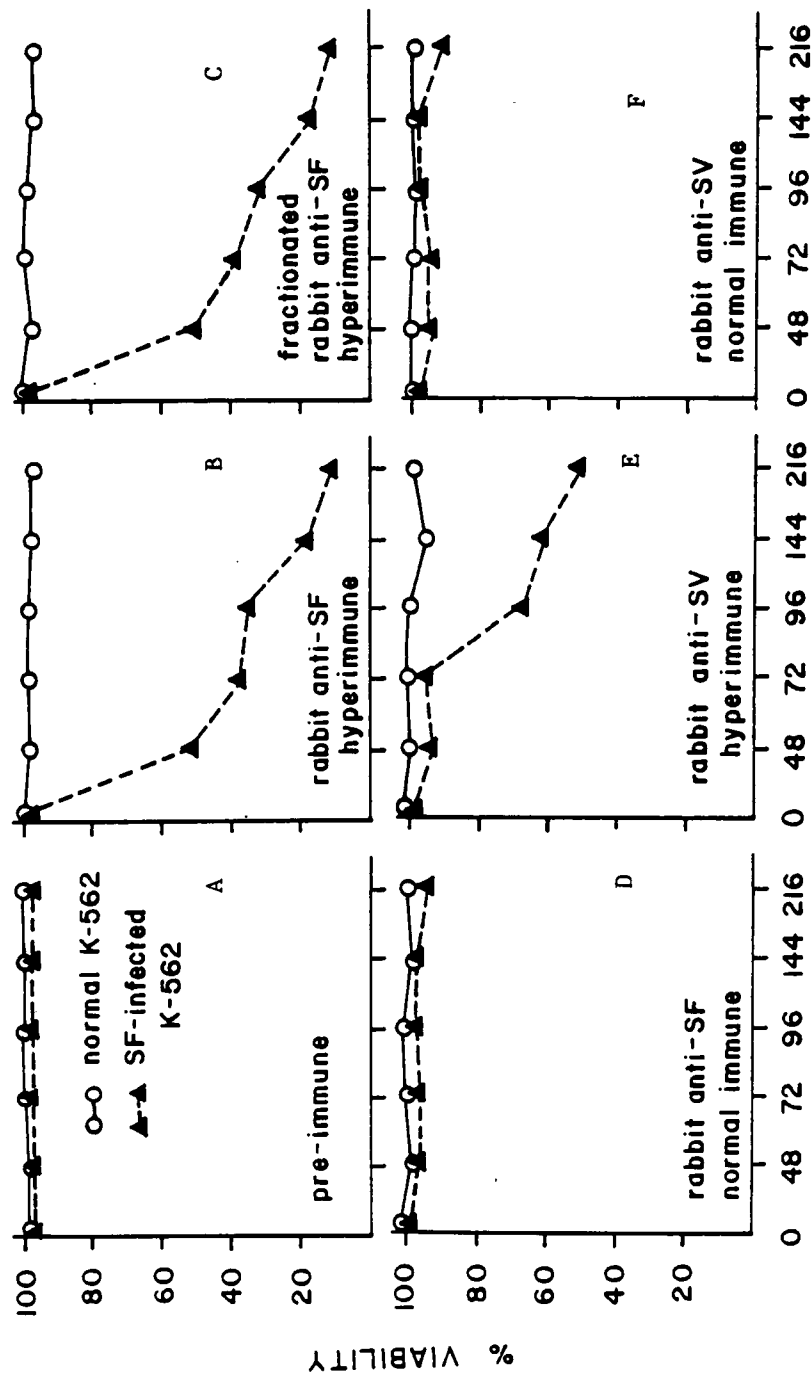


Figure 16. % Residual Viability of SF-infected K-562 Cells with Rabbit Anti-viral Sera or Fractions

K-562 cells were infected with SF (MOI 1 pfu/cell) or mock infected and treated with a) rabbit preimmune serum; b) rabbit anti-SF hyperimmune serum; c) rabbit anti-SF hyperimmune gamma globulin; d) rabbit anti-SF normal immune serum; or f) rabbit anti-SIN normal immune serum (SF-infected Δ - Δ , control $\circ$ - $\circ$).

CHAPTER V

DISCUSSION

Exposure of the human leukemia cell line, K-562, with SF leads to persistent infection and the process is characterized by several unique features. Firstly, establishment appeared to develop in three stages: an early stage in which virus was produced in relatively high titer; an intermediate stage when virus was produced at a decreasingly low level; and, a persistent stage when no infectious virus could be detected and cell growth was much less than in control cultures.

Other characteristics of this persistent infection include:

1. persistence is achieved without high MOI, a high concentration of defective interfering particles, or treatment with interferon. Use of one or more of these methods is usually necessary for development of persistent infection;
2. SF-infected K-562 cells do not undergo an obvious crisis period in which a high percentage of cells are lysed. This is true even in the early stage when virus is being produced in good titer (2.9×10^2 pfu/cell);
3. infected cells do exhibit morphological and metabolic differences from mock-infected cells;
4. the maintenance of persistence intracellularly is independent of the production of any infectious virions;
5. during long term persistence when no infectious virus is detectable, viral antigens are expressed on the cell surface.

Previous reports indicated that persistent infection of L-929 cells with SF or infection of BHK cells with SIN required a virus

preparation rich in defective interfering particles (60,91). Defective interfering particles interfere with the replication of wild type virus enabling the establishment of persistent infections. In the present work, the virus stock was prepared with low passage virus at an MOI of 0.01 to minimize the production of defective interfering particles. In addition, no evidence of interference with virus replication has been noted with this virus stock. Persistent infection of L-929 cells with SF has also been established if interferon (100 units/ml) was added to the cell cultures prior to infection(60). With K-562 cells, persistent infection established with HSV-1, adenovirus 3, poliovirus 1, or measles virus was considered to be possibly influenced by the production of endogenous interferon (6); however, K-562 cells do not produce any interferon when stimulated with Newcastle Disease virus (NDV) (53) and the probability of endogenous interferon is unlikely with these other viruses.

It was of interest to note that even during the early stage of infection when high titer virus was produced, the K-562 cells did not undergo a crisis period in which a high percentage of cells were killed. Such killing has been found in other studies of persistent infection (11,25,26,60) and the cells that survive the crisis comprise the persistently infected culture. The minimal viability of SF-infected K-562 cells throughout a 15-month observation period was 75%.

Although cell viability remains high, the infected K-562 cells do exhibit morphological and metabolic changes as compared to mock-infected control cells. Such changes in the host cell were not found in persistent infection of BHK cells, L-929 cells, and WISH cells

with other RNA viruses (26,60,91), and Cernescu reported that K-562 cells were not altered when persistently infected with HSV-1 or measles virus (6). It is probable that differences in culture and assay methods as well as criteria used to examine the cells may be responsible for the differing interpretations.

The dominant question arising from this investigation is: How can there be a persistent virus infection in the absence of any infectious virus? One possibility is that the viral genome has been replicated by a reverse transcriptase and integrated into the host cell DNA. This seems highly unlikely because there is no precedence for alphavirus genome being so integrated. Further, the virus genome does not code for a reverse transcriptase and this enzyme is lacking in the K-562 cell (54). A second possibility is that a mutation has occurred, such as temperature sensitivity, which allows the synthesis of some viral proteins but not infectious virions. Temperature sensitive mutants of both SIN and SF are produced by persistently infected mosquito cells. This explanation remains to be tested.

The repressed growth rate of K-562 cells during persistent infection with SF, or other viruses (6), suggests that the virus has interfered with cell growth but not in a lethal way. However, the cells do show a number of abnormal characteristics: 1. macromolecular synthesis is depressed as indicated by the low incorporation of ^3H thymidine into DNA and ^3H -amino acids into protein; 2. the metabolic uptake of chromate ion is suppressed; 3. there is extensive vacuolation; 4. the cells are quite fragile when subjected to centrifugation in a cytofuge. All of these features indicate an influence

of persistent virus infection. Further, SF infection interferes with typical K-562 cell proliferation. While cell proliferation does occur, it is at a highly reduced rate which suggests that the cells are surviving the infection, with little proliferation, for long periods of time. Thus, the apparent persistent infection would depend upon the presence of the virus genome and viral mRNA in the cells originally infected and segregated to daughter cells at mitosis. In this instance, all cell progeny would be able to express viral antigens to some extent. This is consistent with the metabolic and immunologic studies of these cells and to the persistence of viral antigens in the absence of detectable infectious virus.

Although infectious virions were not detected during the persistent infection stage, viral antigens were produced as noted by the susceptibility of the cells to virus specific antibody in ADCMC assays. Even more surprising was the killing effect of antibody alone on infected cells. It has been presumed that antibody directed to K-562 cell antigens behaved as ligand to cell receptors but, since the ligand was not the correct one, the signal to the cell led to cell death (99). It is difficult to understand how viral antigen could serve as receptor and function in a similar manner. Perhaps a series of experiments at varying concentrations of antibody would define a signalling event to the cell, (for example, would small concentrations of antibody serve to stimulate cell growth?).

This study has introduced a number of questions and presented opportunities for continued research to understand a well-defined persistent virus infection. The fact that this cell line is a

pluripotent hematopoietic stem cell leukemia line may be important in this understanding. There are several features associated with this cell line (for example, expression of a proto-oncogene product such as abl, the enhanced expression of lambda light chain intracellularly, the unusual translocations and other chromosomal aberrations) that might play a role in virus persistence. In any event, continued study of this system should be beneficial for defining persistent virus infections.

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