PERSISTENT VIRAL INFECTIONS AS MODELS FOR RESEARCH IN VIRUS CHEMOTHERAPY

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I. Introduction

The acute systemic virus infection is commonly used as an experimental model in chemotherapy research despite the fact that the

37

chance for an effective chemotherapy of acute virus infections is small. In most acute infections virus multiplication is well advanced before the disease is expressed and treatment will in many cases come too late (Fenner and White, 1970; Wecker, 1971). Control by chemotherapy might, however, be promising for persistent virus infections where, owing to the slow progression of the disease, sufficient time for treatment is available.

Although there are various ways in which viruses can persist in their hosts, comparative studies *in vitro* and *in vivo* reveal common features which shall briefly be reviewed in Sections II and III,A. They include (1) low pathogenicity of the virus and/or restriction of virus multiplication by various cellular and viral factors and (2) failure of host defense to eliminate the virus. In addition, virus-induced immunopathogenic mechanisms, which are considered to be responsible for the pathology of persistent infections, are discussed (Section III,B).

Animal models with persistent virus infections are usually difficult to experiment with because of the varying length of the incubation period brought about by the complex relationship between virus replication, immune reactions, and disease. It therefore appeared appropriate to study the activity of antiviral compounds first in persistently infected cell cultures (Section V). Such cultures proved to be very useful for the selection of effective and well tolerated antiviral compounds. Subsequent studies *in vivo* were carried out with the lymphocytic choriomeningitis (LCM) virus (Section VI). Its natural host is the mouse where the virus causes either an acute or a persistent tolerant infection (Hotchin, 1962b, 1971). Since the acute disease of the adult mouse is not caused by direct virus-induced injury of cells but results from an immunopathologic disorder (Hotchin, 1958) disease can be abolished either by inhibition of virus multiplication or by suppression of the immune response of the host. Both possibilities of treatment will be considered.

II. PERSISTENCE OF VIRUS IN CULTURED CELLS

A. Mechanism of Virus Persistence

1. Cell-Specific Factors

a. Cell Type. Whether a cytocidal or a persistent infection of cell cultures develops is dependent to a large extent on the type of the host cell which is used for the experiments. When BHK-21 cells are infected with simian virus (SV)-5 extensive cell fusion with subsequent disintegration of the syncytia occurs but only small amounts of infectious

virus are released. In contrast, the infection of rhesus monkey kidney (MK) cells with SV-5 leads to high virus titers. However, despite the large production of infectious virus only little cell destruction is induced and the MK cells continue to multiply almost unimpaired (Holmes and Choppin, 1966). Marked differences in the interactions with various types of host cells have also been described for Newcastle disease (NDV) (Alexander *et al.*, 1973; Hecht and Summers, 1974) and influenza virus (Klenk *et al.*, 1975; Lazarowitz and Choppin, 1975) where the failure of host cell dependent cleavage of viral glycoproteins may lead to persistently infected cells in which viral maturation to a pathogenic form does not take place (Holland and Levine, 1978).

   b. *Cell Cycle.* Virus multiplication and the development of cytopathic effects (CPE) also depend on the stage of the cell cycle at which the infection occurs. In cultures of human conjunctiva cells infected with mumps virus, a persistent infection with little release of infectious virus develops when the cells are kept in growth medium; the transfer to maintenance medium with reduced serum content, or crowding of the cell culture results, however, in increased virus titers (Walker and Hinze, 1962; Walker *et al.*, 1966). Schwöbel and Ahl (1972) made similar observations with BHK-21 cells infected with Sindbis virus. In colonies derived from recovered cells virus-induced cell destruction occurs more readily in the center of the colonies, which consist predominantly of contact-inhibited cells, than at the periphery where rapidly dividing cells are dominant. In contrast, Lehmann-Grube (1971) observed more pronounced virus-induced CPE in LCM virus-infected proliferating L-929 mouse fibroblasts as compared to resting cells.

   c. *Genetically Resistant Cells.* Although it is well established that the resistance of cell lines to oncornaviruses is genetically controlled (Jolicoeur, 1978) the role of genetically resistant cells in other virus–cell systems is less clear. Many cell lines carry latent viruses or viral genomes (Chipman *et al.*, 1969; Weiss *et al.*, 1971). Therefore, in persistently infected cells, it is often difficult to discriminate between genetically controlled resistance and unresponsiveness due to inapparent virus infection. The development of increased resistance (due to host cell mutants?) was frequently observed in cell cultures persistently infected with enteroviruses (Vogt and Dulbecco, 1958; Takemoto and Habel, 1959; Pàsca, 1961; Walker *et al.*, 1966). However, experiments with other viruses (e.g., paramyxoviruses) indicate that genetically resistant cells may not be a major factor in controlling persistent infections in cell cultures (Rima and Martin, 1976).

   d. *Interferon.* Interferon can usually be detected in persistently infected cells in the initial stage of infection, which, in some virus–cell

systems, is characterized by a cyclic rise and fall of virus titers. Wiktor and Clark (1972) showed in mammalian and reptilian cells infected with rabies virus, and Inglot *et al.* (1973) with Sindbis virus-infected mouse cells that this pattern of rising and falling levels of viral infection is probably mediated by interferon. In contrast, no interferon was found in HeLa cells or human lung cells persistently infected with measles virus (Rustigan, 1966; Norrby, 1967) or in other cell lines persistently infected with different viruses (Walker and Hinze, 1962; Fernandes *et al.*, 1964; Desmyter *et al.*, 1968; Kawai *et al.*, 1975). Thus, in some virus–cell systems interferon might be important for the initiation of persistent infections; but its role in maintaining virus persistence remains to be established.

In addition to interferon, other virus-induced regulators of virus multiplication have been isolated from persistently infected cell cultures (ter Meulen and Martin, 1976).

2. *Virus-Specific Factors*

a. Temperature-Sensitive (ts) Mutants. The frequent isolation of *ts* mutants from persistently infected cell cultures and the observed increase in virus production and/or virus-induced CPE at the lower temperature (Norrby, 1967; Nagata *et al.*, 1972; Lewandowski *et al.*, 1974; Kimura *et al.*, 1975) indicate that *ts* mutants may play an important role in the initiation and maintenance of persistent viral infections in cell cultures. However, little is known about the control mechanisms which are mediated by *ts* mutants. Temperature-sensitive mutants of vesicular stomatitis virus (VSV) can be highly effective inducers of interferon at both nonpermissive (40.5°C) and semipermissive (37°C) temperatures (Sekellick and Marcus, 1979). Since interferon can also protect cells from destruction by VSV, Sekellick and Marcus (1979) suggest that interferon induced by *ts* mutants may partially be responsible for the initiation and maintenance of VSV persistence in cultured cells.

b. Defective Interfering Virus Particles. Defective interfering (DI) virus particles, which have been found in many preparations of DNA and RNA viruses, are characterized by the following properties: They (1) possess an incomplete genome, (2) contain the same structural proteins as infectious virus, (3) replicate only in the presence of helper virus, and (4) interfere specifically with the replication of homologous infectious virus (Huang and Baltimore, 1970; Huang, 1973, 1977). Holland and Villareal (1974) showed that in BHK-21 cells persistently infected with VSV, infectious virus free from DI particles will cause a lytic infection while an infection with DI particles and infectious virus

will usually result in persistence of the virus. Thus, DI particles obviously play a role in the initiation and maintenance of persistent infections in cell cultures; but—analogous to *ts* mutants—the molecular control mechanisms, mediated by DI particles in persistently infected cells, are still obscure. DI particles of VSV containing covalently linked, complementary RNA are potent inducers of interferon and therefore might act in a similar way to *ts* mutants (Marcus and Sekellick, 1977; Sekellik and Marcus, 1978). Holland and Villareal (1974) found a depression of translational activity of cells coinfected with DI particles and infectious virus. However, according to Rima and Martin (1976) other control mechanisms (e.g., at the level of transcription or posttranslational processing of viral proteins) have to be considered too.

c. *Proviral DNA as a Mechanism of Virus Persistence.* Zhdanow *et al.* (1974b) found virus-specific DNA sequences in the genome of HEP-2 cells persistently infected with the tick-borne encephalitis (TBE) virus. In addition to infection with TBE virus, the HEP-2 cells were found to be persistently infected with an oncornavirus. Zhdanow *et al.* (1974a) suggested that during the long-term double-infection of HEP-2 cells with both TBE and oncornavirus, reserve transcriptase of the oncornavirus transcribed not only the RNA of the tumor virus but also the genome of the TBE virus and both were integrated into the cellular genome. Similar observations were made with cells infected with either oncornavirus and measles virus (Zhdanow and Parfanovich, 1974) or oncornavirus and respiratory syncytial virus (Simpson and Iiuma, 1975). However, these results could not be confirmed by others (Friedman and Costa, 1976; Holland *et al.*, 1976).

3. *Stages of Virus Persistence*

Different mechanisms apparently exist for the initiation and maintenance of virus persistence. These mechanisms may operate in parallel or in series. They also may change during the course of infection, Schwöbel *et al.* (1975), who studied BHK-21 cells persistently infected with Sindbis virus over several years, observed the following stages of virus infection:

Stage 1 is typified by extensive virus-induced cells destruction with only a few surviving cells (approximately 10^{-7}) in which the virus usually persists. Virus-neutralizing antibodies can eliminate the virus from such cultures. The cured cells are as susceptible to Sindbis virus or other viruses as the noninfected control cells.

Stage 2 is characterized by cycles of virus multiplication and cell

recovery. Gradually, virus-induced cell destruction becomes less marked and finally disappeared after prolonged maintenance although infectious virus was released into the culture medium. After virus decontamination the cells were less susceptible to reinfection with Sindbis virus than the noninfected control cells. Resistant cell mutants might perhaps be responsible for this change in virus susceptibility. This is supported by the finding that the karyotype of the permanently infected cell lines deviated considerably from the karyotype of the original cell line (Schwöbel *et al.*, 1977). In addition, after several passages the virus population changed; virus mutants and/or variants which formed smaller plaques developed.

Stage 3. The infectivity titer of the Sindbis virus-infected cell line decreased gradually and finally after prolonged cultivation no infectious virus could be detected; but the apparently virus-free cells were resistant to reinfection with Sindbis virus.

B. Relationship between the Persistence of Virus in Cell Cultures and Virus Persistence in Mammals

Experiments with persistently infected cell lines have shown that virus infection can persist as long as virus multiplication is restricted to a small number of cells with only minor cellular damage. This appears to be similar to the persistence of virus in the organism (Strohl and Schlesinger, 1965). Moreover, experimental evidence indicates that the mechanisms which restrict virus multiplication and favor persistent infections in cell cultures (e.g., the development of DI virus particles and *ts* mutants) might also be responsible for the initiation and maintenance of persistent virus infections in mammals (NIH Workshop, 1976; Popescu and Lehmann-Grube, 1977; Friedman and Ramseur, 1979). However, in addition to the control mechanisms which are common to both persistent virus infections *in vitro* and *in vivo,* the defense system of the host plays a decisive role in the establishment of virus persistence *in vivo.*

III. Persistence of Virus in Mammals

A. Factors Which Contribute to Virus Persistence in Vivo

1. Deficient Humoral and Cellular Immunity

When virus infections occur *in utero* or neonatally, at a time when the immune competence is lacking or just developing, disease is often

not expressed. Mice infected from birth with LCM or murine leukemia virus (MLV) support virus multiplication but do not respond with an overt disease during the first few months of life. Evidence exists that tolerance of the cellular immune response is induced in these early infections (Hotchin, 1971; Huebner et al., 1971). Humoral antibodies appear as virus–antibody complexes (Hirsch et al., 1969; Oldstone and Dixon, 1971). The occurrence of immune complexes shows that the B cells respond to a presumably T-cell-independent antigen in these neonatal infections. The virus–antibody complexes can still be infectious (Notkins et al., 1966; Hirsch et al., 1969; Porter et al., 1969; Daniels et al., 1975). They can infect other target cells or may be engulfed by macrophages where the virus is able to persist and even replicate. In this way, virus elimination by macrophages might be prevented and consequently virus persistence initiated (Mims, 1974; Oldstone, 1975).

Moreover, virus-induced immunodepression plays an important role. Depressed antibody responses have been observed after infection of experimental animals with oncornaviruses (Dent, 1972), arenaviruses (Mims and Wainwright, 1968; Bro Jørgenson, 1978), herpesviruses (Osborne and Medearis, 1967; Purchase et al., 1968), arboviruses (Craig et al., 1969), paramyxoviruses (Medzon and Vas, 1964), Aleutian mink disease virus (Porter et al., 1965; Lodmell et al., 1971), and other infectious agents of slow virus diseases. Transient depression of cellular immunity was observed in humans after vaccination with live virus or after natural infections (Woodruff and Woodruff, 1975). These clinical observations have been confirmed by experimental data; mice infected with NDV or Gross leukemia virus showed prolonged survival of allogeneic skin transplants as compared to noninfected mice (Dent et al., 1965; Woodruff and Woodruff, 1974).

The following mechanisms have been proposed for virus-mediated immunosuppression: (1) Virus-induced destruction or functional changes of lymphoid or myeloid cells and/or their precursors, (2) antigenic competition, and (3) immunodepression as a result of increased adrenocortical secretion. Furthermore, immunodepression, which is observed after virus infection, might partially be mediated by interferon (Braun and Levy, 1972; Chester et al., 1973; Johnson, 1978).

Experiments in cell cultures showed that the cytotoxic reaction between virus-infected cells and committed T lymphocytes can be reduced or abolished by the addition of specific antibodies. Such phenomena have been observed in cells infected with myxo-, paramyxo-, oncorna-, and other viruses (Speel et al., 1968; Porter, 1971). However, the significance of these blocking antibodies for virus infections in vivo remains to be established (Mims, 1974).

Insufficient defense against viral infections could also be caused by modulation of viral antigen expression on the cellular membrane. Lampert *et al.* (1975) observed "capping" and "stripping" of viral antigens from the surface of measles virus-infected cells after addition of specific antibodies to the culture medium. The removal of cellular antigens from the cellular surface occurred rapidly and was associated with a reduced antibody-mediated complement lysis of these cells. The "stripped" cells, however, continued to synthesize and express viral antigen which could be detected in the cytoplasm. With regard to the elevated antibody titer against measles virus in patients with subacute sclerosing panencephalitis (SSPE), Joseph and Oldstone (1975) suggest that antigenic modulation, as observed *in vitro,* may play a central role in the pathogenesis of SSPE and other persistent virus infections with high-titer antibody response.

2. Defective Interferon Response

The functional deficiency of the immune system observed after neonatal virus infection apparently includes also a reduced production and/or response to interferon. In experiments with Coxsackie B1 virus (Henneberg *et al.*, 1964) adult mice produced interferon in all tissues infected. Suckling mice produced small amounts of interferon only in their livers. Similar results were obtained by Baron and Isaacs (1961) who injected four different viruses into chickens of different age groups. Viruses which tend to cause persistent infections are not usually potent inducers of interferon in their natural host. For instance, varicella zoster and cytomegalovirus induce little or no interferon in human cell lines; these viruses are also resistant to interferon (Glasgow *et al.*, 1967; Armstrong and Merigan, 1971). No interferon was found in mice persistently infected with LCM virus; however, the infected mice responded to other viruses with formation of interferon (Mims and Subrahmanyan, 1966). Thus, the inability to produce interferon is apparently restricted to the virus which causes the persistent infection (Mims, 1974).

3. Low Cytopathogenicity

Viruses inducing persistent infections usually have no severe destructive effects on cells and cause minimal changes in cell metabolism. This is valid for budding viruses with long incubation periods. However, cytolytic viruses may also cause persistent infections when the virus-induced cell destruction is restricted to a relatively small number of cells whose loss will not severely affect the organism (Fenner *et al.*, 1974; Mims, 1974).

4. Regulation of Virus Replication by Defective Interfering Virus Particles and Temperature-Sensitive Virus Mutants

Among the factors which limit virus multiplication and induce persistent infections, DI virus particles and *ts* mutants seem to play an important role (Huang, 1973). According to Huang and Baltimore (1970) the following virus–host interactions may take place under conditions where DI virus is produced:

1. Host cells which are infected by standard (infectious) virus particles produce infectious virus which is transmitted to other cells. New cycles of virus replication will follow and finally result in an acute illness.

2. Cells which are infected only with DI particles produce no progeny.

3. However, when a simultaneous infection with standard virus and DI particles takes place, the infectious virus functions as a helper virus and both types of particles are produced. Their replication is, however, limited by interference. With time an equilibrium is reached between the formation of infectious virus and DI particles which permits the continuation of virus replication at a relatively low rate with little or no apparent damage to the organism. Such interactions might be relevant for the initiation and maintenance of persistent virus infections (Welsh, 1971; Popescu and Lehmann-Grube, 1977).

In addition to DI virus particles *ts* virus mutants may regulate virus replication and thus determine the course of the infection. Fields (1972) infected newborn rats with either wild-type or *ts* mutants of reovirus type 3 and found that animals infected with wild-type virus died of acute encephalitis within 1 to 2 weeks whereas the *ts* mutants caused a fatal encephalitis only after administration of high virus concentrations. At lower concentrations the majority of animals survived the infection and appeared clinically healthy. However, several weeks or months later histological examinations revealed degeneration of the cerebral cortex with secondary enlargement of the cerebral ventricules. Changes of neurovirulence were also observed in mice infected with *ts* mutants or measles or vesicular stomatitis viruses (Haspel and Rapp, 1975; Stanners and Goldberg, 1975).

B. Immunopathogenic Mechanisms of Persistent Virus Infections

Signs and symptoms of a virus infection emerge primarily from cell destruction either caused directly by the virus or mediated by immune

mechanisms. The virus-specific immune reactions may trigger effector and amplification mechanisms (e.g., the complement system) resulting in local inflammatory processes and systemic disturbances. The following possibilities of interactions between virus and host defense are derived from experimental and clinical investigations (Allison *et al.*, 1972a,b).

1. Humoral Immune Response

a. Antibody-Mediated Cytotoxicity. When virus replication is accompanied by the expression of new antigens on the cell surface, antibody and complement may interact with these virus-induced cellular surface antigens. Virus-infected cells are destroyed as "innocent bystanders" as a consequence of the virus-specific immune reaction (Coombs and Gell, 1968). This has been shown with cells infected with herpes simplex, vaccinia, influenza, Newcastle disease, and other viruses (Brier *et al.*, 1971). Passive administration of viral antibodies to animals infected with respiratory syncytial, Japanese B encephalitis, or Aleutian mink disease virus produces or intensifies viral lesions (Allison *et al.*, 1972a). This indicates that antibody-mediated cytotoxicity plays a role in the pathogenesis of viral infections.

b. Formation of Immune Complexes. Persistent virus infections with a high output of viral antigen may lead to virus–antibody complex deposition. The soluble immune complexes are usually phagocytozed by circulating macrophages. However, when the amount of immune complexes exhausts the phagocytozing capacity of the macrophage system the immune complexes are precipitated at preferential sites (e.g., the glomeruli). After activation of complement by these immune complexes polymorphonuclear leukocytes are attracted which, subsequent to phagocytosis of the immune complexes, release lysosomal enzymes and other mediators of the acute inflammatory reaction. This inflammatory reaction increases and causes tissue injury. Dependent on the site of virus–antibody complex deposition, immune complex diseases such as glomerulonephritis, vasculitis, arteritis, or arthritis may develop. For instance, glomerulonephritis has been observed in mice persistently infected with LCM virus and in patients with hepatitis B (Almeida and Waterson, 1969; Oldstone and Dixon, 1970).

2. Cellular Immune Response

Cell-Mediated Cytotoxicity. T lymphocytes recognize virus-induced antigens present at the cell surface. This is followed by blastogenesis, generation of lymphokines, and destruction of the infected cells. The resulting chronic inflammatory response is characterized by the ac-

cumulation of mononuclear cells. Cellular immune reactions play a decisive role in the development of virus-induced exanthemas which are observed after infections with measles, rubella, and canine distemper virus, and in the delayed hypersensitivity reactions of the skin caused by poxviruses (Allison *et al.*, 1972b). However, the best example for virus-induced cellular immunopathogenic mechanisms is the LMC virus infection of the mouse which leads in adult mice to a lethal choriomeningitis within 7 to 10 days whereas in neonatal mice a persistent tolerant infection develops (Hotchin, 1958).

3. Virus-Induced Immunodepression

The depression of humoral and/or cellular immune reactions, which have been observed after infection of mammals with a variety of RNA and DNA viruses, is discussed in Section III,A,1.

4. Other Possibilities of Immunopathogenic Involvement

Viral genes in somatic cells may lead to the emergence of neoantigens, either virus-coded or virus-induced host-coded. Moreover, complexes consisting of virus-coded or virus-induced and normal cell surface antigens may result in new antigenic determinants with overlapping specificity. In consequence, these complex antigens may lead to autoimmune processes involving noninfected cells at distant sites by cross-reaction with the normal, noncomplexed cell surface antigen. Another possibility for virus-induced immunopathogenic mechanisms may be derived from the loss of normal cell surface antigens. This might result in the expression of cryptantigens which are normally not accessible to antibodies or committed lymphocytes and, therefore, may be recognized as "foreign." In addition, neoantigens may be shed from the infected cells and induce systemic immunopathological reactions which either are or mimic autoimmune processes.

IV. VIRUS CHEMOTHERAPY AND CHEMOPROPHYLAXIS

In persistent virus infections where immune mechanisms play a decisive role in the expression of disease two possible approaches to chemotherapy can be considered: (1) modulation (i.e., suppression) of the immune response and (2) abolishment of the antigenic stimulus by inhibition of virus multiplication or production of viral components.

Signs and symptoms of certain chronic virus infections (e.g., LCM) and chronic diseases with prominent immunopathologic processes and possible virus etiology (e.g., systemic lupus erythematosus, rheumatoid arthritis) can be greatly reduced by immunosuppression.

However, immunosuppressive treatment can be applied only for a limited period. Furthermore, latent viruses (e.g., herpes simplex, herpes zoster, cytomegalo, or oncornaviruses) might be activated and the risk of infection by nonviral agents is considerably increased. Therefore, inhibition of virus multiplication by antiviral agents appears to be a more promising approach for the control of the majority of chronic virus diseases than immunosuppressive treatment. Similar to the chemotherapy of most bacterial and fungal infections virus replication should be inhibited by antiviral compounds to the level where the immune system is able to eliminate the residual virus. However, it is often uncertain whether the immune system really eliminates the virus from the organism after an acute, apparent, or nonapparent infection.

Following intraperitoneal infection of adult mice with LCM virus a mild disease with uncharacteristic symptoms develops which lasts a few weeks (Haas, 1954). After that the mice recover and LCM virus can only rarely be isolated. When these apparently healthy mice are treated with antilymphocytic serum a viremia develops with high virus titers (Volkert and Lundstedt, 1968). Thus, LCM virus is still present several weeks after recovery of the host. The observation that varicella, measles, mumps, and several encephalitis viruses may induce life-long immunity in the organism indicates that a variety of viruses may persist in the host after an acute, apparent, or nonapparent infection. Virus replication is obviously reduced to a minimum by the host's defense system and an equilibrium between infectious agent and host is established which is clinically nonapparent. When this equilibrium is disturbed by the occurrence of a deficiency in the immune system then the virus starts to replicate; a high rate of virus multiplication will lead to an acute, low rate to predominantly chronic diseases. The objective of a virus chemotherapy would, therefore, be the restoration of an equilibrium between the virus and the defense mechanisms of the host rather than a "*therapia magna sterilans.*"

A. Characteristics of Antiviral Drugs

An antiviral agent can be defined as any substance which inhibits virus replication and thus cures or reduces signs of disease in the virus-infected host (Becker, 1976). Such a substance should not possess cytotoxic, cytostatic, or immunosuppressive activities in effective concentrations. The compound should be water-soluble and reach the target organ in the required concentration. The chemotherapeutic index (the ratio between the highest nontoxic and lowest effective concentration) should be high (*in vitro* > 100; *in vivo* > 10). An

antiviral agent should not possess mutagenic activities, i.e., the occurrence of resistant mutants during treatment should not exceed the natural mutation rate. The effect of the compound should be virus-specific.

The following modes of action can be envisaged: Inhibition of (1) virus attachment, penetration, or uncoating, (2) viral nucleic acid synthesis, (3) viral translation, (4) virus-specific posttranslational cleavage of proteins, and (5) virus maturation. Inhibitors of virus attachment, penetration, and uncoating can prevent virus infection of cells and thus inhibit the spreading of the virus within the host. Since these inhibitors interfere with early phases of the replication cycle they appear suitable for prophylactic treatment. Inhibitors of viral nucleic acid synthesis (e.g., antimetabolites), which are incorporated into the viral DNA, are often mutagenic (Prusoff and Ward, 1976). When these compounds also inhibit the DNA synthesis of dividing cells their use in chemotherapy is limited. A better chance for a successful chemotherapy might, however, be provided by compounds which are activated by virus-induced enzymes (Chen *et al.*, 1976) or by compounds which specifically inhibit virus-coded enzymes required for virus synthesis (Overby *et al.*, 1977; Helgstrand and Öberg, 1977).

B. Assay for Antiviral Activity

The search for antiviral compounds is usually carried out in cell cultures. Inhibition of viral CPE, virus plaque formation, or suppression of virus-induced inhibition of cell metabolism are used as indications for antiviral activity (Salk *et al.*, 1954; Rada and Zawada, 1961; Schwöbel, 1963). However, in these cell culture tests it is often difficult to discriminate between effects on cellular processes and specific antiviral effects. Therefore, tests with persistently infected cell cultures have been established which provide more precise information on the antiviral and cytotoxic activities of compounds than the usual cell culture screening tests based on the acute infection of cells (Schwöbel and Streissle, 1976a,b).

V. The Activity of Antiviral Compounds in Persistently Infected Cell Cultures

A. The Persistent Infection Cell Culture (PICC) Test

Persistent infections have been initiated in various BHK-21 cell lines which differ with respect to growth characteristics and virus

susceptibility. After infection, the cells were subcultured at various intervals and supplemented with new medium until the rate of virus and cell multiplication permitted a continuous cultivation of these cells. No viral antibodies or inhibitors of virus multiplication were used to establish the persistent infections. However, one BHK-21 subline, which has been used for the experiments with vaccinia virus, had previously been infected with Sindbis virus. After the Sindbis virus-infected cells had been subcultured for four years the virus seemed to have disappeared from this cell line. Different viruses destroyed the cells of the original BHK-21 line completely but caused, without adaptation, a persistent infection in the subline (Schwöbel and Streissle, 1976a).

The assay for antiviral activity is carried out according to the following scheme (Fig. 1): The cultures are seeded with a defined number of persistently infected cells. At intervals of 3 or 4 days the cells are subcultured and fed with medium containing the test compound in
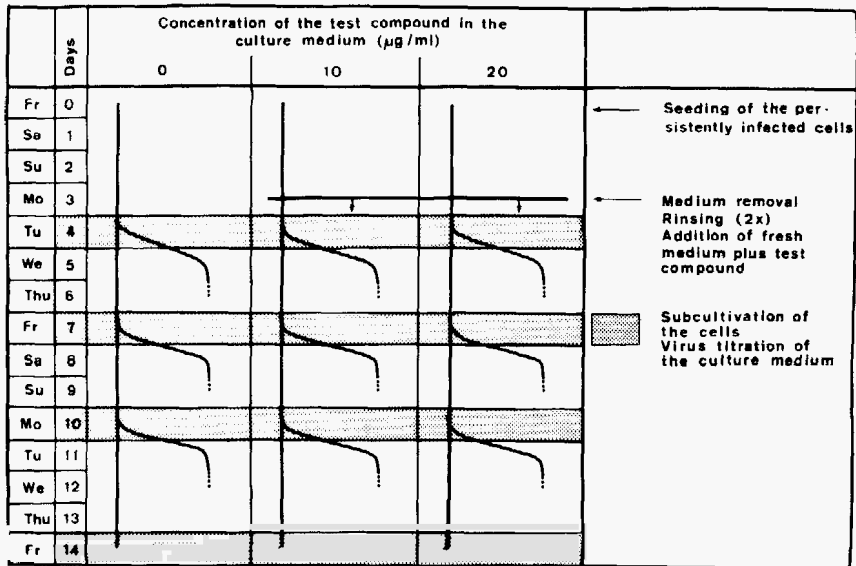
FIG. 1.   Design of the persistent cell culture test. Three days after seeding of the persistently infected cells the medium is removed. The cultures are rinsed twice with phosphate-buffered saline and fed with culture medium containing the test compound. After incubation for 1 day the medium is removed and tested for infectivity. The cells are subcultivated and again fed with compound-containing medium. Further virus titrations and cell subcultivations are made at intervals of 3 or 4 days. The cell cultures are observed microscopically for virus-induced cell destruction and drug-induced cytotoxic effects. From Schwöbel and Streissle (1976a).

various concentrations. The subcultivations are continued until the most effective, noncytotoxic concentrations of the test compound had been established. Decrease of virus-induced cell destruction and/or reduction in virus titer are used as criteria for inhibition of virus multiplication by the test compound.

B. Effect of 5-Iodouridine-deoxyriboside (IUdR) on the Multiplication of Vaccinia Virus

Low concentrations of IUdR (Fig. 2, I) reduced the titer of vaccinia in the plaque reduction (PR) test (Herrmann, 1961) and in persistently
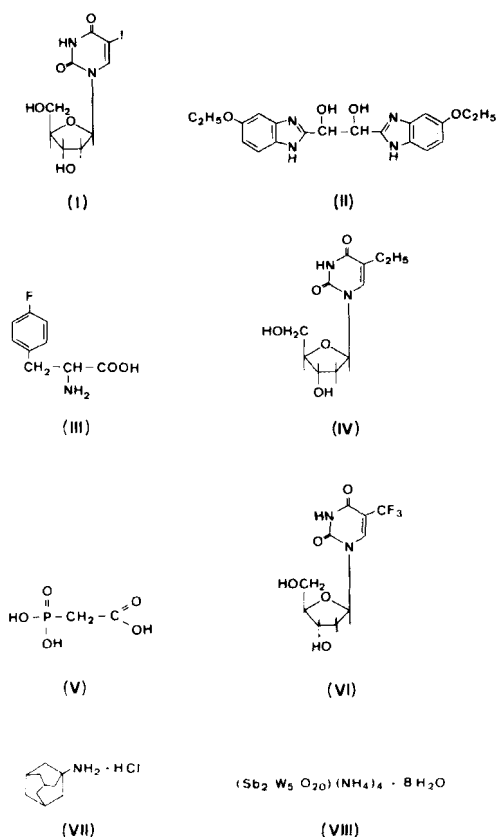

Fig. 2. Formulae of the antiviral compounds: (I) 5-iodouridine-deoxyriboside; (II) 1,2-bis(5-ethoxy-2-benzimidazolyl)-1,2-ethandiol; (III) p-fluorophenylalanine; (IV) 5-ethyluridine-deoxyriboside; (V) phosphonoacetic acid; (VI) trifluorothymidine; (VII) amantadine hydrochloride; (VIII) ammonium pentatungstodiantimonate.

infected cells (Table I). In the PICC test, however, cytotoxic effects occurred at 19 μg/ml whereas in the PR test no such effects were detected at 400 μg/ml. Virus-induced and therapy-induced cytotoxic effects could easily be distinguished by their different morphology (Fig. 3). The increase in plaque number after 7 or 11 days of treatment may indicate the development of drug-resistant mutants or variants. The optimal concentration of IUdR for the treatment of persistently infected cells appeared to be 9 μg/ml since neither virus-induced CPE nor drug-related cytotoxic effects occurred. A few days after treatment the confluency of the cell layer increased to 100% and remained unchanged during the observation period of 14 days. However, even at the optimal concentration the cultures could not be cured of the infection; infectious virus could always be isolated from the culture medium (Schwöbel and Streissle, 1976a).

The persistently infected cell cultures are characterized by a cyclic rise and fall of the virus titer due to periodic fluctuations of virus-induced cell destruction and cell recovery. As a consequence of this oscillation of the virus titer the results which are obtained with treated and untreated cultures differ at various times. This was mainly observed with low concentrations of the test compound. For instance, 1 to 2 μg/ml IUdR reduced the number of virus plaques during the first few days of treatment (Table I). However, due to a pronounced decrease of the virus titer of the control on day 11 the plaque numbers of the treated cultures were now higher than that of the control. Therefore, it is important for the evaluation of antiviral compounds in the PICC test to compare treated and untreated cultures over a period of several days.

C. Effect of 1,2-bis(5-Ethoxy-2-benzimidazolyl)-1,2-ethandiol on the Multiplication of Foot and Mouth Disease (FMD) Virus

After treatment of BHK-21 cells persistently infected with FMD virus, type C, with various concentrations of the bis-benzimidazol derivative 1,2-bis(5-ethoxy-2-benzimidazolyl)-1,2-ethandiol (Fig. 2, II) an increased release of infectious virus into the culture medium was observed. No increase in activity was found in the PR test. The greater release of FMD virus occurred at lower (1–5 μg/ml) and higher concentrations (75–400 μg/ml) of the test compound. The effect, which was associated at the higher concentrations with cytotoxic activities, was observed only in those cell cultures with 15% calf serum in the nutrient medium. Lower concentrations of serum did not permit this stimulation. Similar results were obtained with FMD virus subtype A_2

TABLE I

EFFECT OF IUdR ON VACCINIA VIRUS MULTIPLICATION IN THE PERSISTENT INFECTION CELL CULTURE TEST[a,b]

Days after start of the experiment		Concentration of IUdR in the culture medium (μg/ml)									
		0	1	2	5	9	19	38	75	150	300
1	I	14,800	3,200	6,200	5,000	3,800	6,000	6,600	10,400	6,000	6,200
	II	65	65	65	65	65	65	65	65	65	65
	III	V	V	V	V	V	V	V	V	V	V
4	I	16,800	1,600	2,000	1,500	712	640	1,700	3,600	1,300	1,800
	II	20	90	90	90	100	100	90	30	70	30
	III	V	V	V	V			C	C	C	C
7	I	76,600	15,000	9,100	2,200	94	77	129	135	126	198
	II	5	40	50	90	100	100	80	5	5	2
	III	V	V	V	V			C	C	C	C
11	I	9,900	62,200	24,400	3,100	152	11	59	11	45	0
	II	60	20	30	90	100	100	15	2	2	2
	III	V	V	V	V			C	C	C	C

[a] From Schwöbel and Streissle (1976a).
[b] I, Number of plaques per 0.1 ml of culture medium; II, confluency of the cell sheet in percent; III, status of the cells; V, virus-induced cytopathic effects; C, cytotoxic effects caused by IUdR.
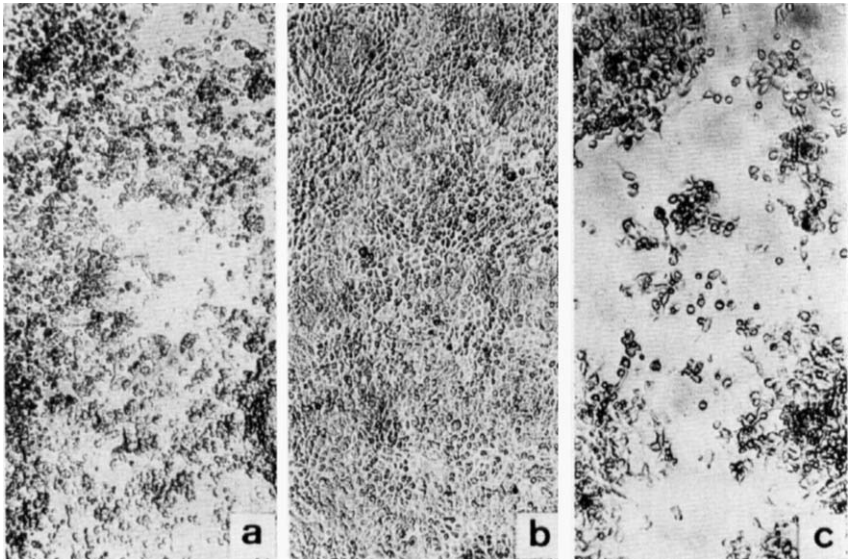
FIG. 3.   Effect of IUdR on BHK-2l cells persistently infected with vaccinia virus. (a) Untreated control culture with virus-induced cytopathic effects. (b) The same culture 7 days after treatment with 9 μg/ml of IUdR. (c) Cell culture with cytotoxic effects after treatment with 150 μg/ml of IUdR. From Schwöbel and Streissle (1976a).

(Schwöbel and Streissle, 1976a). Therefore, in contrast to the PR test, where inhibitory activities are recognized, the PICC test is also suited to detect stimulatory effects of chemical compounds on the virus multiplication. This is important in view of a possible activation of latent virus infections by antiviral compounds.

D. Effect of Heparin, p-Fluorophenylalanin (FPA), 5-Iodouridine-deoxyriboside (IUdR), and 5-Ethyluridine-deoxyriboside (EtUdR) on the Multiplication of Herpes Simplex Virus (HSV)

In these experiments the confluency of the monolayer relative to the available surface area 12 days after the start of the experiments was used as criterium for the antiviral activity of the test compounds. During this period the infected cells were subcultured three times. FPA (Fig. 2, III) was least effective and exhibited cytotoxic effects. Heparin was active and well tolerated by the cells. IUdR and EtUdR (Fig. 2, I and IV) showed pronounced antiviral effects at relatively low concentrations (1-2 μg/ml). Cytotoxic effects have been observed with

IUdR and EtUdR at 19 and 75 μg/ml and above, respectively. Infec-
tious virus was present in all cultures, also in those that were protected
from virus-induced cell destruction (Schwöbel and Streissle, 1977).

E. Effect of Heparin and IUdR on the Multiplication of HSV

Although single antiviral compounds caused a significant decrease
in virus titer and loss of virus-induced CPE in persistently infected
cells, cure of cells from infection was never observed. In contrast, a
complete decontamination of HSV-infected cells was achieved by using
combinations of antiviral compounds such as heparin and IUdR. Hepa-
rin and IUdR have different mechanisms of action. IUdR is incorpo-
rated into the viral DNA in place of thymidine and, thus, prevents new
synthesis of viral DNA (Prusoff et al., 1965). Heparin inhibits viral
attachment to the cell membrane (Hochberg and Becker, 1968) and can
also cause the release of virions already adsorbed to the cell (Nahmias
and Kibrick, 1964). Each compound inhibits significantly the replica-
tion of HSV in cell cultures. However, the antiviral activity could be
increased by combined application of both compounds. In HSV-infected
cell cultures, which were treated with cell-tolerated concentrations of
500 μg/ml heparin and 9 μg/ml IUdR, no infectious virus was found
and the cells remained virus-free after eight subcultivations in nutri-
ent medium without the test compounds. This shows that the PICC test
permits the differentiation of compounds that can cure a cell culture
from virus infection and compounds that can only temporarily retard
virus production (Schwöbel and Streissle, 1977).

F. Development of Drug Resistance

The emergence of drug-resistant virus mutants or variants has been
described for the majority of antiviral agents (Renis and Buthala,
1965; Herrmann and Herrmann, 1977). The formation of drug-
resistant virus mutants should therefore be considered in the evalua-
tion of any antiviral drug. The development of a therapy resistance can
easily be studied in persistently infected cell cultures. BHK-21 cells
persistently infected with HSV have been cultivated for 12 days in the
presence of 150 μg/ml EtUdR. This is the highest concentration of
EtUdR under which the virus replicated. Untreated virus from the
same batch served as a control. The susceptibility of the untreated and
EtUdR-pretreated virus population to the antiviral action of EtUdR
was compared using PR tests. The results are shown in Fig. 4. In the

Concentration of EtUdR in the culture medium (µg/ml)																
0	0.04	0.07	0.1	0.3	0.6	1	2	5	9	19	38	75	150	300	400	500

Status on day 12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	⊖	⊖	⊖

Percentage of Virus Control

100 80 60 40 20

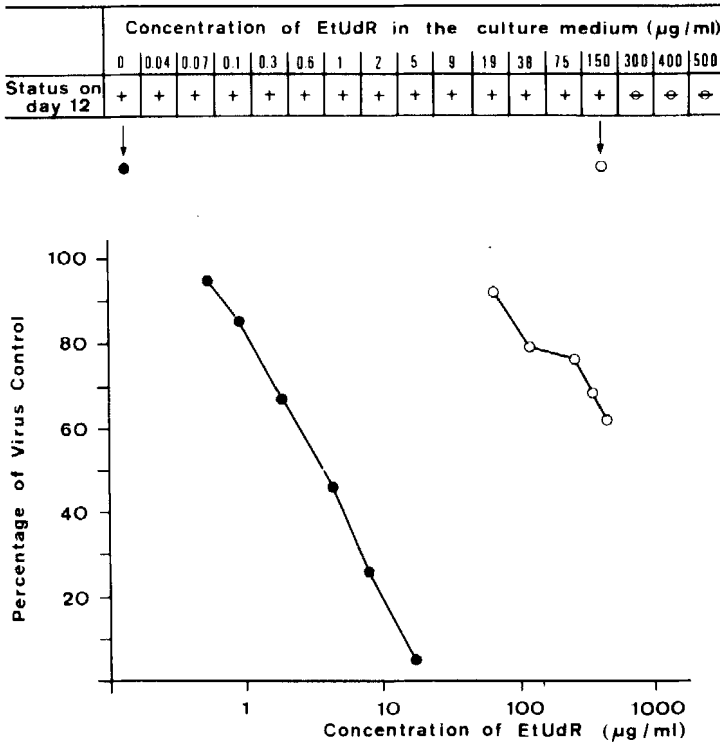1   10   100   1000

Concentration of EtUdR (µg/ml)

FIG. 4. Plaque reduction by EtUdR with two different populations of HSV. The virus populations were obtained from persistently infected cells either untreated (●————●) or pretreated with 150 µg/ml of EtUdR for 12 days (○————○). +, Cultures with infectious virus in the medium; ⊖, no infectious virus in the medium. From Schwöbel *et al.* (1979a).

untreated virus population the concentration of the test compound at which the plaque number is reduced by 50% (ED_{50}) is 7.0 µg/ml. The ED_{50} of the virus which was cultivated in the presence of EtUdR was, however, greater than 300 µg/ml. In further experiments IUdR (Herrmann, 1961), phosphonoacetic acid (Fig. 2, V) (Mao *et al.*, 1975), and trifluorothymidine (Fig. 2, VI) (Kaufman and Heidelberger, 1964) EtUdR-resistant virus was cross-resistant to IUdR but not to phosphonoacetic acid. The EtUdR-resistant virus was, however, more sensitive to trifluorothymidine than the wild-type virus. In this way combinations of antiviral drugs can be found which are much more effective than a single drug (Schwöbel *et al.*, 1979a,b).

VI. Activity of Antiviral Compounds against the Murine Lymphocytic Choriomeningitis (LCM)

The efficacy of a compound is not only dependent on its antiviral activity and lack of cytotoxicity but also on the biovailability of the drug in the target organ (Eggers, 1976). Therefore, subsequent to the detection of antiviral activity of compounds in cell culture assays, experiments in animals are done. However, many viruses require extensive adaption to the laboratory animal and the results obtained in these tests may not be applicable to the original host, since the course of the disease in the laboratory animal may be quite different (Bucknall, 1973). Moreover, there are viruses (e.g., human rhino- and coronaviruses) for which no suitable animal test is available. There-fore, we selected for our studies the LCM virus, the natural host of which is the mouse. The virus causes in this host either an acute or persistent tolerant infection (Lehmann-Grube, 1971).

A fatal choriomeningitis develops after intracerebral injection of LCM virus into immunocompetent mice. Experimental evidence indi-cates that the acute LCM is mediated by committed T lymphocytes which are able to lyse host cells carrying viral antigens on their cellular membrane (Hotchin, 1962b). Following virus injection into the foot pad of mice a marked edematous swelling is observed. The local swelling, which may extend to gross edema of the whole leg, lasts approximately 1 week and is usually followed by a complete recovery. This disorder probably represents an immunopathogenic event too (Hotchin, 1962a; Hotchin and Benson, 1963). A lifelong persistent infection with no apparent signs of disease during the early phases can either be induced by congenital or neonatal infection or by virus inocu-lation into otherwise immunoincompetent mice. Later in life, however, the persistently infected mice may develop a glomerulonephritis which is elicited by virus antigen–antibody complexes (Oldstone and Dixon, 1970). These different forms of disease which are induced by LCM virus were used to study the effect of selected antiviral compounds on LCM virus multiplication and the expression of disease.

A. Effect of Amantadine Hydrochloride, Ammonium Pentatungstodiantimonate, and L-Asparaginase on Acute and Persistent LCM Virus Infections

Although amantadine hydrochloride[1] (Fig. 2, VII) reduced the number of LCM virus plaques in cell cultures (Welsh et al., 1971) no

effect on either the acute or persistent virus infections was observed in the mouse (Table II). The heteropolyanion ammonium pentatungstodiantimonate[1] (Fig. 2, VIII) caused an increased survival rate in mice intracerebrally infected with LCM virus. However, the compound did not reduce the virus titer in the blood of persistently infected mice. L-Asparaginase was also inactive in the persistent infection, but the compound delayed or prevented the disease by immunosuppression, when it was applied 3 days after intracerebral infection (Streissle, 1975). Since none of the antiviral compounds was active in the persistent infection, LCM virus was isolated from persistently infected mice and the effect of amantadine hydrochloride and ammonium pentatungstodiantimonate on the virus isolate was tested in the PR test. The titer of the virus isolated from persistently infected mice revealed no difference in the sensitivity to the antiviral drugs as compared to standard virus.

B. Effect of Ammonium Pentatungstodiantimonate and L-Asparaginase on the Foot Pad Response

In the foot pad response ammonium pentatungstodiantimonate was inactive after intravenous as well as intramuscular application (Streissle, 1977). In contrast, L-asparaginase caused a significant reduction of the paw swelling (Fig. 5). The swelling started in untreated controls on day 7 and reached its peak on day 10 or 11 pi. After intramuscular injection of 10,000 IU of L-asparaginase at the site of infection (3 hours before, 24 and 48 hours after infection) no swelling was noticed until day 8 or 9 pi. However, a few days after the treatment had been terminated a weak swelling dependent on the concentration of the virus inoculum occurred. The effect of L-asparaginase on the foot pad response is dose-dependent and has also been observed after intraveneous treatment.

The activity of L-asparaginase which has been observed in the acute, intracerebral infection and in the foot pad response is obviously caused by its immunosuppressive activities (Maral et al., 1970) since no antiviral effects were recognized either in the cell culture test or in persistently infected mice. The mode of action of ammonium pentatungstodiantimonate remains, however, obscure. In addition to antiviral activity the compound possesses cytotoxic and possibly im-

[1] Amantadine hydrochloride was kindly supplied by Rhone Poulenc, Virty-sur-Seine, France, and ammonium pentatungstodiantimonate by Prof. Dr. O. Glemser, Institute of Inorganic Chemistry, D-34 Göttingen, FRG.

TABLE II

EFFECT OF AMANTADINE HYDROCHLORIDE, AMMONIUM PENTATUNGSTODIANTIMONATE, AND
L-ASPARAGINASE ON THE ACUTE AND PERSISTENT LCM VIRUS INFECTIONS OF THE MOUSE

Acute infection percentage survival				Persistent infection		
Amantadine hydrochloride						
250	100	50	0 mg/kg	250	100	50 mg/kg
Toxic	0	0	0%	Toxic	0[a]	0
Ammonium pentatungstodiantimonate						
250	100	50	0 mg/kg	250	100	50 mg/kg
Toxic	25	25	0%	n.t.[b]	0	0
L-Asparaginase						
10000	2000	400	0 IU/kg	10000	2000	400 IU/kg
75	25	25	0%	0	0	n.t.

[a] Difference in blood virus titer as compared to the untreated control.
[b] Not tested.

munosuppressive activities (Jasmin *et al.*, 1974). The effect on the acute LCM may, therefore, be related to its antiviral or perhaps to its immunosuppressive effects which could also act synergistically. The results of Werner *et al.* (1976) are interesting in this respect since they showed that EMC virus-infected mice could be protected by ammonium pentatungstodiantimonate from a lethal encephalomyelitis despite the fact that treated mice had conparable virus titers in their blood and brain as the untreated controls.

The observation that none of the compounds inhibited LCM virus multiplication in persistently infected mice cannot be conclusively interpreted. It seems unlikely that changes of the virus are responsible for these negative results since virus isolated from persistently infected mice is *in vitro* equally susceptible to treatment with ammonium pentatungstodiantimonate as the standard virus. It rather appears that the persistent LCM virus infection is so well established in the mouse that even with those compounds which are active in other test models no reduction of virus production could be achieved.

Cells persistently infected with HSV could be cured of the virus infection when these cells were treated with two different antiviral compounds. A combined treatment with two or more effective and well tolerated antiviral compounds might also be required to reduce LCM virus replication in persistently infected mice. The PICC test appears well suited to find and characterize such compounds.
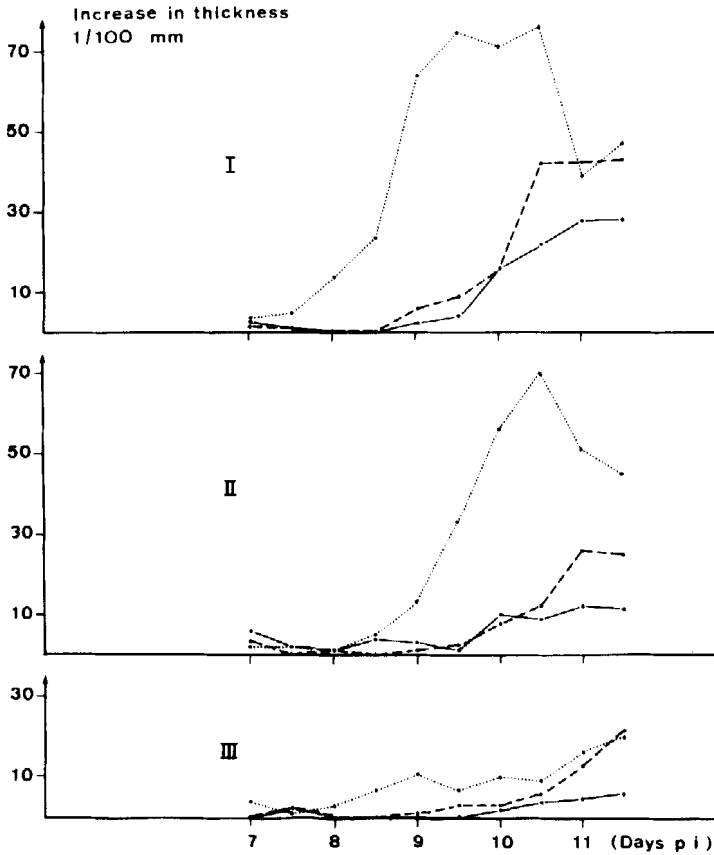
FIG. 5.   Effect of L-asparaginase on the LCM virus-induced swelling of the foot pad. Adult mice were injected into the foot pad with different concentrations of LCM virus and treated with L-asparaginase by injection of the compound at the site of infection 3 hr before, and 24 and 48 hours after infection. Infectious doses (i.c. mice): 1000 LD$_{50}$ (I), 100 LD$_{50}$ (II), 10 LD$_{50}$ (III). Concentrations of L-asparaginase: ● · · ·● nontreated control; ●---● 2000 IU; ●——● 10,000 IU. Each point is the mean of the values from 10 mice.

VII. CONCLUSION

Virus chemotherapy had little success so far and only few clinically effective antiviral drugs are available (Bauer, 1977). This is mainly due to the fact that viruses are obligatory intracellular parasites and completely dependent on the metabolism of the cell for their replication. After virus infection of cells a new entity, the virus–cell complex,

is formed. In this complex virus multiplication and cell metabolism are so intimately linked that any inhibition of virus multiplication without injury to the host cell is difficult to achieve (Whitley and Alford, 1978). However, another reason for the failure of chemotherapy of acute virus infections is due to the fact that virus production usually reaches its maximum before symptoms appear and treatment cannot be initiated in time. In contrast, time for therapy plays no crucial role in persistent virus infections.

Persistent infections can be established *in vitro* and *in vivo* with viruses which might cause chronic diseases in humans. With the aid of the PICC test compounds which are highly effective against such viruses can be selected and subsequently tested for their possible use as antiviral drugs in suitable models *in vivo*. It appears that such an experimental approach provides a better chance for the successful search for antiviral compounds and for a successful chemotherapy than investigations based on acute virus infections.

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