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HERPES SIMPLEX VIRUS

A DISSERTATION

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BY

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Oklahoma City, Oklahoma

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DELAYED HYPERSENSITIVITY TO

HERPES SIMPLEX VIRUS

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DISSERTATION COMMITTEE

ACKNOWLEDGMENTS

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DELAYED HYPERSENSITIVITY TO HERPES SIMPLEX VIRUS

CHAPTER I

INTRODUCTION

From an examination of their ultrastructure, viruses appear to

be the simplest of independent genome-containing systems. Presently,

the events which occur at the virus-host cell level are, at best, only

partly understood. High on the list of host reactions to viral pro-

ducts which merit thorough investigation are those responses which

interrupt the spread of viruses, such as herpes simplex virus (HSV) or

vaccinia virus, from one cell to another and which lead to recovery

from viral disease. Of particular interest in this dissertation is the

paradox of recurrent herpes simplex, in which persons who recover from

primary disease develop high titers of virus neutralizing antibody but

continue to have localized viral lesions.

Disease Caused by Herpes Simplex Virus

A copious literature on the clinical problems associated with

herpes simplex virus awaits the diligent reader, but perhaps the most

concise description of these maladies appears in a treatise by Muller

and Herrmann (67).

Herpes simplex virus is probably the most common viral in-
fection of man and causes a number of clinical syndromes of
skin and mucous membranes that are readily recognized clinically

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and are almost always benign. The site of primary infection
is usually the oropharyngeal tissues, and the infection is
manifested clinically as an ulcerative gingivostomatitis of
variable local severity and systemic reactivity. Probably in
many instances, the stomatitis is mild and passes unnoticed.
Children are usually affected, but results of serologic
studies have indicated an increasing number of adults in the
higher socioeconomic groups who have not been infected.

A high degree of contagiousness of the virus is indicated
by infections frequently affecting more than one member of a
family, by epidemics of herpetic stomatitis among children in
orphanages and of eczema, and by reports of direct inoculation
of herpes virus into the skin by a bite or into an abrasion.
Epidemics of multiple herpes simplex infections of the skin
are common among college wrestlers. The direct venereal
transmission of the disease is also recognized. More serious
herpetic infections may occur in premature and newborn in-
fants inoculated at birth by contact with herpetic lesions of
the birth canal.

Herpetic infections of the fingers (herpetic whitlow),
primarily affecting the soft tissues about the fingernails,
were reported as a form of hospital cross infection by Stern
and associates (93). The infections were noted in hospital
personnel who handled infected saliva, particularly that of
neurosurgical patients who required suction of bronchial
secretions after operation. Dentists are also believed to be
more frequently exposed to such infections.

Primary infection is followed by a latent infection which may persist

for life with the possibility of recrudescent lesions being produced by

many types of stimuli, e.g., menstruation, sunburn, fever, psychic up-

set, and gastro-intestinal distress. Herpes simplex infections give

rise to neutralizing antibodies, which are ineffective in preventing

secondary infection (78). Although antibodies apparently prevent

viremia during secondary infection the virus is able to pass from in-

fected cells to adjoining susceptible cells without coming into contact

with extracellula. antibody. This seems to be the way in which HSV pro-

duces the recurrent fever or cold blister so commonly seen at the

mucocutaneous juncture of the mouth and nose.

Plummer (77) differentiated between two types of HSV, later

known as type I (oral) and type II (genital) strains, in infections.

He demonstrated antigenic differences by the cross neutralization technique. Type I HSV was shown to be neurotropic, less stable in heparin, and proliferated better in rabbit kidney monolayers than did type II virus (79).

Recently, interest has turned to the epidemiology of HSV in association with cervical carcinoma. Josey et al. (50) found 22% correlation between cervical carcinoma and herpes virus infection. Rawls et al. (81) reported an 83% correlation between cervical cancer and antibody to type II HSV, a finding which suggests a relationship between HSV and carcinoma of the cervix.

HSV infection causes alterations of mammalian chromosomes. Hampar and Ellison (34) noted increased translocations, abnormal formation, accentuated constriction and breakage of chromosomes in HSV-infected hamster cells. Later, Stich et al. (94) reported that the chromosomal aberrations in human or Chinese hamster lung cells infected with HSV were restricted to fixed regions of the X-chromosome and chromosome I. Such aberrations were not produced by non-infectious HSV and viral nucleic acid synthesis could not be shown to cause the chromosomal alterations.

In view of the prevalence of HSV infections in the population and the results of surveys indicating good correlation between HSV type II infections and cervical cancer, a more complete understanding of the host responses to HSV and to the products of HSV infection seems imperative.

Delayed Hypersensitivity

In addition to circulating antibody, delayed hypersensitivity to HSV is known to follow primary infection. Hypersensitive reactions

are separable into two categories based upon the time required for manifestation of the reaction following antigenic challenge. Immediate hypersensitive reactions usually begin within minutes after exposure to antigen and become maximal by 4 hr, with the exception of the Arthus phenomenon in which hemorrhage and necrosis may not peak until 24 hr (88). Other examples of immediate reactions include atopy, serum sickness, and anaphylaxis. Delayed hypersensitive reactions usually do not first become apparent until 3 to 6 hr after challenge with antigen and may take from 24 to 72 hr to become maximal.

A second criterion which differentiates between immediate and delayed hypersensitivities is revealed in passive transfer studies. Whereas skin reactivity in immediate hypersensitivity is transferable with serum, lymphoid cells are required for transfer of the delayed hypersensitive state (88). Chase (II) demonstrated that delayed skin reactivity to tuberculin could be systemically transferred in guinea pigs by lymphoid cells from peritoneal exudates, spleen, and lymph nodes of donor animals. In addition to intact lymphoid cells, lysates of lymphoid cells prepared by sonic vibration have been shown to transfer passively skin reactivity to 2,4 -dinitrochlorobenzene (48) and tuberculin (12). Two fractions from sensitized cell lysates which were capable of passively transferring tuberculin hypersensitivity were prepared by Dunn and Patnode (19). The first fraction, which was estimated by gel filtration to have a molecular weight (mol wt) of 50,000 or less was characterized as protein. A smaller RNA-like fraction, having a mol wt of about 10,000 was also reported to transfer tuberculin hypersensitivity passively. Local passive transfer of delayed skin hypersensitivity can be demonstrated by the intracutaneous injection of

lymphoid cells and subsequent challenge with antigen at the same site or by the intravenous administration of antigen (65). In a recent study (75), as few as 100,000 lymphoid cells were shown to transfer local reactivity to vaccinia virus in guinea pigs. The authors utilized peritoneal exudate cells obtained from guinea pigs sensitized by infection with vaccinia virus. Twenty-four hours after intracutaneous injection of the exudate cells into normal animals, inactive viral antigen was administered intravenously and subsequently, the skin sites were examined for erythema and induration. The local passive transfer technique has been used to transfer delayed allergy to diphtheria toxoid (87), sheep erythrocytes (42), and 2,4-dinitrochlorobenzene (9).

The nature of the cellular infiltrate 24 hr after skin testing also distinguishes immediate from delayed skin reactions. In the Arthus reaction and other immediate hypersensitivities, the predominate cell type in the infiltrate at 4 and 24 hr is the polymorphonuclear neutrophile. Mononuclear cells are seen in Arthus reactions at 24 hr but they are attributed to the stage of healing following hemorrhage and ischemia. In contrast, mononuclear cells predominate in the delayed hypersensitive reaction at 24 hr after skin testing. Occasionally, polymorphonuclear leukocyte counts may reach 60% at 24 hr, but usually many more mononuclear cells are present (26, 27, 16). Dienes and Mallory (16) suggested that the degree of necrosis seen may be a result of the number of polymorphonuclear leukocytes in the skin test site at 24 hr. Features which distinguish cell-mediated hypersensitivity from serum-mediated hypersensitivity include the following in vitro reactions which are characteristic of delayed allergy: 1) cyto-toxic effect of

sensitized lymph node cells upon syngeneic fibroblasts in the presence

of specific antigen (84); 2) inhibition of sensitized macrophage migra-

tion in the presence of the sensitizing antigen (14, 19, 28).

Delayed Hypersensitivity in Viral Infections

In his review of viral infections which give rise to delayed

hypersensitivity to skin test antigens, Beveridge (7) listed vaccinia,

herpes, mumps, influenza, measles, swine fever, contagious ecthyma of

sheep, ectromelia, and lumpy skin disease of cattle. He postulated

that delayed hypersensitivity probably occurred in all virus infections.

Allison (1) quoted from Edward Jenner's original description of

the delayed reaction appearing at the site of variola inoculation in

the arm of Mary Barge, a farm girl who had cowpox. Jenner's descrip-

tion on page 13 of An Inquiry into the Causes and Effects of the

Variolae Vaccinae, originally published in 1798, is as follows:

> An efflorescence of a palish red colour soon appeared about
> the parts where the matter was inserted and spread itself
> rather extensively, but died away in a few days without pro-
> ducing any variolous symptoms....It is remarkable that vario-
> lous matter, when the system is disposed to reject it, should
> excite inflammation on the part to which it is applied more
> speedily than when it produces the Small Pox. Indeed it be-
> comes almost a criterion by which we can determine whether
> the infection will be received or not. It seems as if a
> change, which endures through life, had been produced in the
> action, or disposition to action, in the vessels of the skin;
> and it is remarkable too, that whether this change has been
> effected by the Small Pox, or the Cow Pox, that the disposition
> to sudden cuticular inflammation is the same on the application
> of variolous matter.

The principle of the skin test was put to use in 1909 when von Pirquet

(76) developed the diagnostic tuberculin skin test.

In early studies of delayed hypersensitivity following viral

infection, crude antigens were often employed. For example,

lymphogranuloma venereum (LGV) skin test antigen consisted of heat-inactivated pus from a bubo of an infected person.

Andervont and Rosenau (4) described a delayed reaction in persons immunized with vaccinia virus which could be elicited by antigen heated for I hr at 120 C. The heated preparation produced reactions in vaccinated persons only, and the skin test material was shown to be the vaccinia virus particle.

In 1944, Beveridge and Burnet (8) reported that influenza types A and B, proliferated in embryonated eggs, produced a heat-stable skin test antigen which was not inactivated by exposure to 120 C for 30 min. Injection of this preparation into human adults often elicited erythematous areas with diameters as large as 30 mm.

Persons immunized with inactivated measles vaccines develop an early, indurated response to intradermal injection of measles antigen, while persons with a history of natural disease or infective measles immunization exhibit little or no response (46, 56).

In addition to its use as a skin test antigen (73), soluble antigen of vaccinia virus is also effective in inhibiting migration of sensitized peritoneal exudate cells in vitro (103). Studies of the specificity of the soluble antigen of vaccinia virus are needed. Recently, skin test reactivity to vaccinia virus particles was transferred to normal guinea pigs with as few as 10^5 peritoneal exudate cells from sensitive donors. This showed the high degree of sensitivity attainable with whole virus sensitization and challenge (75).

Enders et al. (21) noted that persons who responded to mumps antigen with a positive skin test did not develop the disease as often

as non-reactors. Later, delayed hypersensitivity to mumps was demon-
strated by inhibition of migration of peritoneal exudate cells in vitro
(22), in vivo skin test reactivity (102, 30), passive transfer by cells
(102), and tissue culture cytotoxicity (33).

Henle et al. (38) prepared a skin test antigen for infectious
hepatitis from amniotic fluid of infected chick embryos. Following
intradermal injection of UV-irradiated antigen, reactions of erythema
and induration which measured up to 45 mm in diameter were observed in
100% of persons with known contact with infectious hepatitis, whereas
only 11% of presumed non-exposed persons showed skin reactivity. In
an epidemiological study, persons previously skin tested with the same
antigen had only a 1.6% attack rate of infectious hepatitis, while non-
skin tested persons in the same institution had an attack rate of 13.6%.
The authors suggested that the lower attack rate in the skin tested
persons was due to an immunizing effect of the skin test material (18).

Contrary to results of earlier studies, Smith (89) reported the
skin test antigen of vaccinia virus to be inactivated by autoclaving.
In addition much of the skin test activity of the preparation was re-
moved by filtration through a 9 mμ filter but not by filters of larger
pore size. Vaccinated persons responded to the active skin test anti-
gen with areas of erythema measuring 20-30 mm in diameter, whereas un-
vaccinated persons showed no reactions. The reaction was specific for
vaccinia; HSV preparations did not elicit skin reactions in vaccinia-
sensitized animals.

Noren et al. (73) confirmed the skin reactivity of a soluble
vaccinia antigen and noted that this material was a better skin test

antigen than was the purified, whole virion. The degree of response was dose dependent, since diameters of erythema in the skin tests of guinea pigs and human volunteers were directly proportional to the concentration of antigen injected.

A linear relationship between inhibition of migration of peritoneal exudate cells and cellular transfer of local cutaneous hypersensitivity to inactive vaccinia virions in guinea pigs was shown by Pilchard et al. (75), who noted that delayed skin reactions first appeared on the fourth day following infection with vaccinia virus. Tompkins et al. (101) showed a direct relationship between inhibition of migration and skin test diameters to fibroma virus in rabbits. Skin test antigens in the psittacosis-lymphogranuloma venereum group are known to have a common group antigen (40) and cross reactions occur in sensitized animals (80). Lymphogranuloma venereum (LGV) patients exhibit positive skin reactions to the following skin test antigens prepared in yolk sacs: LGV, feline pneumonitis, meningo-pneumonitis, psittacosis, ornithosis and mouse pneumonitis. The skin test antigens in psittacosis and LGV have been shown to be resistant to degradation by both trypsin and potassium periodate. Complement fixing (CF) antigens from these agents are trypsin resistant, but they are inactivated by periodate (6), an indication that they are carbohydrate in nature or a complexed carbohydrate. Jorgenson et al. (49) presented evidence which indicated that the CF antigen of ornithosis virus was a lipopolysaccharide.

Sensitization to various viruses may follow natural infection, skin testing, vaccination, or injection of viral antigen alone or in

adjuvant. The skin test or challenge antigens and their antigenic character are as varied as the methods of sensitization. Both whole virus particles, infective or inactivated, and fractions thereof have been shown to be good skin test antigens.

Antigens of Herpes Simplex Virus

Spear and Roizman (91) reported that HSV-infected HEp-2 cells synthesized at least 25 proteins at various times during the growth cycle. The antigenicity of these proteins was not studied. Their size estimates of these detectable proteins ranged from 29,000 to 125,000 daltons. The application of polyacrylamide electrophoresis to herpes-infected cells revealed the existence of 12 specific viral antigens which stimulated production of precipitating antibodies in rabbits (105).

By gel immunodiffusion, Tokumaru (100) demonstrated three soluble antigens of HSV in addition to the virion. The soluble antigens were estimated to have molecular weights of 90,000, 170,000, and 1,000,000; while the viral antigen was estimated to have a mol wt greater than 3×10^6.

Brown (10) reported that washed, whole virus particles adsorbed the neutralizing component, but not the CF components of antiserum. The reverse situation was also found to hold true, that is, antisera prepared to whole viral particles lacked CF ability but possessed neutralizing ability. Heat treatment at 56 C for 1 hr destroyed the CF activity of the soluble antigen, but did not affect it as a skin test antigen. Roane and Roizman (83) found that, when treated later with complement and HSV antiserum, HEp-2 cells infected 24 hr previously

with HSV were lysed, but were not altered morphologically by comple-
ment or antiserum alone. These data suggest that a distinct surface
antigen, specific for HSV is produced in HSV-infected cells within
24 hr.

Nine proteins located in the coat of HSV were estimated by
Olshevsky and Becker (74), to have mol wt ranging from 24,000 to
14,000 daltons. It appeared that a protein of 110,000 mol wt, in
combination with a 30,000 mol wt particle, comprised much of the
virus capsid.

Kaplan (52) noted that HSV-infected cells yielded a soluble
CF antigen which was separable from the viral particle by differential
centrifugation. It has been suggested that the CF antigen is excess
structural protein which was not incorporated into the complete virions.
More recently, Spear et al. (92) demonstrated that HSV infection of
HEp-2 cells elicited the production of three or four viral glycoproteins
which became incorporated into cell membranes. The mol wt of these
proteins ranged from 0.5 to 1.0 x 10^6 daltons.

Skin Test Antigens of Herpes Simplex Virus

Since 90% of persons above four years of age exhibit an erthe-
matous reaction to skin test material prepared from HSV-infected
chorioallantoic membranes, HSV infections must be widespread in the
population (68). In 1951, Jawetz et al. (47) showed that high speed
centrifugation of HSV-infected chick embryo allantoic fluid yielded a
supernatant fluid containing a soluble antigen which was a more
effective skin test antigen than was the virus-containing sediment. In
these early studies with human subjects, the skin test antigen was

prepared from amniotic or allantoic fluids harvested from infected
embryonated hen's eggs, and the preparations were inactivated by ultra-
violet irradiation before use.

Other workers have employed inactivated HSV in skin testing
(10, 99) but a comparison of viral skin test antigen and soluble skin
test antigen showed the latter to be more satisfactory since it
elicited smaller non-specific reactions (3, 10). Brown (10) demon-
strated that HSV hyperimmunized guinea pigs responded to HSV skin test
material with large areas of induration and erythema. Repeated intra-
peritoneal injections of infective HSV induced animals to develop in-
durated skin test reactions to the soluble antigen. In a study by
Lausch et al. (55) which characterized the skin test reaction following
live virus sensitization, guinea pigs were skin tested with soluble
antigen from heat-treated HSV-infected chick embryo fibroblasts. The
erythematous reaction was found to be maximal at 24 hr after testing
and histological examination revealed a predominance of mononuclear
cells. Passive transfer of the skin reactivity was accomplished by use
of peritoneal exudate cells but not of serum. From these data, the
authors concluded that this was a delayed type of hypersensitivity
without a consistent induration. Later studies by Swyers et al. (97)
revealed that delayed hypersensitivity reactions could be produced
in the corneas of HSV-infected guinea pigs. They suggested that a
similar reaction may contribute to disciform keratitis in man.

While the above studies indicated that a delayed-type of skin
reactivity follows infection with HSV, the relationship of the skin
test to antibody titers was not examined. Good correlation between

erythema, which peaked at 24 hr, and titer of CF antibody in human sub-
jects skin tested with ultraviolet light-inactivated soluble antigen
was reported by Yamamoto (107). Although this would suggest that the
skin test is mediated by CF antibody, it should be noted that the skin
test material used was a crude preparation which could have contained
many different antigens acting concomitantly to produce many different
responses. However, this study did emphasize that delayed skin re-
activity is a frequent occurrence following natural infection with HSV.

To date, it has been shown that the skin test antigen of HSV
is heat stable (3, 10, 55), non-dialyzable (55), not sedimented by
ultracentrifugation at speeds of 79,000 x g for 3 hr (3) and, most
importantly, does not elicit significant reactions in non-sensitized
animals (10).

Factors Involved in Recovery from Viral Disease

Antibody

According to Fenner (23), the role of antibody in viral in-
fections is the prevention of the attachment and penetration of virus
into susceptible cells. Once inside the cell, the virus is protected
from neutralizing antibody. Apparently, the mode of release of virus
from infected cells, as well as the mechanism of transfer of infective
virus to adjacent cells, determines the effectiveness of neutralizing
antibody in virus infections. HSV and vaccinia virus are able to pro-
duce lesions in the presence of high titers of neutralizing antibody
(23). Persons subject to recurrent herpes labialis have circulating,
neutralizing antibody. Recurring infections do not necessarily stimu-
late detectable increases in antibody titer (17).

Inactivated HSV vaccines will protect mice and guinea pigs
against intracerebral challenge with high doses of virus. However,
Kaplan (52) noted that the administration of similar vaccines in man
did not stimulate circulating antibody production of the magnitude
elicited in animals. These vaccines were shown to be ineffective in
preventing the disease in man; even the administration of antiserum to
patients with active disease has been of questionable value (52).

Interferon

Viral interference was shown by Isaacs and Lindenmann (44) to
be mediated by a protein produced by host cells in response to viral
infection. Isaacs and Lindenmann (44) and Isaacs et al. (45) named the
protein-containing substance interferon and characterized it as being
heat stable (56 C for 1 hr), sensitive to the action of trypsin, not
inactivated by anti-viral serum, stable over a pH range of 1 to 11,
host species specific, non-dialyzable, not sedimented by centrifugation
at 100,000 x g for 1 hr, and effective against viruses other than those
inducing its production.

Fungi and bacteria, as well as viruses, induce interferon syn-
thesis. In addition, certain nucleic acids and simple copolymers of
nucleic acids, such as poly IC, a synthetic copolymer of inosinic and
cytidylic acids, induce interferon formation (64). Neither the exact
mechanisms involved in interferon production, nor its mode of action,
are understood. It is known that interferon can act by preventing
formation of viral coat protein, inhibiting viral DNA or RNA replica-
tion, and hampering complete virion production (64), presumably by
interfering with translation of viral messenger RNA into protein (39).

Recently, it has been demonstrated that the same mechanism involved in delayed hypersensitive reactions may stimulate interferon production both in intact animals (95) and _in vitro_ (33). Mice previously infected with _Mycobacterium_ _tuberculosis,_ strain BCG, produced significant levels of interferon upon challenge with purified protein derivative (95). Lymphocytes from persons whose skin tests were positive to PPD produced large amounts of interferon upon stimulation of the lymphocyte cultures with purified protein derivative (33). Similar cultures from persons previously immunized with tetanus toxoid or diphtheria toxoid also contained significantly larger amounts of interferon upon challenge with these antigens than were contained in control lymphocyte cultures (33). These findings illustrate the problems involved in critical analysis of the comparative importance of interferon and delayed hypersensitivity in recovery from viral infections. An excellent review of studies on this subject is presented by Glasgow (29).

Mice infected with HSV exhibit maximum of interferon production at the site of the viral lesion five days post-infection, with maximal virus production occurring on the sixth day (98). This may be interpreted as an indication of recovery attributable to interferon but conclusive data in support of this hypothesis are lacking. Persons with vaccinia gangrenosa are often unable to develop delayed hypersensitivity to the virus and often die with this viral disease (37). These data clearly indicate that while interferon may be a very important factor in recovery from viral infection, the role of other cellular phenomena, such as delayed hypersensitivity, must be elucidated.

Cellular Immunity

Studies of certain deficiencies of the immune system suggest that the ability to manifest delayed hypersensitive reactions is necessary for recovery from viral infections. Males with congenital, sex-linked agammaglobulinemia have few plasma cells and do not produce detectable levels of antibody. Such persons can develop delayed hypersensitivity and handle many viral infections in a normal fashion (15, 88). A second type of immune-deficiency disease, Swiss-type agammaglobulinemia, is characterized by the absence of antibodies, plasma cells, and lymphocytes. These persons are extremely susceptible to viral and bacterial diseases. Keidan et al. (53) and later, Somers (90), reported cases of Swiss-type agammaglobulinemia in which immunization with vaccinia virus resulted in generalized vaccinia and death. Persons with Swiss-type agammaglobulinemia have also died following inoculation with Mycobacterium tuberculosis, strain BCG (61).

In the Wiskott-Aldrich syndrome, characterized by defects in cellular immunity in varying degrees but exhibiting normal levels of immunoglobulins (31, 96), fatalities due to normally benign viral infections have been reported. Fatalities due to HSV infection have occurred in persons with the Wiskott-Aldrich syndrome, and in these cases, neutralizing antibodies to HSV were either absent or at extremely low levels (60, 32).

Fulginiti et al. (24) described three children with inability to develop skin test reactions to mumps virus or monilial antigens. One child died of generalized vaccinia, and in another, varicella lesions persisted for eight months. All three children died of infections

before 16 months of age, even though some of them had almost normal
levels of immunoglobulins.

Three patients who were unable to develop skin reactions to
monilial antigens and who subsequently died of morbilli were described
by Lipsey et al. (58). The patients' sera contained four times the
level of immunoglobulins seen in individuals with Swiss-type agamma-
globulinemia. Autopsy disclosed an atrophic thymus, but normal levels
of lymphocytes and plasma cells were observed. Immune globulin was
administered in all three cases without success. Nahmias et al. (69)
described a four month-old patient with normal levels of immunoglobulins
who expired with measles virus pneumonia. At autopsy, no thymic tissue
could be detected.

Surgical removal of the thymus or treatment with anti-lymphocyte
serum has also indicated the importance of cellular immunity in recovery
from viral disease. In mice with persisting lymphocytic choriomenin-
gitis virus (LCM) infection, administration of anti-lymphocyte serum
produced a viremia which disappeared when the anti-lymphocyte serum was
discontinued (104). The titer of CF antibody to LCM was unaffected
during this treatment.

In HSV infections of mice, Nahmias et al. (70) demonstrated
that treatment with anti-thymocyte serum produced higher mortality rates
in mice infected intraperitoneally or intragenitally than in untreated
animals. Mice challenged intracerebrally and treated with the same
anti-thymocyte serum exhibited an increased survival rate. The role of
interferon was not mentioned in this study. Treatment with anti-
lymphocyte serum causes a decrease in the interferon levels in mice
subsequently challenged with an inducer of interferon (5).

After immunization with HSV, neonatally thymectomized mice became more susceptible to challenge with HSV than were normal mice (66). The possible effects of interferon upon the results were not ascertained in this study. The results were interpreted as strong evidence in support of the role of cellular factors in immunity to HSV.

Hirsch et al. (41) demonstrated that treatment with anti-thymocyte serum allowed establishment of persisting lymphocytic choriomeningitis virus infection in mice which ordinarily would have died of the challenge dose of virus. Furthermore, high titers of circulating antibody and virus were detected in the blood of these animals. Large reticular cells infiltrated kidney, lung, and liver of the mice. Viral antigens were detected in kidney tissue by the fluorescent antibody technique.

Treatment with anti-mononuclear cell serum apparently results in an increased susceptibility to viral diseases in experimental animals. Injection of young mice with anti-lymphocyte serum prior to inoculation with 10^4 plaque forming units of HSV produced 100% mortality with symptoms of encephalitis and meningitis but little hepatic involvement (108). The same dose of HSV in normal mice produced death in only 18% of the animals. Administration of anti-macrophage serum prior to injection of HSV resulted in 93% mortality with symptoms of severe hepatitis. Both the amount and duration of viremia increase in HSV infections following treatment with anti-mononuclear cell sera (70, 108). In addition to increasing the severity of infection with HSV, pox-viruses, and other viral agents; immunosuppresive drugs increase mortality to viral infections (29). Cortisone has been shown to increase

the severity of HSV infections. Since immunosuppresive drugs usually decrease both delayed hypersensitive   reactions and circulating antibody formation, the conclusions that can be drawn from reports of their effects are of limited value in assessing the importance of delayed hypersensitivity in viral disease.

Goals of the Research

Recent reports of a high correlation between infection with herpes simplex virus, type II, and cervical carcinoma (79, 81) indicate the need for further study of host cell-virus relationships in the more common viral infections.

Current interest in the significance of delayed hypersensitivity in viral disease has necessitated an examination of methods of induction of skin test reactivity and responses to antigenic challenge other than antibody formation. To initiate a study of the significance of delayed hypersensitivity in a viral disease, the role of delayed hypersensitivity must be separated from that of antibody and interferon. Achievement of this goal will require purification and characterization of an antigen which is capable of eliciting delayed hypersensitive reactions. Most importantly, separation of the skin reactive material from intrinsic interferon inducing materials, such as high molecular weight nucleic acids or endotoxins, is essential.

In the present investigation, the primary goal was the partial purification and chemical characterization of a soluble skin test antigen of herpes simplex virus. Secondarily, characterization of the soluble skin reactive antigen necessitated a comparison of various methods of sensitization of guinea pigs to herpes simplex virus.

CHAPTER II

MATERIALS AND METHODS

Solutions

Water used for preparation of all solutions and media and for final rinsing of all glassware was demineralized[1] and glass distilled. Reagent grade chemicals were used in the preparation of all solutions unless otherwise stated.

Hanks' balanced salt solution (HBSS), used for cell culture media, diluents, and tissue and cell culture rinses, was prepared according to the method of Hanks and Wallace (36) and sterilized by Millipore[2] membrane filtration. For this purpose, and in subsequent experiments in this study, a 0.45μ pore membrane filter was used for sterilization.

Phosphate buffered saline (PBS), which was calcium- and magnesium-free, was prepared and sterilized by autoclaving at 121 C, 15 lb pressure for 20 min according to the technique of Schmidt (86). PBS was used for the preparation of trypsin solutions and for washing of cell cultures.

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[1]Deeminite L-10, Crystal Research Laboratories, Hartford, Connecticut.

[2]Millipore Corporation, Bedford, Massachusettes.

Biologicals

Penicillin[3] and Streptomycin[4] were prepared in sterile water as stock solutions and stored in 1 ml amounts at -25 C until added to culture media or solutions. Final concentrations were 100 units of Penicillin and 100 µg of Streptomycin per milliliter of growth medium.

Horse serum[5] and newborn calf serum[6] were heat-inactivated at 56 C for 30 min and stored at -25 C. For use, they were thawed and added to cell culture media in appropriate concentrations and the complete media were sterilized by membrane filtration.

A 10% stock solution of trypsin[7] was prepared in PBS, centrifuged at 700 x g, sterilized by membrane filtration, and stored at -25 C. For dispersing cells in culture and preparing primary cell cultures, 0.1% or 0.2% trypsin solutions were prepared in PBS from the concentrated stock solution. In addition, a 1% solution of stock trypsin was prepared in 0.1 M phosphate buffer, pH 7.6, and used to treat soluble antigen preparations in the characterization procedure.

A 50% chick embryo extract (CEE) in HBSS was prepared from nine-day incubated Honegger white Leghorn eggs according to the method of Schmidt (86), and stored at -25 C. Prior to use, the preparation

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[3]Potassium Penicillin G, E.R. Squibb & Sons, New York, N. Y.

[4]Streptomycin sulfate, Chas. Pfizer & Co., Inc., New York, N. Y.

[5]Microbiological Associates, Bethesda, Md., Lot 7163214403.

[6]Newborn calf serum, Hyland, Div. of Travanol Laboratories, Inc. Los Angeles, Calif., Control 3102H007A1.

[7]Trypsin 1:250, Difco Co., Inc., Detroit, Mich.

was thawed and centrifuged 10 min at 700 x g for clarification. A 4%

concentration of CEE was added to the outgrowth medium for cultivation

of chick embryo fibroblasts.

Culture Media

Eagle's Minimum Essential Medium (MEM) used as growth medium

for cell cultures was prepared by dissolving a dehydrated commercial

preparation[8] in water (20). Unless otherwise specified, this medium

was supplemented with 9% horse serum (MEM_{91} H_9) or 9% calf serum

(MEM_{91} C_9) and sterilized by membrane filtration.

In order to use a colorimetric protein determination procedure,

a growth medium was needed which did not contain an indicator. Minimum

Essential Medium, 1X concentration which lacked phenol red, was pre-

pared by adding to HBSS appropriate amounts of the following concen-

trates[9]: 1) MEM vitamin solution, 100X; 2) solution of MEM amino acid

mixture, 50X; and 3) solution of MEM non-essential amino acid mixture,

100X. L-Glutamine[10] was added to a final concentration of 0.292 g per

liter and the medium was then filter sterilized. This medium is here-

after referred to as MEM-PR.

A second growth medium used to proliferate L cells for viral

titration and routine cell maintenance consisted of HBSS supplemented

with 0.5% lactalbumin hydrolysate[11], 0.1% yeast extract[12], 0.45%

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[8]Grand Island Biological Co., Grand Island, N.Y., Control 674551.

[9]Microbiological Associates, Bethesda, Md.

[10]Sigma Chemical Co., St. Louis, Mo.

[11]Nutritional Biochemicals Co., Cleveland, Ohio.

[12]Difco Laboratories, Detroit 1, Mich.

dextrose[13], and 8.0% inactivated newborn calf serum. This medium was sterilized by membrane filtration. It was designated as LHYE C.

Growth medium for chick embryo fibroblasts (CEF) was a modified LHYE and was designated as LHYE for CEF. It was prepared by supplementing phenol-red-free HBSS with 0.25% lactalbumin hydrolysate, 2% chick embryo extract, 0.07% $NaHCO_3$, and 2% inactivated, newborn calf serum.

Cell Lines and Culture Techniques

Primary Cell Culture Preparation

CEF cultures were prepared according to the method of Schmidt (86). Eleven-day-old Honegger white Leghorn embryos were harvested aseptically; eyes, wings, legs and beaks were removed and discarded. After they were rinsed three times in HBSS, the torsos were finely minced and placed in a 250 ml trypsinizing flask. The minced tissue was washed once in HBSS, allowed to sediment, and the supernatant fluid was decanted. Two hundred milliliters of 0.25% trypsin were added per 10 embryos and the mixture was stirred with a magnetic stirrer for 1 hr at room temperature. After trypsinization, the supernatant fluid was decanted through six layers of sterile gauze into a 250 ml centrifuge bottle and centrifuged at 80 x g for 10 min in a graduated 50 ml centrifuge tube. The supernatant fluid was decanted and LHYE for CEF was added to bring the cell concentration to 0.5%. This cell suspension was dispensed in 10 ml volumes per 150 ml bottle and the bottles were

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[13]Fisher Scientific Co., Fair Lawn, New Jersey, Lot 774383.

incubated at 36 C for 48 hr. LHYE for CEF was then removed and 10 ml

MEM-PR was added. Cultures were incubated at 36 C until used.

Continuous Cell Culture Propagation

To provide a cell culture which would be less likely to cross

react with chick embryo antigens, a strain of mouse fibroblasts, L-929

(85), was used to proliferate pools of HSV. This cell line was obtained

from Microbiological Associates, Bethesda, Md., and was maintained in

this laboratory either by routine passage at weekly intervals or by

storage at -70 C in 5% glycerol in MEM_{80} C_{15}, according to the procedure

of Schmidt (86). The cell cultures were treated periodically with

Kanamycin sulfate[14] (200 µg/ml) for a 7-day period to control possible

contamination with pleuropneumonia-like organisms.

Viruses

Herpes Simplex Virus

The HF strain of herpes simplex virus (HSV), which was originally

obtained from the National Communicable Disease Center, Atlanta, Ga.,

was used in these studies. The virus had been propagated previously in

this laboratory through several passages in HeLa cells and in embry-

onated eggs. To prevent carry-over of specific antigens of HeLa or

chick embryo cells in which the virus had been propagated, virus was

passed twice in L cells before preparation of seed inoculum. Certain

of these viral preparations were used to sensitize experimental animals.

Similarly, virus was passed three times in CEF before preparation of

soluble antigen.

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[14]Bristol Laboratories, Syracuse, N. Y., Control 61L 1135.

Vaccinia Virus

Vaccinia virus was obtained originally from the American Type Culture Collection (#VR325). It had been maintained in this laboratory by passage on the chorioallantoic membrane of embryonated eggs and, just prior to use, was passed once in CEF monolayers grown in LHYE for CEF. Forty-eight hours after inoculation, the infected monolayers were subjected to three freeze-thaw cycles and the cell lysates were centrifuged at 700 x g for 30 min. The supernatant fluid was the seed inoculum for growth of vaccinia viral pools which were used for the preparation of soluble antigen.

Virus Titration

Fifty Per Cent Infectivity

Mouse fibroblast monolayers, maintained in LHYE C, in roller tubes (16 x 150 mm) were infected with 0.1 ml of a HSV suspension. Quadruplicate monolayers were used for each $\log_{10}$ dilution of virus. After a 1 hr adsorption period at 36 C, 1 ml of growth medium was added and the tubes were incubated on a roller drum at 36 C for 6 days before final infectivity readings were recorded and viral titers were determined. Titers were calculated by the method of Reed and Meunch (82) and were expressed as the reciprocal $\log_{10}$ of the highest dilution of HSV giving 50% infection of the monolayers ($TCID_{50}$).

Plaque Forming Unit

Assay of HSV by the plaque forming unit (PFU) method was performed according to the technique of Garabedian and Scott (25). Monolayers of L cells maintained in MEM_{91} C_9 were inoculated with $\log_{10}$

dilutions of virus and overlaid with MEM_{91} C_9 plus 1.5% methyl cellulose. Four flasks were used for each log_{10} dilution. For the viral dilution giving 30-300 plaques per monolayer, the average number of plaques was calculated and expressed as PFU per ml of undiluted inoculum.

Animals

Young adult, male, albino guinea pigs, of the Hartley strain, weighing 400-550 g each, were obtained from Camm Research Institute and maintained on a diet of water, laboratory food blocks, and leafy green vegetables. After a short period of acclimatization, the animals were used in the skin test experiments to be described.

Embryonated Honegger white Leghorn eggs which had been incubated at 39 C for 9 to 11 days were obtained from a local hatchery and were used for preparing chick embryo fibroblast cell cultures and for proliferation of HSV in preparation of soluble antigen (SA).

Skin Testing Procedures

Sensitization to Viral Antigen

Guinea pigs were sensitized by the intracutaneous injection of 0.1 ml of antigen (either active virus, inactive virus, or SA) into a single site on the dorsal thorax. Virus was inactivated by heating the HSV preparation ($10^{6.5}$ $TCID_{50}$/ml prior to inactivation) at 56 C for 1 hr. After inactivation, no infectivity could be demonstrated by inoculation of the preparation into L cell monolayer cultures. In certain experiments, a second injection of antigen was administered 14 days after the primary injection to determine its effectiveness in

sensitizing animals to HSV SA. When Freund's complete adjuvant[15] was employed, the antigen was mixed with adjuvant in a 1:1 ratio and injected in 0.1 ml volumes in two separate sites on the dorsal thorax.

Detection of Sensitivity to Viral Antigens

Fourteen days after sensitizations, animals were skin tested by injection of 0.1 ml of antigen intracutaneously into the shaved dorsum. Control and test antigen injections were placed in transverse sites on opposing sides of the back. Skin test diameters were recorded in millimeters as the average of two readings taken at right angles to one another. In pilot studies which will not be described in detail, typical erythema was evident as early as 4 to 8 hr and progressed to maximal intensity by 16 hr. When induration was present, the time of maximal intensity closely paralleled that of the erythematous response. Based upon these findings, observation times of 0.5, 4, 16, 24, 48, and 72 hr were selected. In studies where induration was not marked, only the diameters of erythema were recorded. Color photographs were made of skin test sites 0.5 hr and 24 hr after injection of antigens.

Microscopic sections of biopsied skin test sites were fixed in buffered formalin, stained with hematoxylin and eosin, and examined for cellular infiltration. The results of microscopic examination were recorded in color photomicrographs. Photomicrographs of hematoxylin and eosin stained sections of skin test sites were made with a Leitz

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[15]Difco Laboratories, Detroit 1, Mich.

microscope fitted with a 35 mm Leica camera and a Micro-Ibso attach-
ment.[16] Kodachrome II professional film (Type A)[17] was used for the
photomicrography.

Photographs of the gross skin test sites were taken with a
35 mm Yashica camera[18] utilizing Kodachrome II daylight type film.

Statistical Analysis of Skin Test Data

In experiments involving three or more skin test sites per
animal, comparisons of erythematous and/or indurative responses for
each time interval were made by Duncan's new multiple range test (57).
Data from certain experiments did not fulfill all criteria for
application of this statistical test since a large number of zero re-
actions prohibits use of normal theory analysis. In comparisons of
only two test sites and when the data qualified, Student's "t" test
was applied and significance at the 1% or 5% levels was recorded in
each instance (57).

Herpes Simplex Virus Soluble Antigen Preparation

Treatment of Lysates

Sonication of virus-infected cells. To disrupt cell membranes,
thus releasing intracellular products, and to determine the effect on
HSV SA, pools of infected cells were sonicated for 15 min at 10 kc in

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[16]E. Leitz, Inc., New York, N. Y.

[17]Eastman Kodak Co., Rochester, N. Y.

[18]Yashica Co., LTD., Tokyo, Japan.

a sonic oscillator.[19] Uninfected control cells were treated in a similar fashion. After sonication, the materials were centrifuged at 600 x g for 10 min. The sediment was discarded and the supernatant fluid was stored at -70 C.

Ultracentrifugation procedures. Both HSV-infected and control preparations were centrifuged[20] at 66,000 x g for 0.5 hr at 10 C. The supernatant fluids were removed by decantation and stored at -70 C. The pellet from HSV-infected monolayers was resuspended in fresh growth medium and stored at -70 C until used as viral antigen.

Protein determination. The protein content of antigens prepared in media containing phenol red was determined by the method of Lowry et al. (59). When antigens were prepared in phenol-red-free media, their protein content was determined spectrophotometrically by the method of Kalckar (51). Protein levels determined by both methods were expressed as mg/ml of antigen preparation.

Lyophilization procedure. Materials to be stored or concentrated were frozen in an ethanol-dry ice bath prior to lyophilization on a Virtis lyophilizer.[21] Following lyophilization, antigen preparations were stored at 5 C until used. Antigens were reconstituted with either distilled water or PBS as required.

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[19]Raytheon Manufacturing Co., Waltham, Mass.

[20]Type 30 rotor, Model L2-65B centrifuge, Beckman Instruments, Inc., Spinco Div., Palo Alto, California.

[21]Virtis Research Equipment, Gardiner, N. Y.

L Cell-Prepared Herpes Simplex Virus Soluble Antigen

Complete monolayers of L cells were prepared in 250 ml bottles employing $MEM_{91} H_9$ which contained antibiotics. Monolayers were washed once with HBSS to remove contaminating sera or other proteins and were infected with membrane-filtered lysate from HSV-infected cells which had a titer of 1 x 10^5 $TCID_{50}$/ml. To rid the seed inoculum of contaminating proteins, the virus was propagated through three serial passages in L cells proliferated in $MEM_{91} H_9$ prior to the making of the viral pool. The culture medium for L cells was supplemented with horse serum in an attempt to obviate cross reactions in skin tests due to common serum or cell components in both sensitizing and skin test antigens. One milliliter of viral inoculum was placed on each culture and allowed to adsorb for 1 hr at 36 C before addition of 10 ml of $MEM_{91} H_9$. These infected monolayers were incubated for 72 hr at 36 C and were then subjected to five freeze-thaw cycles using temperatures of 37 C and -70 C to release intracellular virus. After lysis, the pH of the lysate was adjusted to 7.0 by addition of IN NaOH and the preparation was centrifuged at 700 x g at 4 C for 30 min to remove cellular debris. The resulting supernatant liquid was centrifuged further at 66,000 x g for 30 min. The soluble antigen (SA) fraction remained in the liquid portion and was concentrated five-fold by lyophilization. The lyophilized SA was resuspended in 80 ml of distilled water and dialyzed against PBS at pH 7.0 for 24 hr at 4 C. This partially purified and concentrated SA was stored at -70 until used. The sediment from the ultracentrifugation procedure was resuspended in 1/8 the original volume of $MEM_{91} H_9$,

designated viral antigen (VA), and stored at -70 C in 1 ml amounts for

later use.

Chick Embryo Fibroblast-Prepared Herpes Simplex
Virus Soluble Antigen
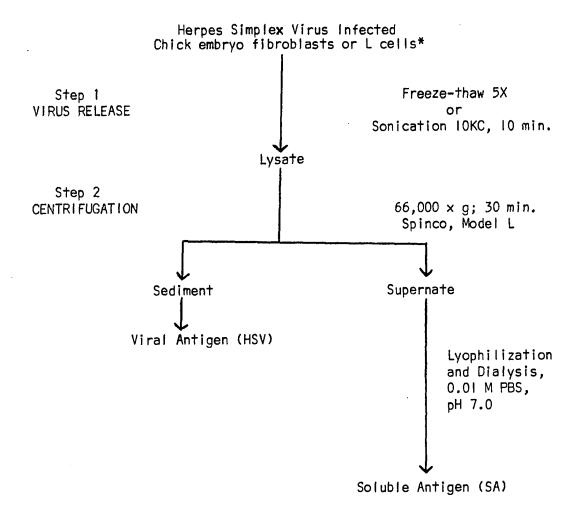
Complete monolayer cultures of chick embryo fibroblasts were

proliferated in LHYE for CEF in 250 ml bottles (86). The growth medium

employed contained 2% calf serum[22], but it lacked phenol red. The CEF

monolayers were maintained 48 hr on MEM-PR before inoculation with 0.5

ml of CEF-proliferated HSV which had a titer of $10^{5.5}$ $TCID_{50}$/ml. Ad-

sorption for 1 hr at 35 C was allowed before 10 ml of MEM-PR was added

to each monolayer. An equal number of uninfected monolayers were kept

as controls for later studies. The antigen treatments are described

in flow diagram form in Figure I. Uninfected monolayers were subjected

to a similar treatment to obtain control antigen.

Chick Embryo-Prepared Herpes Simplex Virus Soluble Antigen

Herpes simplex virus SA was prepared in chick embryos (CE) ac-

cording to the method of Jawetz et al. (47). Embryonated eggs, incu-

bated 10 days, were infected with HSV via the chorioallantoic membrane

and incubated four days before the allantoic fluid was harvested.

Pools of HSV-infected CE allantoic fluid and control CE allantoic fluid

were centrifuged at 17,000 x g for 1 hr at 5 C before supernatant

fluids were collected and stored at -70 C. The sediment of HSV-infected

CE was labelled HSV CE and retained at -70 C for later use. Prior to

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[22]Newborn calf serum, Hyland, Div. of Travanol Laboratories, Inc. Los Angeses, Calif., Control 3102H007A1.

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Herpes Simplex Virus Infected
Chick embryo fibroblasts or L cells*

Step 1
VIRUS RELEASE

Freeze-thaw 5X
or
Sonication 10KC, 10 min.

Lysate

Step 2
CENTRIFUGATION

66,000 x g; 30 min.
Spinco, Model L

Sediment

Supernate

Viral Antigen (HSV)

Lyophilization
and Dialysis,
0.01 M PBS,
pH 7.0

Soluble Antigen (SA)

*Uninfected cells received identical treatment to obtain control
antigens.

Figure 1. Summary of preparation methods for antigens
from either viral-infected or uninfected cells.

use as skin test antigens, allantoic fluid antigen pools were heated at 56 C for 1 hr.

Vaccinia Soluble Antigen Preparation

Chick embryo fibroblast monolayers in 250 ml bottles were infected with 1.0 ml volume containing $10^{5.5}$ TClD$_{50}$ of CEF proliferated vaccinia virus. A two-hour adsorption period at 36 C was allowed before addition of 9 ml of MEM-PR per culture bottle. Monolayers were incubated at 36 C for 48 hr and the virus was then released by three freeze-thaw cycles. The resulting lysate was centrifuged at 700 x g to remove cellular debris, and the supernatant fluid resulting from centrifugation at 66,000 x g for 30 min at 10 C was designated the vaccinia soluble antigen (VSA).

Treatment of Herpes Simplex Virus Soluble Antigen

In order to characterize further the SA, it was subjected to enzyme action, ether extraction, dialysis, and ultrafiltration procedures before being used as skin test antigen.

Trypsin

A 1% solution of trypsin[23] was prepared in 0.1 M sodium phosphate buffer, pH 7.6 as described earlier. The proteolytic activity of trypsin was determined by measuring the increase in ultraviolet light absorbance at a wavelength of 280 mμ after treatment of 1% casein with unheated and heated (100 C, 10 min) trypsin (54). A unit of trypsin is defined as that amount of activity which produces an increase, under

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[23]Trypsin 1:250, Difco Co., Inc., Detroit, Mich.

the conditions described, of one unit/min in the absorbance at 280 mμ. The unheated trypsin preparation used in this study had an activity of 0.012 units/ml/min and a specific activity of 0.001 units/mg. Heated trypsin showed no proteolytic activity under these conditions. One milliliter amounts of CE proliferated soluble antigen were incubated for 2 hr at 37 C with equal volumes of heated and unheated trypsin. Control allantoic fluid was incubated with unheated trypsin under the above conditions. Following trypsin treatment, control and soluble antigen preparations were stored at -70 C until used as skin test antigens. Before testing, the antigens were heated at 70 C for 2 hr to reduce trypsin activity. Following treatment at 70 C for 2 hr, the activity of the trypsin preparation was too low to measure by this technique.

Pronase

A 0.5% solution of pronase[24] was prepared in 0.1 M sodium phosphate buffer, pH 7.4. The proteolytic activity of unheated and heated (100 C, 10 min) pronase was determined by the same method as used for trypsin, except that the assay was done at pH 7.4. The activity of unheated pronase was 0.0046 units/ml/min. Heated pronase showed no activity under these conditions. The specific activity of the unheated pronase was 2.4×10^{-3} units/mg. The remainder of the procedure was the same as that used in the trypsin treatment.

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[24]Pronase B grade, Calbiochem, 45,000 PUK units/gram, Lot 801929.

Deoxyribonuclease

A stock solution of deoxyribonuclease (DNase)[25] was prepared

in 0.2 M sodium phosphate buffer, pH 7.0, at a concentration of 12.5

mg/ml. DNase activity was measured by determining the increase in

absorbance at a wavelength of 260 mµ after treatment of deoxyribo-

nucleic acid with unheated and heated (70 C, 2 hr) stock DNase (63).

The activity of DNase is defined as that amount of activity which

gives an increase in absorbance at 260 mµ of one unit/min. The ac-

tivities of the heated and unheated DNase preparations were 0.018

units/ml/min and 22.5 units/ml/min, respectively. Specific activities

were 1.5×10^{-3} units/mg for heated DNase and 1.8 units/mg for un-

heated DNase. One milliliter of the stock DNase solution was added to

4 ml of SA and L cell control preparations, respectively, and both were

incubated at 37 C for 16 hr. In addition, a 4 ml aliquot of SA was

incubated with 1 ml of inactive DNase (heated 70 C, 2 hr) under the

same experimental conditions as above. Both SA and L cell control

preparations contained 290 µg of protein per ml. Following the incu-

bation period, the preparations were heated at 70 C for 2 hr to in-

activate remaining enzyme before lyophilization to concentrate

the antigen preparations for skin testing. Antigen preparations were

adjusted to 1.5 mg of SA or control protein/ml prior to animal chal-

lenge.

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[25]Deoxyribonuclease, beef pancreas, Grade 1, Miles Labora-
tories, Inc., Elkhart, Ind., Control #1307C.

Ribonuclease

A 1% stock solution of Ribonuclease (RNase)[26] was prepared in

0.1 M sodium acetate buffer, pH 5.0. Ribonuclease activity was assayed

by determination of the increase in absorbance at 260 mμ after treat-

ment of ribonucleic acid with unheated and heated (70 C, 2 hr) RNase

(62). A unit of RNase is that amount of activity which causes an in-

crease in absorbance of one unit/min under standard conditions.

Unheated RNase had an activity of 0.0048 units/ml/min. The specific

activity of the preparations was 0.48 units/mg for unheated RNase and

2.4×10^{-3} units/mg for heated RNase. With the exceptions of enzyme

and buffers used, the remainder of the RNase treatment was the same as

the above for DNase.

Following incubation and concentration as above, the protein

contents of the treated preparations were adjusted to 2.5 mg/ml for

use in skin testing of sensitized animals.

Ether Extraction of Soluble Antigen

Fifty milliliter portions of HSV SA or CEF control preparations

were each extracted three times with 50 ml volumes of ethyl ether.

Following ether extraction, the preparations were heated at 37 C under

flowing nitrogen to remove residual ether before being subjected to

ultrafiltration and used as skin test antigens.

Dialysis of Soluble Antigen

CEF SA and control CEF preparations were dialyzed against dis-

tilled water for 48 hr at 5 C. When precipitates were noted in the

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[26]Ribonuclease, RNase-A, Protease free, Type XII-A, Bovine Pan-
crease, Sigma Chemical Co., St. Louis, Mo., Lot 27B-8550.

dialysis bags, the contents were centrifuged at 5 C for 15 min at
700 x g. The supernatant fluids were saved and used as skin test anti-
gens following adjustment of protein concentrations to 150 µg/ml.
Sediments were resuspended in PBS, pH 7.0, and the protein concentra-
tion was adjusted to 150 µg/ml. This material was used as a skin test
antigen.

When antigen preparations were dialyzed against PBS, treatment
was carried out at 5 C for 48 hr.

Ultrafiltration of Soluble Antigen

Size estimates of the SA fraction responsible for eliciting
skin test reactions were made by filtration of CEF SA through XM-100
and XM-50 ultrafiltration membranes.[27] Following filtration through
the membrane, HSV SA preparations were concentrated by lyophilization
and the protein contents were adjusted to 1.5 mg/ml by spectrophoto-
metric methods before use. These antigens were used in testing of
both HSV sensitized and L cell sensitized guinea pigs.

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[27]Amicon Corporation, Lexington, Mass.

CHAPTER III

RESULTS

Antigen Preparations

For the purpose of clarity in presentation, a catalogue of
antigens used in this study, including information on the nature of
viral, soluble and control antigens and cell cultures, listing cells,
media, and sera used in the production of antigen pools is presented
in Table 1. Herpes simplex virus (HSV) antigens were also prepared
from infected chick embryo allantoic fluid. In order to prevent un-
desirable cross reactions several viral preparations from L and chick
embryo fibroblast cell cultures were prepared. Likewise, the sera
used to supplement growth media were varied to obviate cross reactivity
due to use of the same serum in both sensitizing and challenge anti-
gen preparations. The HSV sensitizing antigen prepared in L cells and
the vaccinia virus sensitizing antigen prepared in CEF had infectivity
titers of $10^{6.5}$ and $5 \times 10^{5.3}$ $TCID_{50}/ml$, respectively. Since the
amount of protein in these antigens varied from 1.5 to 11 mg/ml, the
protein concentrations were adjusted to approximately 150 µg/0.1 ml
before use.

TABLE 1

ANTIGENS USED IN SKIN TEST STUDIES

Antigen		Source			$TCID_{50}{}^a$	Protein (mg/ml)	
Nature	Use	Cell	Medium	Serum		Lowry et al. (59)	Kalckar (51)
HSV	S^b	L-929	MEM_{91} H_9	Horse	$10^{6.5}$	-	-
HSV SA	S	L-929	MEM_{91} H_9	Horse	-	11.0	-
HSV SA	C^c	CE	Embryo	None	-	-	3.98
Control	S/C	L-929	MEM_{91} H_9	Horse	-	10.0	-
Control	C	CE	Embryo	None	-	-	5.57
VV	S	CEF	LHYE	Calf	$5 \times 10^{5.3}$	-	-
VSA	C	CEF	LHYE	Calf	-	-	2.0
HSV SA	C	CEF	LHYE	Calf	-	-	1.5
CEF Control	C	CEF	LHYE	Calf	-	-	1.5

[a]All abbreviations explained in Chapter II.

[b]Antigen used to sensitize animals.

[c]Antigen used to challenge animals.

Sensitization of Guinea Pigs to Herpes Simplex Virus Antigens

Infective Herpes Simplex Virus

One injection. The skin test reactions in five guinea pigs which were sensitized by the intradermal injection of 0.1 ml of HSV ($10^{5.5}$ TCID$_{50}$) and challenged 14 days later with CE prepared SA, which had been dialyzed against PBS, are presented in Table 2. Neither erythema nor induration were seen in the skin reactions to SA at 2 hr (Figure 2). Slight erythema was noted by 4 hr, but discernable induration was not present (Table 2). Maximal erythema occurred at 16 hr, but induration noted at this time was of borderline intensity and, therefore, not readily measurable. Thus, only average diameters of erythema are reported. Both erythematous and indurative reactions (Figure 3) were somewhat smaller at 24 hr than at 16 hr, had diminished greatly by 48 hr, and had disappeared by 72 hr. Erythema and induration in the CE control antigen site were negligible. The response to CE control antigen at 2 hr was zero (Figure 2). A slight reaction became evident by 4 hr and was maximal at 16 hr. The control reaction disappeared completely by 24 hr and never attained the intensity of erythema noted at the SA site in these animals. Induration was never observed at the CE control antigen sites.

Two injections. Six animals which had received two sensitizing injections of infectious HSV ($10^{5.5}$ TCID$_{50}$/0.1 ml) on days 0 and 14 were challenged on day 28 with SA and CE control antigens. The largest responses at the SA challenge sites in these animals occurred at 16 hr (Table 3). Erythema and induration were of comparable size at 16 and 24 hr and were still evident at 48 hr. No reactions were seen at 72 hr.

TABLE 2

SKIN TEST REACTIONS IN GUINEA PIGS SENSITIZED[a] WITH ONE INJECTION
OF HSV AND CHALLENGED WITH SOLUBLE ANTIGEN

Time (Hours)	Antigen	
	SA[b]	Control[c]
0.5	0[d]	0[d]
4	3.6	0.9
16	12.1	1.1
24	7.5	0
48	3.3	0
72	0	0

[a]Animals sensitized by single injection HSV, $10^{5.5}$ $TCID_{50}$; challenged 14 days later.

[b]Soluble antigen prepared from HSV infected CE allantoic fluid.

[c]Control antigen prepared from uninfected CE allantoic fluid.

[d]Average diameter of erythema in mm; 5 animals.

Figure 2. Skin reactions of an infective HSV sensitized guinea pig 2 hr after challenge with CE control antigen (left side) and HSV SA (right side), 1/3 normal size.
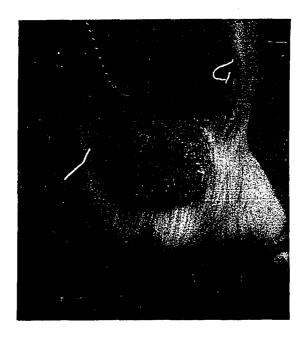
Figure 3. Skin reactions of an infective HSV sensitized guinea pig 24 hr after challenge with CE control antigen (left side) and HSV SA (right side), 1/3 normal size.

TABLE 3

SKIN TEST REACTIONS IN GUINEA PIGS SENSITIZED[a] WITH TWO INJECTIONS
OF HSV AND CHALLENGED WITH SOLUBLE ANTIGEN

Time (Hours)	Erythema		Induration	
	SA[b]	Control[c]	SA[b]	Control[c]
0.5	0[d]	0[d]	0[d]	0[d]
4	11.3	7.6	5.7	3.7
16	15.4	2.8	11.2	0
24	14.5	2.5	8.4	0
48	10.4	0.5	5.0	0
72	0	0	0	0

[a]Animals sensitized by two injections HSV, $10^{5.5}$ $TCID_{50}$ on days 0 and 14; challenged on day 28.

[b]Soluble antigen prepared from HSV infected CE allantoic fluid.

[c]Control antigen prepared from uninfected CE allantoic fluid.

[d]Average diameter in mm; 6 animals.

While central blanching of the lesions occasionally occurred, necrosis was not observed with the concentration of antigen employed. The reactions to CE control antigen in these animals were transient. Erythema was maximal at 4 hr and decreased to less than one-half its former level by 16 hr. Induration was detectable only at 4 hr and disappeared by 16 hr. The area of erythema was smaller and the intensity of induration was less at the CE control sites than at SA sites. The diameters of both erythema and induration were larger at the SA sites than at the CE control antigen sites at 4, 16, 24, and 48 hr after challenge. Reactions to both antigens were zero at 0.5 and 72 hr.

Heated Herpes Simplex Virus

Guinea pigs inoculated with heated HSV preparations ($10^{5.5}$ $TCID_{50}$ prior to inactivation), which contained no detectable infective virus, received 0.1 ml of material on days 0 and 14 and were challenged with SA and CE control antigens on day 28. Diameters of erythema at SA sites were larger than those at CE control antigen sites at 24 and 48 hr (Table 4). SA skin test sites developed larger diameters of induration than CE control antigen challenge sites at 16, 24, and 48 hr. These results demonstrate the ability of non-infective HSV to sensitize animals to a skin test antigen of HSV.

Herpes Simplex Virus Soluble Antigen

A single injection of HSV SA prepared in L cells (1.1 mg of protein/0.1 ml) sensitized guinea pigs to HSV SA (400 μg/0.1 ml) prepared from infectious allantoic fluid (Table 5). Animals so sensitized showed maximal erythematous responses to both CE control and soluble

TABLE 4

SKIN TEST REACTIONS IN GUINEA PIGS SENSITIZED[a] WITH
HEATED HSV AND CHALLENGED WITH SOLUBLE ANTIGEN

Time (Hours)	Erythema		Induration	
	SA[b]	Control[c]	SA[b]	Control[c]
0.5	0[d]	0[d]	0[d]	0[d]
4	9.9	7.9	5.2	4.3
16	8.4	4.3	4.6	1.6
24	9.8	0	5.5	1.0
48	7.5	0	2.5	0
72	0	0	0	0

[a]Animals sensitized by two injections heated HSV (prior to inactivation, $10^{5.5}$ TCID$_{50}$) on days 0 and 14; challenged on day 28.

[b]Soluble antigen prepared from HSV infected CE allantoic fluid.

[c]Control antigen prepared from uninfected CE allantoic fluid.

[d]Average diameter in mm; 6 animals.

TABLE 5

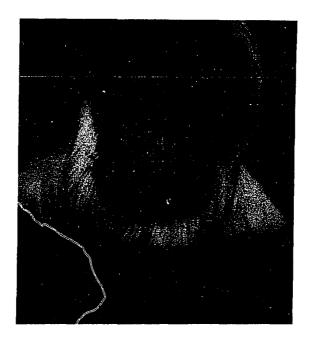SKIN TEST REACTIONS IN SOLUBLE ANTIGEN SENSITIZED
GUINEA PIGS[a] CHALLENGED WITH SOLUBLE ANTIGEN

Time (Hours)	Antigen	
	SA[b]	Control[c]
0.5	0[d]	0[d]
4	1.4	0.6
16	7.9	1.4
24	4.2	0.5
48	2.2	1.1
72	0	0

[a]Animals sensitized by single injection of HSV SA, 1.1 mg; challenged 14 days later.

[b]Soluble antigen prepared from HSV infected CE allantoic fluid.

[c]Control antigen prepared from uninfected CE allantoic fluid.

[d]Average diameter of erythema in mm; 21 animals.

antigens at 16 hr. Diameters of erythema at the SA sites were larger
than at CE control antigen sites at 16, 24, and 48 hr after challenge.
No erythema was seen 0.5 hr after skin testing, but by 2 hr, a faint
redness was observed occasionally (Figure 4). A comparison of the
response to SA at 24 hr in SA sensitized animals (Figure 5) with that
in HSV sensitized animals (Figure 3) reveals a more intense reaction
in the latter animals.

Comparison of Sensitization Methods

One injection of soluble antigen and infectious herpes simplex
virus. Since in the studies described above, the results of which are
presented in Tables 2 and 5, the animals had been skin tested with the
same SA preparation, it was of interest to compare the resulting re-
actions. The results of this comparison can be seen in Table 6.
Average diameters of erythema were larger in HSV infected animals from
4 through 48 hr of examination. No reactions were detected at 0.5 and
72 hr. The reactions in both groups were maximal at 16 hr and dimin-
ished slightly by 24 hr. The only time at which HSV sensitized animals
responded with significantly greater skin reactions than SA sensitized
animals was at 16 hr. No induration was noted in any of the animals.
From these results it may be concluded that infective HSV is a better
sensitizing agent than SA. These data and earlier results with ani-
mals immunized with heated HSV (Table 4) demonstrate that infective HSV
is not required to produce delayed hypersensitivity to SA.

Two injections of active herpes simplex virus and heated herpes
simplex virus. When guinea pigs were sensitized with two injections
of either infectious (Table 3) or heated (Table 4) HSV and skin tested

Figure 4. Skin reactions of an HSV SA sensitized guinea
pig 2 hr after challenge with CE control antigen (left side) and
HSV SA (right side), 1/3 normal size.
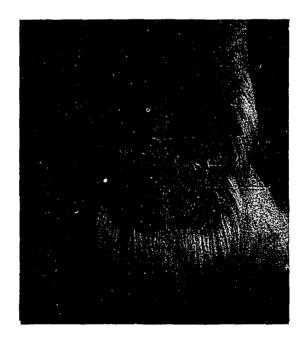
Figure 5. Skin reactions of an HSV SA sensitized guinea
pig 24 hr after challenge with CE control antigen (left side) and
HSV SA (right side), 1/3 normal size.

TABLE 6

AVERAGE DIAMETERS OF ERYTHEMA IN GUINEA PIGS SENSITIZED WITH HSV
SOLUBLE ANTIGEN[a] OR ACTIVE HSV[b] AND CHALLENGED
WITH HSV SOLUBLE ANTIGEN

Time (Hours)	Antigen		
	SA[c]	HSV	Significance
0.5	0[d]	0[e]	
4	1.4	3.6	
16	7.9	12.1	+[f]
24	4.2	7.5	
48	2.2	3.3	
72	0	0	

[a]Animals sensitized by single injection of HSV SA, 1.1 mg protein, challenged 14 days later.

[b]Animals sensitized by single injection HSV, $10^{5.5}$ $TCID_{50}$; challenged 14 days later.

[c]Soluble antigen prepared from HSV infected CE allantoic fluid.

[d]Average diameter of erythema in mm; 21 animals.

[e]Average diameter of erythema in mm; 5 animals.

[f]Statistically significant ($p < 0.05$) difference, Student's "t" test.

later with SA, larger reactions were produced with SA than with CE control antigen. Both sensitizing antigens were prepared in L cell fibroblasts proliferated in MEM_{91} H_9. The challenge preparation was prepared from HSV-infected CE allantoic fluid. Data from the two studies have been combined in Table 7. Except for zero readings at 0.5 and 72 hr, larger diameters of erythema and induration were observed in guinea pigs sensitized with infective virus than in animals injected with the same material which had been heated for 2 hr at 56 C. At 16 hr, diameters of both erythema and induration in HSV-infected animals were significantly larger than those in animals immunized with heated HSV. At 24 hr the diameters of erythema were significantly different while the diameters of induration were not. These results indicate that infective HSV is a better sensitizing antigen than heated HSV.

Comparison of various methods of sensitization. In the previous studies, infective HSV (Table 3), heat-inactivated HSV (Table 4), and the SA (Table 5) were found to be capable of specifically sensitizing animals to HSV. The average diameters of erythema and induration produced following challenge with SA in animals sensitized by infective HSV, heated HSV and HSV SA are shown in Table 8. Average diameters of induration and erythema were at maximum by 16 hr. The average diameters of erythema and induration can be ranked according to the method of sensitization. Reactions were largest in animals sensitized with two injections of infective HSV. The second largest reactions were observed in animals sensitized with two injections of inactivated-HSV. Smallest reactions to SA were noted in animals sensitized with a single injection of SA. The SA sensitized animals

TABLE 7

SKIN TEST REACTIONS IN ANIMALS SENSITIZED WITH ACTIVE[a] OR HEATED[b]
HSV AND CHALLENGED WITH SOLUBLE ANTIGEN[c]

Time (Hours)	Erythema			Induration		
	Active HSV	Heated HSV	Significance	Active HSV	Heated HSV	Significance
0.5	0[d]	0[d]		0[d]	0[d]	
4	11.3	9.9		5.7	5.2	
16	15.4	8.4	+[e]	11.2	4.6	+
24	14.5	9.8	+	8.4	5.5	
48	10.4	7.5		5.0	2.5	
72	0	0		0	0	

[a]Animals sensitized by two injections HSV, $10^{5.5}$ TCID$_{50}$, on days 0 and 14; challenged on day 28.

[b]Animals sensitized by two injections heated HSV on days 0 and 14; challenged on day 28.

[c]Soluble antigen prepared from HSV infected CE allantoic fluid.

[d]Average diameter in mm; 6 animals.

[e]Statistically significant (p < 0.01) difference, Student's "t" test.

TABLE 8

SKIN TEST REACTIONS TO SOLUBLE ANTIGEN IN GUINEA PIGS
SENSITIZED BY THREE METHODS

Time (Hours)	Erythema			Induration		
	Active HSV[a]	Heated HSV[b]	HSV SA[c]	Active HSV[a]	Heated HSV[b]	HSV SA[c]
4	11.3[d]	9.9[d]	1.4[d]	5.7[d]	5.2[d]	0[d]
16	15.4	8.4	7.9	11.2	4.6	0
24	14.5	9.8	4.2	8.4	5.5	0
48	10.4	7.5	2.2	5.0	2.5	0

[a]Six animals in group, sensitized by two injections HSV, $10^{5.5}$ TCID$_{50}$, challenged with CE HSV SA.

[b]Six animals in group, sensitized by two injections of heated HSV, challenged with CE HSV SA.

[c]Twenty-one animals in group, sensitized by single injection soluble antigen, 1.1 mg protein, challenged with CE HSV SA.

[d]Average diameter in mm.

did not respond with induration (Table 3). The largest skin reactions, in which considerable induration was consistently produced, were observed in the animals receiving two injections of infective HSV.

Specificity of the Skin Test Reaction

When six guinea pigs were sensitized with $5 \times 10^{4.3}$ $TCID_{50}$ of vaccinia virus and skin tested 14 days later with vaccinia soluble antigen (VSA) and L cell prepared HSV SA, neither erythema nor induration was noted in any of the animals. In addition, one group of six animals was sensitized with L cell control antigen plus 50% FCA; another group was sensitized with infective HSV plus 50% FCA. When these animals were skin tested with VSA, reactions of equal size were seen in the two groups. When HSV SA was used as the skin test antigen, much larger reactions were seen in the HSV sensitized animals than in animals sensitized with L cell control. No erythema was noted at 2 or 24 hr in L cell control antigen-sensitized animals challenged with HSV SA (Figures 6 and 7).

Nature of Cellular Infiltrate in Skin Test Sites

L cell control sensitized animals. Very little leucocytic infiltration was noted in histologic sections from L cell control sensitized animals at either CE control (Figure 8) or SA (Figure 9) test sites 0.5 hr after challenge. CE control sites examined 24 hr after challenge contained a few mononuclear and polymorphonuclear leucocytes, but had no perivascular infiltration (Figure 10). However, HSV SA skin test sites showed focal mononuclear cell infiltrations with large numbers of lymphocytes at 24 hr (Figure 11).

Infective herpes simplex virus sensitized animals. In photomicrographs of histologic sections of skin test sites from infective
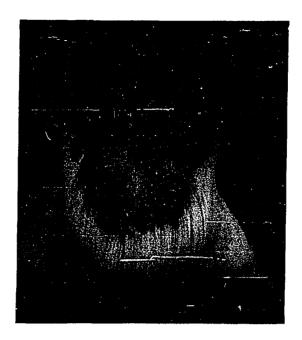
Figure 6. Skin reactions of an L cell control antigen sensitized guinea pig 2 hr after challenge with CE control antigen (left side) and HSV SA (right side), 1/3 normal size.
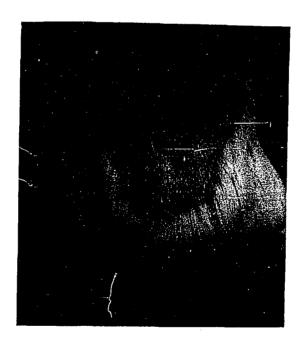
Figure 7. Skin reactions of an L cell control antigen sensitized guinea pig 24 hr after challenge with CE control antigen (left side) and HSV SA (right side), 1/3 normal size.
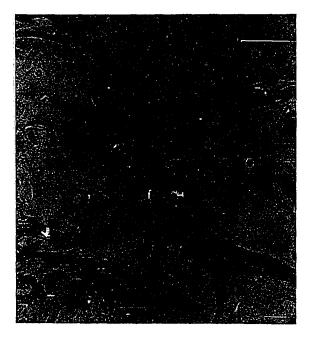
Figure 8. Hematoxylin and eosin stained section of tissue from skin test site of L cell control sensitized guinea pig 0.5 hr after challenge with CE control antigen, 200X.

Figure 9. Hematoxylin and eosin stained section of tissue from skin test site of L cell control sensitized guinea pig 0.5 hr after challenge with HSV SA , 200X.

Figure 10. Hematoxylin and eosin stained section of tissue from skin test site of L cell control sensitized guinea pig 24 hr after challenge with CE control antigen, 200X.

Figure 11. Hematoxylin and eosin stained section of tissue from skin test site of L cell control sensitized guinea pig 24 hr after challenge with HSV SA, 200X.

HSV sensitized guinea pigs taken 0.5 hr after challenge with CE control antigen (Figure 12) and HSV SA (Figure 13), a minimal cellular infiltrate consisting of a few mononuclear and polymorphonuclear leucocytes was observed. By 24 hr, the CE control sites (Figure 14) had more cellular infiltrate than at 0.5 hr, but when compared with the SA sites (Figure 15) the amount of infiltration was negligible. The SA skin test sites contained predominantly mononuclear cells at 24 hr. The high degree of mononuclear cell infiltration at 24 hr in the SA site parallels the amount of erythema noted in these animals (Figure 3 and Table 2).

Herpes simplex virus soluble antigen sensitized animals. No cellular infiltration was noted in control antigen sites from HSV SA sensitized guinea pigs examined 0.5 hr after challenge (Figure 16). The SA test site (Figure 17) contained a small number of polymorphonuclear leucocytes. At 24 hr the control antigen site remained essentially unchanged, with only an occasional mononuclear or polymorphonuclear leucocyte (Figure 18). Perivascular infiltration of polymorphonuclear leucocytes with a predominating mononuclear cell infiltrate was seen in SA test sites at 24 hr (Figures 19 and 20). This infiltrate closely resembled that seen in SA sites at 24 hr in HSV infected animals (Figure 15). On the basis of the cell type observed at 24 hr in SA sensitized animals, the response to SA in guinea pigs so sensitized appears to be a delayed hypersensitivity reaction.

Characterization of Herpes Simplex Virus Soluble Antigen

Dialysis

The precipitate formed when SA was dialyzed against distilled water at 5 C for 48 hr and the supernatant fluid left when this
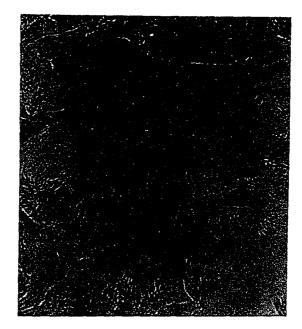
Figure 12. Hematoxylin and eosin stained section of tissue from skin test site of infective HSV sensitized guinea pig 0.5 hr after challenge with CE control antigen, 200X.
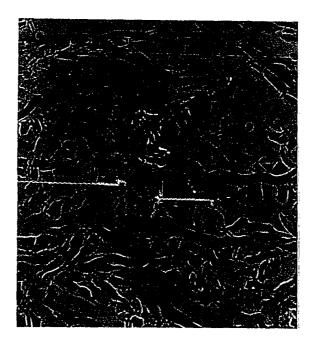
Figure 13. Hematoxylin and eosin stained section of tissue from skin test site of infective HSV sensitized guinea pig 0.5 hr after challenge with HSV SA, 200X.
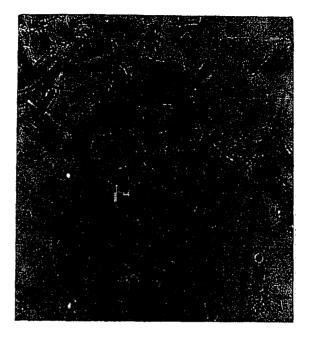
Figure 14. Hematoxylin and eosin stained section of tissue from skin test site of infective HSV sensitized guinea pig 24 hr after challenge with CE control antigen, 200X.
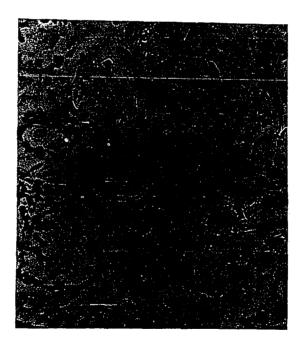
Figure 15. Hematoxylin and eosin stained section of tissue from skin test site of infective HSV sensitized guinea pig 24 hr after challenge with HSV SA, 200X.
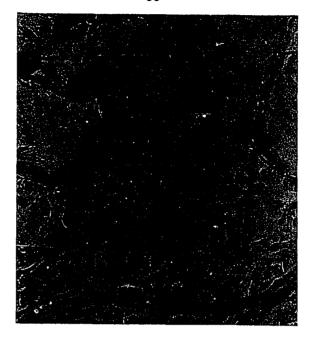
Figure 16. Hematoxylin and eosin stained section of tissue from skin test site of HSV SA sensitized guinea pig 0.5 hr after challenge with CE control antigen, 200X.
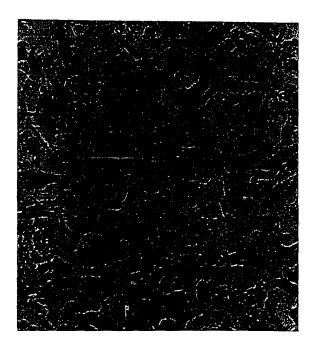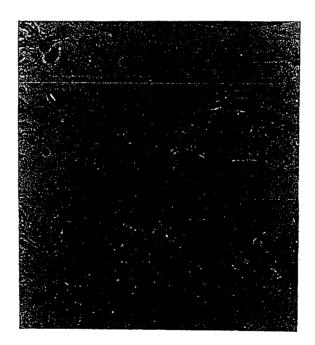

Figure 17. Hematoxylin and eosin stained section of tissue from skin test site of HSV SA sensitized guinea pig 0.5 hr after challenge with HSV SA, 200X.
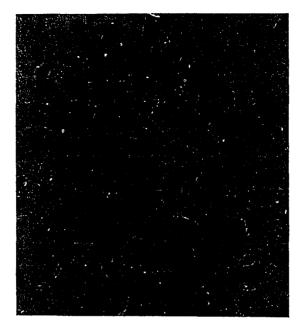
Figure 18. Hematoxylin and eosin stained section of tissue from skin test site of HSV SA sensitized guinea pig 24 hr after challenge with control antigen, 200X.
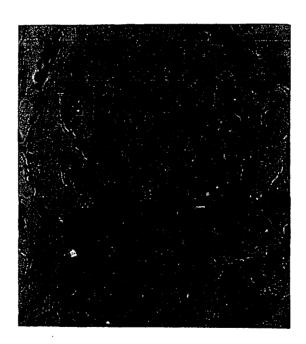
Figure 19. Hematoxylin and eosin stained section of tissue from skin test site of HSV SA sensitized guinea pig 24 hr after challenge with HSV SA, 200X.
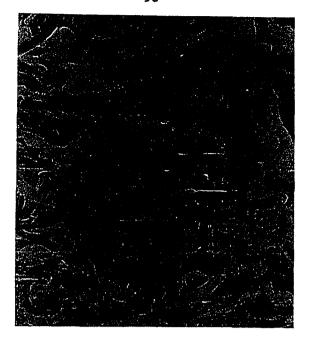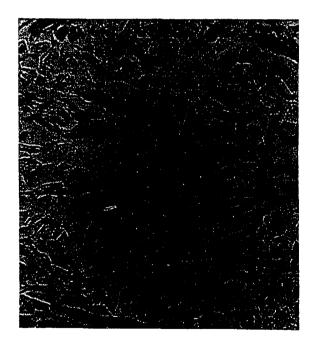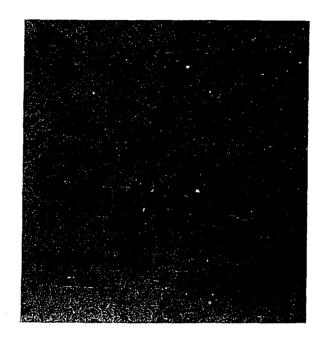
Figure 20.   Hematoxylin and eosin stained section of tissue from skin test site of HSV SA sensitized guinea pig 24 hr after challenge with HSV SA, 100X.

precipitate was removed were both used as challenge antigens to determine which fraction contained skin test activity.

Supernatant fluid. Since other investigators (99, 22) used adjuvants successfully with viral sensitizing antigens, animals were sensitized with 50% infective HSV in Freund's complete adjuvant (FCA). When the supernatant fluid was used to skin test these guinea pigs, larger diameters of erythema were seen with HSV SA material at 16, 24 and 48 hr than were noted with CEF control material dialyzed in the same manner (Table 9 and Figure 21). Likewise, at 16 and 24 hr larger areas of induration were observed in response to SA than control antigen.

The reactions elicited in response to the SA and CEF control supernatant fluid in L cell control sensitized guinea pigs can be seen in Table 10. SA did not elicit appreciably larger skin test reactions than the CEF preparation. It can be concluded that the active portion of the skin test antigen is not completely precipitated by dialysis in low ionic strength solutions. Since animals sensitized with infective HSV plus FCA showed somewhat comparable diameters at the SA test site to those observed with a different SA in animals sensitized with two injections of infective HSV (Table 8), this sensitization method was used for subsequent studies.

Precipitated material. When the precipitated fraction of dialyzed HSV soluble antigen was resuspended in PBS and used as the skin test antigen in HSV sensitized guinea pigs, the areas of erythema seen at 16 and 24 hr were much larger than those observed in response to CEF control antigen (Table 11 and Figure 21). Only minimal induration was

TABLE 9

SKIN TEST REACTIONS IN HSV SENSITIZED GUINEA PIGS[a]
CHALLENGED WITH DIALYZED HSV SOLUBLE ANTIGEN

Time (Hours)	Erythema		Induration	
	HSV SA[b]	CEF Control[c]	HSV SA[b]	CEF Control[c]
0.5	0[d]	0[d]	0[d]	0[d]
4	9.4	6.8	2.5	2.5
16	13.4	4.0	6.0	0
24	13.0	3.6	4.5	0
48	11.5	0	0	0

[a]Animals sensitized by single injection HSV, $10^{5.5}$ TCID$_{50}$, in 50% FCA; challenged 14 days later.

[b]Supernatant fluid from soluble antigen dialyzed against distilled water; prepared from HSV infected CEF; protein content was 150 μg per 0.1 ml.

[c]Control antigen supernatant fluid prepared from uninfected CEF.

[d]Average diameter in mm; 4 animals.
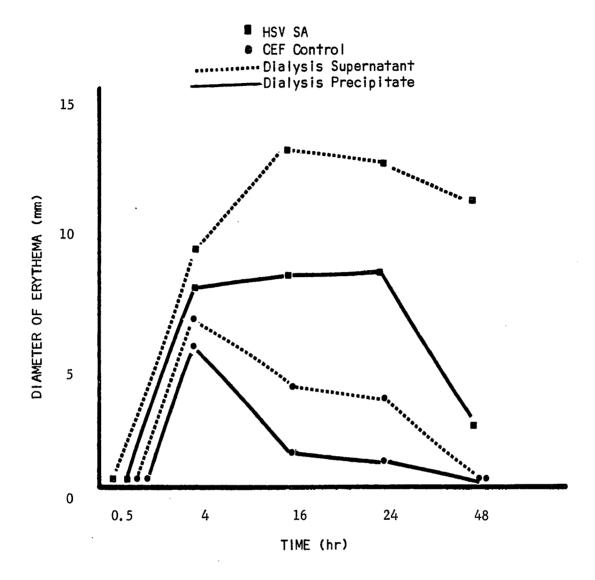
Figure 21. Average diameters of erythema in guinea pigs sensitized with active herpes simplex virus (HSV) plus Freund's complete adjuvant and challenged with fractions (precipitate and supernatant) of dialyzed soluble antigen.

TABLE 10

SKIN TEST REACTIONS IN L CELL CONTROL SENSITIZED GUINEA PIGS[a]
CHALLENGED WITH DIALYZED HSV SOLUBLE ANTIGEN

Time (Hours)	Erythema		Induration	
	HSV SA[b]	CEF Control[c]	HSV SA[b]	CEF Control[c]
0.5	0[d]	0[d]	0[d]	0[d]
4	5.0	9.0	1.2	1.2
16	6.1	5.5	1.7	1.7
24	5.1	5.5	1.5	1.7
48	0	0	0	0

[a]Animals sensitized by single injection L Cell control antigen in 50% FCA; challenged 14 days later.

[b]Supernatant fluid from soluble antigen dialyzed against distilled water; prepared from HSV infected CEF; protein content adjusted to 150 μg per 0.1 ml.

[c]Control antigen supernatant fluid prepared from uninfected CEF.

[d]Average diameter in mm; 4 animals.

TABLE 11

SKIN TEST REACTIONS IN HSV SENSITIZED GUINEA PIGS[a]
CHALLENGED WITH DIALYSIS PRECIPITATE

Time (Hours)	Erythema		Induration	
	HSV SA[b]	CEF Control[c]	HSV SA[b]	CEF Control[c]
0.5	0[d]	0[d]	0[d]	0[d]
4	8.0	5.9	1.0	0
16	8.4	1.3	2.0	0
24	8.5	1.0	2.5	0
48	2.6	0	0	0

[a]Animals sensitized by single injection HSV, $10^{5.5}$ TCID$_{50}$, in 50% FCA; challenged 14 days later.

[b]Precipitate from soluble antigen dialyzed against distilled water; prepared from HSV infected CEF; protein content was 150 µg per 0.1 ml.

[c]Control antigen precipitate prepared from uninfected CEF.

[d]Average diameter in mm; 4 animals.

elicited by the precipitated antigen. Although activity remained in
the precipitated SA, the amount of insoluble material in the precipi-
tate of both SA and CEF control preparations indicates partial removal
of CEF material from SA. Skin testing of HSV-FCA sensitized guinea
pigs with SA supernatant fluid evoked much larger erythematous and in-
durated reactions than did challenge with SA precipitated material
(Figure 21). Neither the dialysis-precipitated SA nor the CEF control
antigen induced erythema or induration in L cell control sensitized
guinea pigs. While treatment of SA by dialysis against distilled water
served to precipitate some of the skin test antigen, most of the ac-
tivity remained in the supernatant fluid.

Ultrafiltration of Herpes Simplex Virus Soluble Antigen

XM-100 ultrafiltration and ether extraction. Soluble antigen
which had been thrice extracted with ether and filtered through an
XM-100 ultrafiltration membrane retained its skin test activity (Table
12 and Figure 22). It elicited larger diameters of erythema than its
corresponding control antigen in HSV sensitized animals at 16, 24,and
48 hr. Induration was not seen with either skin test material and
negligible degrees of erythema and induration were observed in L cell
control sensitized animals upon challenge with the XM-100 filtrates.
Determination of the protein content of the SA preparation before and
after XM-100 ultrafiltration revealed that 98% of the protein passed
the filter.

XM-50 ultrafiltration. Most of the skin test active fraction
of the SA preparation was removed by the XM-50 ultrafiltration membrane
(Table 13 and Figure 22). Only traces of erythema and induration were

TABLE 12

SKIN TEST REACTIONS IN HSV SENSITIZED GUINEA PIGS[a] CHALLENGED WITH
ETHER EXTRACTED AND XM-100 ULTRAFILTERED SOLUBLE ANTIGEN

Time (Hours)	Erythema		Induration	
	HSV SA[b]	CEF Control[c]	HSV SA[b]	CEF Control[c]
0.5	0[d]	0[d]	0[d]	0[d]
4	4.6	6.0	0	0
16	7.1	3.8	0	0
24	7.6	2.6	0	0
48	5.4	0	0	0

[a]Animals sensitized by single injection HSV, $10^{5.5}$ $TCID_{50}$, in 50% FCA; challenged 14 days later.

[b]Water soluble fraction of ether extracted, XM-100 filtered SA; prepared from HSV infected CEF; protein content was 150 µg per 0.1 ml.

[c]Control antigen ether extracted, XM-100 filtered material; prepared from uninfected CEF.

[d]Average diameter in mm; 4 animals.
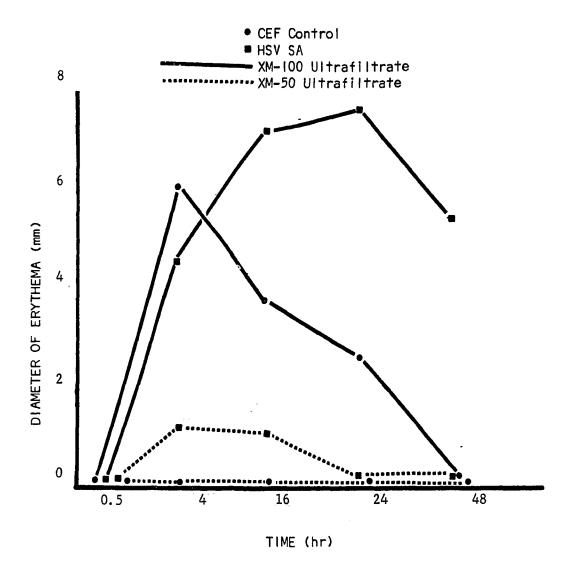
Figure 22. Average diameters of erythema in guinea pigs sensitized with active herpes simplex virus (HSV) plus Freund's complete adjuvant and challenged with SA and CEF control antigens which had been ether extracted and filtered through Amicon XM-50 and SM-100 ultrafiltration membranes.

TABLE 13

SKIN TEST REACTIONS IN HSV SENSITIZED GUINEA PIGS[a] CHALLENGED WITH
XM-50 ULTRAFILTERED SOLUBLE ANTIGEN

Time (Hours)	Erythema		Induration	
	HSV SA[b]	CEF Control[c]	HSV SA[b]	CEF Control[c]
0.5	0[d]	0[d]	0[d]	0[d]
4	1.3	0	0.9	0
16	1.1	0	0.9	0
24	0	0	0	0
48	0	0	0	0

[a]Animals sensitized by single injection HSV, $10^{5.5}$ TCID$_{50}$, in 50% FCA; challenged 14 days later.

[b]Soluble antigen prepared from HSV infected CEF monolayers and filtered through XM-50 membrane; protein content was 150 µg per injection.

[c]Control antigen XM-50 filtered material; prepared from uninfected CEF.

[d]Average diameter in mm; 4 animals.

observed at the SA skin test sites in HSV-FCA sensitized animals and
L cell-FCA sensitized animals developed no erythema or induration to
the filtered preparation.

Enzyme Treatment of Soluble Antigen

To characterize further the SA preparation, it was treated with
two peptidases (trypsin and pronase) to determine if it contained pro-
tein. Treatment of the SA with two nucleases (DNase and RNase) was
performed to determine DNA or RNA content.

Trypsin treatment. When HSV sensitized guinea pigs were skin
tested with the trypsin-treated antigen and control preparations, larger
erythematous reactions were seen at 4, 16, 24, and 48 hr in response to
inactive trypsin-treated SA than to active trypsin-treated SA (Table
14 and Figure 23). Diameters of induration were larger at the inactive
trypsin-treated SA site than at the active trypsin-treated SA site at
16, 24, and 48 hr. Larger reactions were seen in inactive trypsin-
treated SA sites than in active trypsin-treated control antigen sites
throughout the experimental period. Testing of L cell sensitized ani-
mals with the three trypsin treated materials resulted in erythematous
or indurated reactions which were notably different only at the 4 hr
period (Table 15 and Figure 24). At all other time intervals the re-
actions were of comparable size. These results demonstrate that the
reactions seen in the HSV sensitized guinea pigs were specific.

Pronase treatment. In HSV sensitized guinea pigs, somewhat
larger erythematous reactions were evoked at 24 and 48 hr in response
to inactive pronase-treated SA than to active pronase-treated SA
(Table 16 and Figure 25). Diameters of induration were larger in

TABLE 14

SKIN TEST REACTIONS IN HSV SENSITIZED GUINEA PIGS[a] CHALLENGED
WITH TRYPSIN TREATED SOLUBLE ANTIGEN[b]

Antigen Treatment	Erythema (mm) (Hours)					Induration (mm) (Hours)				
	0.5	4	16	24	48	0.5	4	16	24	48
Active (unheated) trypsin + SA	0[d]	7.6	8.1	8.2	4.3	0[d]	5.0	3.5	4.3	0
Inactive (heated) trypsin + SA	0	9.2	10.3	12.4	6.3	0	5.0	5.8	5.8	4.0
Active trypsin + control antigen[c]	0	5.0	0	0	0	0	0	0	0	0

[a]Animals sensitized by single injection of HSV, $10^{5.5}$ TCID$_{50}$, in 50% FCA; challenged 14 days later.

[b]Soluble antigen prepared from HSV infected CE allantoic fluid.

[c]Control antigen prepared from uninfected CE allantoic fluid.

[d]Average diameter in mm; 3 animals.

HSV SENSITIZED ANIMALS

■———■ Trypsin-Treated SA
■··········■ Trypsin (Inactive)-Treated SA
■·—··—■ Trypsin-Treated Control Allantoic Fluid

DIAMETER OF ERYTHEMA (mm)

14

12

10

8

6

4

2

0

0.5   4   16   24   48   72

TIME (hr)

Figure 23. Average diameters of erythema in guinea pigs sensitized with active herpes simplex virus (HSV) plus Freund's complete adjuvant and challenged with trypsin treated soluble antigen.

TABLE 15

SKIN TEST REACTIONS IN L CELL SENSITIZED GUINEA PIGS[a]
CHALLENGED WITH TRYPSIN TREATED SOLUBLE ANTIGEN[b]

Antigen Treatment	Erythema (mm) (Hours)					Induration (mm) (Hours)				
	0.5	4	16	24	48	0.5	4	16	24	48
Active (unheated) trypsin + SA	0[d]	7.0	2.3	0	0	0[d]	4.9	0	0	0
Inactive (heated) trypsin + SA	0	5.5	0	0	0	0	0	0	0	0
Active trypsin + control antigen[c]	0	0	0	0	0	0	0	0	0	0

[a]Animals sensitized by single injection of L cell control in 50% FCA; challenged 14 days later.

[b]Soluble antigen prepared from HSV infected CE allantoic fluid.

[c]Control antigen prepared from uninfected CE allantoic fluid.

[d]Average diameter in mm; 4 animals.

L CELL SENSITIZED ANIMALS

O———O Trypsin-Treated SA

■■■■■■■■■■■ Trypsin (Inactivated)-Treated SA

C—·—·—C Trypsin-Treated Control Allantoic Fluid

DIAMETER OF ERYTHEMA (mm)

10

8

6

4

2

0

0.5   4   16   24   48   72

TIME (hr)
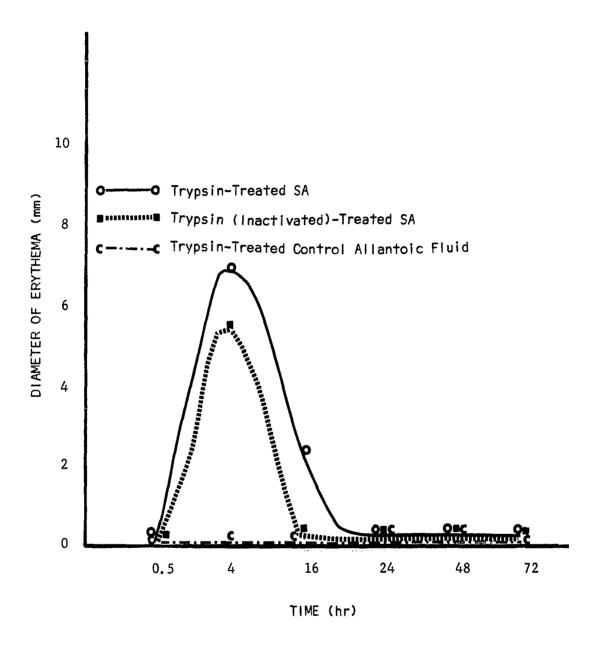
Figure 24. Average diameters of erythema in guinea pigs sensitized with L cell control antigens plus Freund's complete adjuvant and challenged with trypsin treated soluble antigen.

TABLE 16

SKIN TEST REACTIONS IN HSV SENSITIZED GUINEA PIGS[a] CHALLENGED
WITH PRONASE TREATED SOLUBLE ANTIGEN[b]

Antigen Treatment	Erythema (mm) (Hours)					Induration (mm) (Hours)				
	0.5	4	16	24	48	0.5	4	16	24	48
Active (unheated) pronase + SA	0^d	8.0	9.5	8.8	2.7	0^d	6.7	5.3	5.3	4.1
Inactive (Heated) Pronase + SA	0	9.8	10.8	11.5	8.2	0	3.0	5.3	5.6	4.5
Active Pronase + control antigen[c]	0	6.6	8.2	6.0	1.4	0	7.0	5.1	4.9	0

[a]Animals sensitized by single injection of HSV, $10^{5.5}$ TCID$_{50}$, in 50% FCA; challenged 14 days later.

[b]Soluble antigen prepared from HSV infected CE allantoic fluid.

[c]Control antigen prepared from uninfected CE allantoic fluid.

[d]Average diameter in mm; 4 animals.
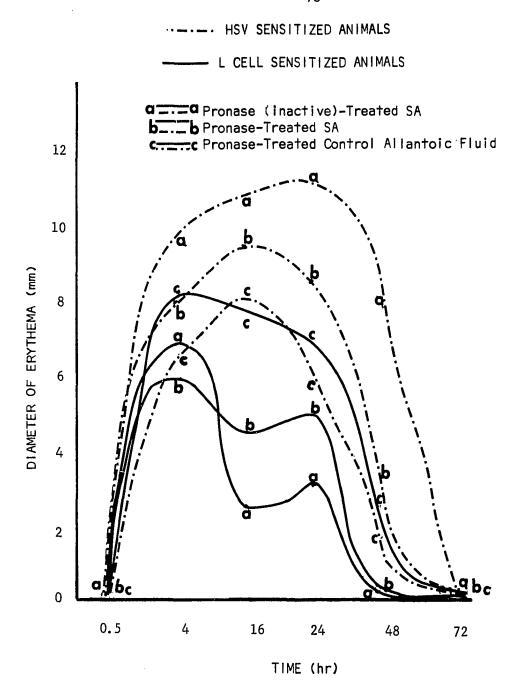
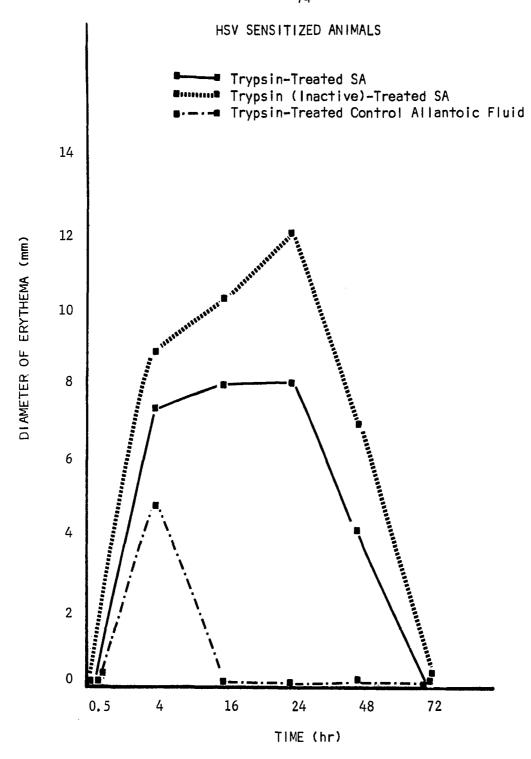Figure 25. Average diameters of erythema in guinea pigs
sensitized with active herpes simplex virus (HSV) plus Freund's
complete adjuvant or L cell control antigen plus Freund's complete
adjuvant and challenged with pronase treated soluble antigen.

response to active pronase-treated SA than to inactive pronase-treated SA at 4 hr but the reactions were not greatly different at the other time periods. Control CE antigen plus pronase elicited smaller skin reactions at 24 and 48 hr than either of the SA preparations. This demonstrates that the reactivity seen in these animals is directed toward a component in the SA preparation. In L cell control sensitized guinea pigs reactions to inactive pronase-treated SA were smaller than reactions to either active pronase-treated SA or active pronase plus control antigen, indicating that SA did not elicit an HSV specific skin reactivity in L cell control sensitized animals (Table 17 and Figure 25).

Deoxyribonuclease treatment. HSV sensitized guinea pigs responded to skin testing with inactive DNase-treated SA with erythematous responses which were considerably larger at 16 hr than those observed with active, DNase-treated SA (Table 18). Average diameters of induration in the former group were comparable in size to those with the latter antigen treatment (Table 18). Skin test reactions to SA injected sites were larger than those to control antigen at 16, 24 and 48 hr. L cell sensitized animals responded to all three skin test materials to essentially the same degree (Table 19). The only exception was in the diameters of induration seen at 48 hr. These results reveal the specificity of the SA preparation for HSV antigens and suggest that active DNase degraded the SA.

Ribonuclease treatment. Skin test reactions to both active RNase-treated SA and inactive RNase-treated SA were similar at all time intervals in HSV sensitized guinea pigs (Table 20). At 16 and

TABLE 17

SKIN TEST REACTIONS IN L CELL SENSITIZED GUINEA PIGS[a]
CHALLENGED WITH PRONASE TREATED SOLUBLE ANTIGEN[b]

Antigen Treatment	Erythema (mm) (Hours)					Induration (mm) (Hours)				
	0.5	4	16	24	48	0.5	4	16	24	48
Active (unheated) pronase + SA	0[d]	6.0	4.6	5.0	0	0[d]	5.5	4.1	4.0	0
Inactive (heated) pronase + SA	0	7.0	2.5	3.3	0	0	1.0	1.0	0	0
Active pronase + control antigen[c]	0	8.1	7.4	7.1	1.7	0	6.2	4.4	4.2	0.7

[a]Animals sensitized by single injection of L cell control in 50% FCA; challenged 14 days later.

[b]Soluble antigen prepared from HSV infected CE allantoic fluid.

[c]Control antigen prepared from uninfected CE allantoic fluid.

[d]Average diameter in mm; 4 animals.

TABLE 18

SKIN TEST REACTIONS IN HSV SENSITIZED GUINEA PIGS[a] CHALLENGED
WITH DNASE TREATED SOLUBLE ANTIGEN[b]

Antigen Treatment	Erythema (mm) (Hours)					Induration (mm) (Hours)				
	0.5	4	16	24	48	0.5	4	16	24	48
Active (unheated) DNase + SA	0[d]	4.0	7.3	6.7	0	0[d]	3.7	4.5	4.4	3.9
Inactive (heated) DNase + SA	0	4.6	11.0	8.6	0	0	3.9	4.7	4.7	4.4
Active DNase + control antigen[c]	0	4.2	6.7	4.7	0	0	3.8	3.8	3.6	0

[a]Animals sensitized by single injection of HSV, $10^{5.5}$ $TCID_{50}$, in 50% FCA; challenged 14 days later.

[b]Soluble antigen prepared from HSV infected CEF.

[c]Control antigen prepared from uninfected CEF.

[d]Average diameter in mm; 6 animals.

TABLE 19

SKIN TEST REACTIONS IN L CELL SENSITIZED GUINEA PIGS[a]
CHALLENGED WITH DNASE TREATED SOLUBLE ANTIGEN[b]

Antigen Treatment	Erythema (mm) (Hours)					Induration (mm) (Hours)				
	0.5	4	16	24	48	0.5	4	16	24	48
Active (unheated) DNase + SA	0[d]	3.3	7.4	0	0	0[d]	4.5	4.8	4.5	3.8
Inactive (heated) DNase + SA	0	1.4	4.8	2.9	0	0	4.4	4.7	4.8	4.2
Active DNase + control antigen[c]	0	1.9	4.5	1.4	0	0	4.5	4.4	4.3	0

[a]Animals sensitized by single injection of L cell control in 50% FCA; challenged 14 days later.

[b]Soluble antigen prepared from HSV infected CEF.

[c]Control antigen prepared from uninfected CEF.

[d]Average diameter in mm; 6 animals.

TABLE 20

SKIN TEST REACTIONS IN HSV SENSITIZED GUINEA PIGS[a] CHALLENGED
WITH RNASE TREATED SOLUBLE ANTIGEN[b]

Antigen Treatment	Erythema (mm) (Hours)					Induration (mm) (Hours)				
	0.5	4	16	24	48	0.5	4	16	24	48
Active (unheated) RNase + SA	0[d]	4.6	11.6	10.4	0	0[d]	4.5	5.0	4.8	0
Inactive (heated) RNase + SA	0	3.9	11.1	10.0	0	0	4.1	4.7	4.6	0
Active RNase + control antigen[c]	0	4.1	7.8	6.9	0	0	3.9	4.3	4.6	0

[a]Animals sensitized by single injection of HSV, $10^{5.5}$ $TCID_{50}$, in 50% FCA; challenged 14 days later.

[b]Soluble antigen prepared from HSV infected CEF.

[c]Control antigen prepared from uninfected CEF.

[d]Average diameter in mm; 6 animals.

24 hr, erythematous reactions were larger in both of these groups than in animals skin tested with RNase-treated control antigen. Diameters of induration were essentially the same in all three groups of animals at all time intervals. L cell sensitized guinea pigs responded to all three skin test materials to essentially the same degree at all time intervals (Table 21).

TABLE 21

SKIN TEST REACTIONS IN L CELL SENSITIZED GUINEA PIGS[a]
CHALLENGED WITH RNASE TREATED SOLUBLE ANTIGEN[b]

Antigen Treatment	Erythema (mm) (Hours)					Induration (mm) (Hours)				
	0.5	4	16	24	48	0.5	4	16	24	48
Active (unheated) RNase + SA	0[d]	1.8	5.9	3.1	0	0[d]	4.9	5.2	5.3	4.5
Inactive (heated) RNase + SA	0	1.7	6.4	3.5	0	0	4.7	5.2	5.0	4.3
Active RNase + control antigen[c]	0	1.5	4.9	2.4	0	0	4.1	5.0	4.9	4.0

[a]Animals sensitized by single injection of L cell control in 50% FCA; challenged 14 days later.

[b]Soluble antigen prepared from HSV infected CEF.

[c]Control antigen prepared from uninfected CEF.

[d]Average diameter in mm; 6 animals.

CHAPTER IV

DISCUSSION

Antigens and Media

A sensitizing herpes simplex virus (HSV) antigen was prepared in L cells proliferated in Eagle's Minimum Essential Medium (MEM) plus horse serum. The skin test antigens were prepared in chick embryos (CE) or in chick embryo fibroblasts (CEF) grown in a calf serum supplemented medium (LHYE C). Different cell lines, media, and sera were used in an attempt to minimize cross reactions which might make it difficult to interpret skin test results (46). Lausch et al. (55) were able to avoid cross reactions by using mouse brain proliferated HSV for sensitization of guinea pigs and by employing CEF-prepared HSV antigen as skin test material. In the present studies, HSV skin test antigen prepared in CE was capable of eliciting reactions in HSV sensitized guinea pigs, as can be seen in Tables 2, 3 and 4. In these three experiments, skin reactions to control allantoic fluid were largest at 4 hr and declined to low levels by 16 and 24 hr, while reactions to soluble antigen (SA) were maximal at 16 to 24 hr. In addition, the reactions to SA were much larger at 16 and 24 hr than were those to control antigen. The use of CEF in the preparation of skin test antigens also allowed the detection of sensitivity to HSV in

guinea pigs (Table 9). CEF control antigen elicited minimal reactions which diminished by 24 hr, while the reactions to HSV SA prepared in CEF were considerably larger and more persistent. Evidence for the specificity of the reaction to SA in these animals is presented in Table 10. Guinea pigs sensitized with L cell control antigen and skin tested with SA responded with reactions that were no larger than those seen at control skin test sites. These results offer proof that the skin reactivity at SA test sites in HSV sensitized guinea pigs is directed against an HSV component and not a component of L cells, MEM, or horse serum.

Methods of Sensitization

Lausch et al. (55) reported sensitization of guinea pigs by a single injection of mouse brain proliferated HSV which contained 2×10^4 PFU of virus/0.1 ml. Skin reactions measuring 15 mm in diameter were observed at HSV SA test sites one and two weeks after sensitization. Maximal reactions (16 mm of erythema) were observed after three weeks. Maximal reactions to SA occurred 24 hr after skin testing. While induration was noted, it was erratic and not as constant as the erythematous response. The SA used by these investigators was prepared in CEF grown in medium containing 5% calf serum. Reactions elicited by CEF control antigen were maximal (6-8 mm) by 4 to 8 hr and diminished to an average of 3 to 5 mm by 24 hr. The SA skin testing preparation contained 98 µg of protein/0.1 ml.

In the present studies, skin reactions at SA test sites two weeks after sensitization with $10^{5.5}$ TCID$_{50}$ of HSV were maximal at 16 hr (12.1 mm) and 24 hr (7.5 mm) (Table 2). The smaller diameters of

erythema observed in the present study, as compared with those reported by Lausch et al. (55), may be attributed to several factors. First, the sensitizing preparations used in the two studies were different. Lausch et al. (55) used 2×10^4 PFU of HSV prepared from HSV-infected mouse brain, while in the present studies $10^{5.5}$ TCID$_{50}$ of HSV prepared from L cell monolayers proliferated in MEM$_{91}$ H$_9$ was employed (Table 2). Since mouse brain contains up to 23% lipid by weight (106), and since lipids are known to act as adjuvants (13), the possibility that material from HSV-infected mouse brain acted as an adjuvant in sensitization of guinea pigs to HSV cannot be overlooked. Data from the present study which support such an interpretation will be discussed later. A second factor which may have played a role in the apparently better sensitizing efficiency of the preparation used by Lausch et al. could have been the quality of the sensitizing HSV preparation. The mouse brain-prepared HSV preparation may have contained large quantities of inactive virus or viral products which could induce a greater degree of sensitivity. While the washed preparation used as sensitizing HSV in the present studies probably contained inactive virus, many of the smaller mol wt products were removed. In addition, data are presented here, and will be discussed later, which demonstrate that inactive HSV (Table 4) and low mol wt viral components (Table 5) are capable of inducing sensitivity to HSV. A third factor which could influence the size of skin test reactions is the protein content of the skin test preparation. The protein content of the skin test antigen used by Lausch et al. was 98 μg/0.1 ml while that used in the present study was estimated to be 400 μg/0.1 ml. Clearly, the

CEF SA preparation of Lausch et al. contained more virus specific

material, if one assumes that the size of the skin reaction is dose

dependent. It should also be noted that large amounts of non-viral

protein might be expected to be contained in allantoic fluid of HSV-

infected embryonated eggs, since control allantoic fluid contained

5.5 mg protein/ml (Table 1). The results seen following sensitization

of guinea pigs by a single injection of HSV prepared in L cells and

skin testing of the same animals two weeks later with CE-prepared SA

suggested that characterization of the soluble antigen of HSV would

require antigen preparations with more virus specific material and

greater degrees of sensitivity in test animals.

Various methods of sensitization of guinea pigs to HSV were

compared. Animals were sensitized by injection of the following:

1) one and two injections of infective HSV ($10^{5.5}$ $TCID_{50}$) prepared in

L cells; 2) two injections of heat-inactivated HSV (which had contained

$10^{5.5}$ $TCID_{50}$ of infective virus) prepared in L cells; and 3) one injec-

tion of HSV SA (1.1 mg of protein) prepared in L cells and shown to

contain no infective HSV. The largest skin test reactions to HSV SA

were seen in animals sensitized with two injections of infective HSV

(Table 8). This finding provides additional proof of a dose dependent

response in sensitization of guinea pigs to HSV. The demonstration that

the second and third sensitization methods (above) were capable of sens-

itizing guinea pigs to HSV confirms the earlier suggestion that non-

infective HSV specific materials can contribute to sensitization. These

findings suggest that the use of a skin test antigen for HSV in human

studies could sensitize patients to HSV, making retesting with SA of no

value. The finding that infective HSV induced greater sensitivity in guinea pigs than an equal amount of heat-inactivated HSV may have been predicted from studies of BCG immunization. Immunization of children with live BCG induced much larger skin reactions to tuberculin than did immunization with heat-killed BCG (71). Larger numbers of live BCG organisms also produced greater sensitivity than smaller numbers, as evidenced by larger skin reactions.

Incorporation of Freund's complete adjuvant (FCA) into the sensitizing HSV preparation resulted in a high degree of sensitivity in guinea pigs, as shown by skin tests with dialyzed SA (Table 9). Since the skin reactions observed in these animals two weeks after sensitization were comparable in size to those seen four weeks after two injections of infective HSV, use was made of HSV in 50% FCA in sensitization of animals for studies on characterization of the SA. The finding that FCA increases the sensitization to HSV lends further support to the hypothesis that mouse brain may act as adjuvant in sensitization of guinea pigs with HSV. Other investigators have reported similar success with the use of FCA in sensitization of animals to mumps virus (102) and HSV (99).

Nature of Skin Test Reaction

Andervont and Rosenau (4) reported that vaccinia virus-infected guinea pigs did not demonstrate skin reactions of significant size upon challenge with HSV skin test antigen. The results of the present studies confirmed this earlier finding and also demonstrated that HSV-sensitized guinea pigs do not respond to vaccinia SA with significant reactions. These findings indicate the specificity of the skin test

reactions in HSV sensitized animals. However, the possibility exists that antigens of other viruses in the herpesvirus group may cross react with the skin test antigen of HSV. Results of studies utilizing simian herpesvirus and neutralizing antiserum to HSV indicated cross reactivity due to shared antigens (35, 77). Further investigation of potential group specific skin test antigens in the herpesviruses would be desirable. Type specific skin test antigen for HSV type 1 and HSV type 2 could possibly be prepared. Correlative studies on the incidence of cervical carcinoma and skin test reactions to HSV type 2 would then be possible (Melnick, J. L., personal communication). Such work could be invaluable in studies concerning the etiology of cancer.

The time of appearance of the maximal response to skin tests with SA in HSV sensitized guinea pigs has been reported to be 24 hr (10, 55), but in no instance was the 16 hr reading reported. Lausch et al. (55) reported maximal erythema in HSV sensitized guinea pigs at 24 hr, but they noted that the size of the eight-hour readings often approached that of the 24 hr readings. Brown (10) found the skin test reaction in HSV sensitized guinea pigs to be much larger at 24 hr than that at 48 or 72 hr. Readings at earlier time periods than 24 hr were not reported. In the present investigation, skin tests applied 14 days after sensitization with a single injection of infective HSV reached maximal intensity at 16 hr and appreciable reactivity persisted at 24 hr (Table 2). There are certain differences in the times at which readings were recorded in this study and those used by Lausch et al. (55). In the latter studies, readings were made at 0.5, 4, 8, 24, 48 and 72 hr after skin testing while in the present investigation readings were taken at 0.5, 4, 16, 24, 48 and 72 hr. It is possible

that Lausch et al. might also have detected maximal skin reactivity at 16 hr if they had examined the test sites at that time. Maximal reactions were observed at 16 hr in guinea pigs sensitized with two injections of infective virus (Table 3) or a single injection of SA (Table 5), but skin reactions seen in animals sensitized with heated HSV were approximately the same at 4, 16, and 24 hr (Table 4). Skin reactivity was also readily apparent at 48 hr, although the 72 hr readings were zero. The time required for maximal skin response to SA is not unlike that reported for tuberculin in Mycobacterium tuberculosis-sensitized guinea pigs (26, 27).

Lausch et al. (55) demonstrated that skin reactivity following infection of guinea pigs with HSV was transferrable by mononuclear cells, but not by hyperimmune serum, was maximal at 24 hr as previously noted, and was characterized by a mononuclear cell infiltrate at the test site 24 hr after challenge with SA. These workers concluded that the cutaneous response to SA in HSV infected guinea pigs was a classical, delayed-type, hypersensitivity reaction.

In the present studies, induration was noted in skin reactions to SA in guinea pigs sensitized with two injections of HSV, either infective or heated, and in animals sensitized with HSV plus 50% FCA (Table 8). These results suggest the attainment of a higher degree of sensitization with these methods than by a single injection of HSV. Since induration is a characteristic of the delayed skin reaction to tuberculin (26, 27), the present findings suggest that the skin reaction to SA is typical of a delayed hypersensitivity response. Earlier, Brown (10) had noted that repeated weekly injections of infective HSV

over a two-month period sensitized guinea pigs to HSV. Diameters of
induration were 14 mm at 24 hr, 5 mm at 48 hr and 2 to 3 mm at 72 hr.
These diameters of induration are somewhat larger than those observed
in the present study (Table 8). These findings support the view that
the degree of sensitization to HSV in guinea pigs is dependent upon
the amount of virus specific antigen injected.

Skin test reactions to CEF and CE control antigens usually
attained maximal erythema at 4 hr (Tables 2 through 5, 9 through 21,
and Figures 21 through 25). The reactions to SA, prepared in either
CE or CEF, were significantly larger at 16 hr in HSV-FCA sensitized
animals, HSV sensitized animals, and SA sensitized animals, than in
L cell control-FCA sensitized animals (Tables 15 and 17. Some excep-
tions occurred in the enzyme treatment studies. Since the response
to both SA and the equivalent control preparations were usually maxi-
mal at 4 or 16 hr in L cell control sensitized animals, these reactions
are considered to be the result of inherent host responses to intra-
cutaneous injection of foreign materials. Lausch et al. (55) also
observed an early erythematous response to CEF control antigen in
HSV sensitized guinea pigs which diminished greatly by 24 hr.

The erythematous nature of the skin reaction to SA at 24 hr
in HSV sensitized animals reported by Lausch et al. (55) was confirmed
in the present studies (Figure 3). In addition, the SA was shown to
elicit no erythema at 24 hr in L cell control sensitized animals
(Figure 9). A comparison of the intensity of erythema at 24 hr in HSV
sensitized animals (Figure 3) with the response at 24 hr in SA sensi-
tized animals (Figure 5) reveals a more intense response in HSV
infected animals. This is interpreted as further demonstration that

the amount of HSV specific material in the sensitizing preparation determines the degree of skin sensitivity attained. Cytologic examination of the SA challenge sites in infective HSV and SA sensitized animals at 0.5 hr revealed small numbers of polymorphonuclear leucocytes (Figures 13 and 17). At 24 hr, the infiltrate was composed of 70 to 80% mononuclear leucocytes and 20 to 30% polymorphonuclear leucocytes (Figures 15 and 19). The mononuclear cell infiltrate at the SA challenge sites was more intense in infective HSV sensitized guinea pigs than in SA sensitized animals. Control preparations taken at 24 hr (Figures 10 and 18) resembled the 0.5 hr sections (Figures 8 and 16) taken from SA challenge sites. Sections of SA challenge sites taken at 24 hr from L cell control sensitized guinea pigs contained scattered mononuclear cells (Figure 11). This indicates that SA itself may stimulate some mononuclear cell infiltration but the number of cells was not as large as that observed in SA sites from SA or HSV sensitized animals. The cellular reactions at SA challenge sites in HSV or SA sensitized guinea pigs are typical of those described in delayed hypersensitivity to M. tuberculosis (16, 27).

These findings indicate that a single injection of SA can sensitize guinea pigs to HSV but that larger amounts of HSV specific material are needed to induce sensitivity characterized by indurated responses with intense erythema and mononuclear cell infiltration.

Characterization of Soluble Antigen

Up to 25 different proteins are known to be present in HSV (91). A summary of the characteristics of the materials known to act as CF or skin test antigen is presented in Table 22. Briefly, the CF

TABLE 22

PROPERTIES OF HERPES SIMPLEX VIRUS SOLUBLE ANTIGEN(S)

Treatment	CF[a]		Skin Test[b]		
	Others	Refs.	Others	Refs.	Present Studies[c]
Heat					
56 C	$-^d$	10, 100	$+^e$	10, 47, 55	+ Table 2
70 C	$-$	10	+	68	+ Table 14
UV Irradiation	N.D.^{f}		+	47	N.D.
Lyophilization	+	107	+	107	+ Table 9 Figure 1
Centrifugation					
66,000 × g	N.D.		+	55, 3	+ Table 9 Figure 1
100,000 × g	+	100	N.D.		N.D.
Dialysis					
PBS	+	100	+	55, 107	+ Table 2 Figure 1
Distilled H_2O					
1. Supernatant fluid	N.D.		N.D.		+ Table 9
2. Precipitate	N.D.		N.D.		+ Table 11
Acid Hydrolysis	$-$	100	N.D.		N.D.
Ether	+	100	N.D.		+ Table 12

TABLE 22--<u>Continued</u>

Treatment	Activity				
	CF[a]		Skin Test[b]		
	Others	Refs.	Others	Refs.	Present Studies[c]
Ultrafiltration					
XM-100	N.D.		N.D.		+ Table 12
XM-50	N.D.		N.D.		− Table 13
Enzymes					
Trypsin	+	100	N.D.		*[g] Table 14
Pronase	N.D.		N.D.		* Table 16
DNase	+	100	N.D.		* Table 18
RNase	+	100	N.D.		+ Table 20

[a]Complement fixing ability with anti-HSV serum.

[b]Skin test reactions produced in HSV sensitized hosts.

[c]Characteristics determined in present investigation.

[d]Inactivation after treatment.

[e]Active after treatment.

[f]Not determined.

[g]Partial inactivation after treatment.

antigen of HSV has been shown to be of low MW; heat labile; resistant

to action of trypsin, DNase, RNase and ether; destroyed by acid hydrol-

ysis; and not sedimented at 100,000 x g (100). The skin test antigen,

prior to the present investigation, had been reported to be stable to

heating to 56 C and 70 C (3, 10, 55), not destroyed by UV irradiation

(47), nondialyzable (55), and not sedimentable by centrifugation at

66,000 x g for 0.5 hr (55).

In the present studies, the observation that dialysis of both

CEF control and CEF HSV preparations resulted in the formation of large

amounts of precipitate suggested that such treatment might provide

partial purification of SA. It was found that most of the skin test

activity in HSV sensitized animals was associated with the supernatant

fluid after dialysis (Table 9). The globulin class of proteins is

characterized by a decreased solubility at low ionic strength so that

precipitation occurs in distilled water or upon addition of methanol

to the solution (106). The fact that active SA was only partially

precipitated by dialysis against distilled water suggests that it is

not a globulin. The SA could be a histone- or a protamine-type protein,

since these classes of proteins are not readily precipitated by

dialysis (106). The activity of the precipitated fraction (Table 11

and Figure 21) may indicate simple absorption of SA to CEF globulins

or it could be interpreted as evidence for SA having properties of both

globulins and other proteins, such as protamines.

In this study it was observed that SA was stable to heating at

56 C or 70 C for 1 hr, stable to lyophilization, non-dialyzable, and

not sedimented by centrifugation at 66,000 x g. These findings confirm

earlier work on the nature of SA (Table 22). Since HSV is a lipid-containing virus (52), it seemed of interest to determine the ether susceptibility of SA. It was shown in this study that SA is not extracted by ether but remains active in the aqueous fractions (Table 12). This finding is interpreted as evidence that the SA is not a lipid material. The observation that ether extraction of SA did not remove its skin test activity may be interpreted as an indication that the SA is not derived from the lipid portion of the HSV envelope. HSV is an enveloped virus containing up to 22% phospholipids, by weight (52). However, a firm conclusion that the SA is not a part of the envelope cannot be made, since various glycoproteins from the viral envelope (92) could be acting as SA. Characterization of the possible structural or accessory proteins of HSV which could act as SA awaits studies utilizing radioactive labelling and/or ultrastructural tagging.

In these studies, SA was retained by size XM-50 ultrafiltration membranes (Table 13) but it passed through size XM-100 membranes (Table 12 and Figure 22). Assuming that SA has filtration properties similar to other materials (2), the mol wt of SA can be estimated to be 50,000 to 150,000. An estimation of mol wt based upon ultrafiltration, however, warrants further consideration. According to data provided by the Amicon Corporation (2), the XM-100 membrane retains 90% of gamma globulin of mol wt 160,000, 20% of aldolase of mol wt 142,000, and no dextran of mol wt 110,000. Retention of greater than 95% of aldolase (mol wt 142,000), 10% of dextran (mol wt 110,000) and 90% of human serum albumin (mol wt 67,000) is reported for the XM-50 membrane. If the SA is a linear molecule with filtration properties similar to dextran, it could be much larger than 150,000 mol wt.

The demonstration that treatment of SA with active trypsin decreased greatly its skin test reactivity (Table 14) is a strong basis for assuming that SA is protein in nature. Tokumaru (100) reported that the CF antigen of HSV was not susceptible to digestion by trypsin but proof of the activity of the enzyme preparation was not presented. In the present study the trypsin used to treat SA was shown to degrade casein. In addition, inactive trypsin was shown to have no activity on casein. While the SA responsible for skin test reactivity need not necessarily be the same material as the CF antigen, such a possibility does exist.

The finding that trypsin inactivates SA may also indicate something of the nature of the protein moiety of SA. Trypsin is a proteolytic enzyme which acts only at peptide, amide, or ester bonds at the carbonyl end of arginine or lysine residues (54, 106). Since trypsin is rather specific in its action, it can be concluded that SA contains a protein having arginine or lysine residues. Olshevsky and Becker (74) isolated an arginine-rich protein associated with the core of HSV which had a mol wt of 37,000. They also described another protein with a mol wt of 110,000, which made up the majority of the virus specified protein. It is possible that this material could serve as a soluble skin test antigen. However, the existence of many other HSV specific proteins (91, 92) makes difficult any correlation of the SA with other known proteins of HSV.

Further support for the conclusion that SA contains a protein moiety is provided by studies with pronase (Table 16). Although active pronase reduced the activity of SA, the non-specific reactions which it

elicited in L cell control sensitized animals (Table 17) point out the need for adequate controls in using this enzyme to characterize a skin test antigen. Inactive pronase did not degrade the skin test activity of SA. The broad specificity of pronase makes it a good choice for degrading protein, since it is reported to act on all peptide bonds (72).

Active DNase reduced the skin reaction elicited by SA at 16 hr but not at 24 hr (Table 18). This finding suggests that the SA contains a deoxyribonucleic acid moiety. The DNase used in this study has been reported to be active against both natural and denatured DNA (63). Since HSV is a DNA-containing virus (52), the existence of a deoxyribonucleoprotein specific for HSV which could act as a skin test antigen is not too surprising. Deoxyribonucleoproteins which elicit the production of antibodies directed against nucleic acid have been reported (43).

Active RNase had no effect on SA (Table 20). This finding suggests that an RNA material does not act as skin test antigen in HSV sensitized guinea pigs. Such a conclusion seems valid since the RNase used was demonstrated to be active against natural RNA.

The results of the present studies agree with those of Lausch et al. (55) in suggesting that more than one HSV antigen may act as a skin test antigen. The results of the enzyme treatment studies may be interpreted as evidence for two separate skin test antigens. In addition, the demonstration of skin test activity in both precipitate and supernatant fluid following dialysis of SA may be interpreted as evidence for two skin test antigens.

It would now be possible to initiate studies with a SA of HSV which might differentiate between specific reactions to HSV and its

antigens, that is, delayed hypersensitivity, and non-specific responses
to interferon-inducing agents.

CHAPTER V

SUMMARY

Guinea pigs were sensitized to herpes simplex virus (HSV) by inoculation with infective virus, heat inactivated virus, or a HSV soluble antigen (SA) preparation. Sensitivity was assayed by means of skin tests with SA. The largest skin reactions (erythema and induration) were observed in guinea pigs which had received two sensitizing injections of infective HSV. Incorporation of Freund's complete adjuvant (FCA) into the infective HSV preparation was also found to be an effective method for sensitizing guinea pigs to SA. Inactive HSV was found to be an effective sensitizing material, suggesting that production of cutaneous hypersensitivity to HSV can occur in the absence of viral infection. A single intracutaneous injection of SA also sensitized animals to a subsequent skin test with SA.

Based upon the timing of their maximal response and the mononuclear nature of the cellular infiltrate at 24 hr, the skin reactions observed in SA sensitized animals are believed to be those of classical delayed hypersensitivity. While the skin reactions seen in these animals were not as large as those in animals sensitized with infective or inactivated HSV, they were shown to be specific for SA. Animals sensitized with infective vaccinia virus or L cell control antigen plus

FCA did not respond to skin tests with SA. Treatment of SA with DNase, pronase, and trypsin greatly reduced its skin test reactivity. Ribonuclease treatment, heating at 70 C for 2 hr, and extraction with ether did not degrade SA. SA was retained by XM-50 but not XM-100 ultrafiltration membranes. Ultracentrifugation at 66,000 x g for 0.5 hr did not sediment the skin test activity of the SA. These findings suggest that SA is a lysine and/or arginine-containing protein or deoxyribonucleoprotein with a mol wt of 50,000 to 150,000.

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