SUSCEPTIBILITY AND RESISTANCE TO COMPLEMENT-MEDIATED ANTIBODY LYSIS IN HERPES SIMPLEX VIRUS-INFECTED CELLS

by

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A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

April 1976

RESISTANCE TO COMPLEMENT-MEDIATED LYSIS IN HSV-INFECTED CELLS

MASTER OF SCIENCE (Biology)

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Susceptibility and Resistance to Complement-mediated Antibody Lysis in Herpes Simplex Virus-infected Cells

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NUMBER OF PAGES: xi, 99

ABSTRACT

Herpes simplex virus-infected cell lines were tested for susceptibility or resistance to complement-mediated antibody lysis by means of the ^{51}Cr release test. Certain human cell lines were found to be resistant to lysis. The resistance to lysis could not be correlated with the ability to produce viral progeny or the expression of viral antigens, as detectable by indirect immunofluorescence assays. Adsorption studies permitted a more precise quantitation of antigenic expression. All cell lines expressed similar quantities of antigen, and adsorbed equal amounts of antibody. Resistant cell lines consumed complement, but less efficiently than did the susceptible lines. Resistance could not be related to the ability to cap, or to the phase of the growth cycle. Resistance to lysis appeared to be a property of the cell membrane, modified by the insertion of viral specific proteins after infection. Treatment of the immunoresistant cells with neuraminidase, an enzyme which, it has been suggested, non-specifically increases the immunogenicity of target cells, resulted in the reversal of resistance to lysis in all four HSV-1-infected human cell lines, and in two of the four HSV-2 infected human cell lines. Neuraminidase did not act by unmasking viral antigens, as has been previously suggested, nor did it facilitate the binding of antibody to antigen. This was shown by adsorption of HSV-specific antiserum with neuraminidase-treated and untreated cells. Cells treated with neuraminidase consumed less complement than did untreated cells.

This suggested that neuraminidase acted by facilating the interaction of complement with the target cell, making complement uptake more efficient.

ACKNOWLEDGEMENTS

I would like to thank Dr. W. E. Rawls, my supervisor. He gave most generously of his knowledge and experience in guiding me in these studies.

I very much appreciate the patient teaching of and discussions with Drs. W. Tompkins and Pradeep Seth.

Mrs. Judy Grenville and Mrs. Terry DeCola worked very hard to type this thesis and I would like to thank them for it.

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INTRODUCTION

The interaction of antibody and complement with viral-specific antigens located on the plasma membrane of virus-infected cells frequently results in cell lysis. This phenomenon is well-established *in vitro*. Cells infected with measles virus (Kibler and TerMeulen, 1975), lymphocytic choriomeningitis (Oldstone and Dixon, 1971), Vaccinia (Singh *et al.*, 1972), herpes simplex (Smith *et al.*, 1972) and Newcastle disease virus (Eaton and Scala, 1969) are examples of systems which have been shown to be susceptible to complement-mediated antibody lysis.

While examining parameters of complement-dependant antibody lysis of cells infected with herpes simplex virus, it was observed that different cell lines varied in their susceptibility to lysis (Rawls and Tompkins, 1975). The present study was undertaken to examine the variations in lysis of cells by antibody and complement in greater detail. Initially, virus replication in the cells and expression of viral antigens at the cell surface were examined and were not found to vary. Subsequently, treatment of the resistant cell lines with neuraminidase was found to render them susceptible to lysis by antibody and complement, thus suggesting that variations in susceptibility to lysis were related to cell surface carbohydrate composition.

BACKGROUND

A. Mechanism of Complement Activation

A great deal of information has accumulated on the mechanism of action of complement (Muller-Eberhard, 1975). The group of proteins called complement is comprised of eleven or perhaps twelve glycoproteins which interact sequentially to produce cell lysis. In the classical pathway of complement activation, the recognition unit of complement, called C1q, is bound to immune complexes which consist of IgM or IgG attached to antigens on the plasma membrane. The multivalent C1q protein is attached to the Fc fragments of either IgM or two molecules of IgG which are in close proximity, resulting in a conformational change. The altered C1q then converts the proenzyme C1r into an activated form which cleaves C1s into $\overline{C1s}$. The stable association of C1q, C1r and C1s is known to require the presence of calcium ions, and is reversible. Once C1 is bound, the pathway usually proceeds to completion.

Assembly of the activation unit occurs next, and involves the sequential formation of C3 and C5 convertase. C3 convertase is produced via the interaction of C1s with C4 and C2. The cleavage products C4b and C2a unite to form C3 convertase, or $\overline{C4b2a}$, which now is bound to a second distinct site on the membrane; the by-products of the reaction, C4a and C2b, remain in the fluid phase. The catalytic site of C3 convertase, which probably resides in the C2a portion of the enzyme, now cleaves the protein C3. A by-product of this reaction, C3a, has anaphylotoxin activity. C3b becomes bound to

the C3 convertase, forming $\overline{C4b2a3b}$, or C5 convertase. C3b may also bind to other sites of an unknown nature on the cell membrane; this is responsible for the immune adherante reaction, facilitates phagocytosis by macrophages.

C5 convertase then cleaves C5 into C5a, also an anaphylotoxin, and C5b, which is then bound to a third membrane site of an unknown nature. The binding of C5b initiates the lytic stage of complement activation. One may then demonstrate the binding of C6 and C7 to form a trimolecular complex $\overline{C5b67}$, and then the binding of C8, which initiates slow lysis. The addition of C9 enhances lysis, and a maximum of six molecules may be bound.

The current hypothesis concerning the mechanism of damage is that the trimolecular complex $\overline{C5b67}$ form a channel through which C8 may enter and so come into direct contact with the cell membrane. It is possible that portions of the C8 molecule may actually extend into the interior, causing breakdown of the structure of the membrane, or perhaps forming a helical channel through the membrane. C9 may then act in the manner of a "syringe", forcing the C8 "needle" more deeply into the membrane. It is believed that only one lesion need be present to result in cell lysis - the so-called one-hit hypothesis.

Membrane damage by complement fixation operates under a variety of controls. The capacity for amplification exists at three places in the activiation of complement. $\overline{C1s}$ may activate many molecules of C4 and C2; similarily, both C3 and C5 convertase may

act repeatedly on the appropriate substrate. This effect is partly counteracted by the very brief half-lives of $\overline{C4b2a}$ and $\overline{C4b2a3b}$. In addition, the C4b portion has only a transient ability to bind to the cell membrane. Three serum inhibitors exist as well. One destroys $\overline{C1}$; another inactivator splits cell-bound C3b or the soluble C3b component of $\overline{C4b2a3b}$ into two inactivate fragments, and C4b is susceptible to a C4b inactivator which acts in a similar manner. It is also possible that C5b may be inactivated by a serum protein.

Concurrent with or alternate to the classical pathway of complement activation, the properdin pathway may be operative. In this reaction sequence, $\overline{C1s4b2a}$ formation is by passed. C3b in this pathway is produced via a series of reactions which is activated by aggregates of IgA. An initiation factor, formed as a consequence of this activation, is believed to convert the protein properdin into an active form that ultimately gives rise to a C3 convertase. This in turn produces the alternate C5 convertase, after which the reaction proceeds as described in the classical pathway. A positive feedback mechanism exerts controls on this alternate pathway.

B. The *in vivo* and *in vitro* Role of Complement-mediated Cell Lysis

An understanding of the mechanism of complement-mediated antibody lysis has been established using *in vitro* procedures. The significance of this mode of lysis *in vivo* is quite unknown. However, by-products of the pathway to activation do possess *in vivo* activity. For example, C3a and C5a, both of which are anaphylotoxins, bind to mast cells and stimulate the release of histamine. This is responsible

for the contraction of smooth muscle fibres and also is a vasodilator. In addition, these fragments have a chemotactic function, and attract polymorphonucleocytes to the site. Both C3a and C5a are susceptible to the action of a serum carboxypeptidase, which removes the carboxyl terminus of an essential arginine residue, and so destroys activity. Finally, as previously mentioned, C3b is responsible for immune adherance, which facilitates phagocytosis.

Actual lysis of target cells by activated complement has never been observed in vivo. The available evidence suggests that damage to target cells is produced indirectly. For example, the pathogenesis of lymphocytic choriomeningitis virus lies mainly in the production of cell lysis. Infected mice, after treatment with cobra venom factor which inhibits the C3 component of complement, show reduced mortality. However, C5-deficient mice are as susceptible to lymphocytic choriomeningitis as normal mice, which suggests that only the early components of complement are involved in pathogenesis, and not the later ones which induce cell lysis in vitro (Oldstone and Dixon, 1971). Incidentally, neither C5-deficient mice or C6-deficient rabbits show an increased susceptibility to disease, which further supports the view that direct cytolysis by complement has no critical function in vivo.

In the light of these facts, it is interesting to consider that several in vitro systems exist in which complement activation fails to result in cell lysis. Moloney virus-transformed lymphocytes are lysed by antibody specific for Moloney leukemia virus and complement

only in the G1 phase of the cell cycle; at all other stages, no lysis occurs, even though both C5 and C8 can be demonstrated on the cell membrane (Cooper, Polley and Oldstone, 1974). Ohanian and Borsos (Ohanian, Borsos and Rapp, 1973) presented evidence that L10 cells, a line of guinea pig hepatoma cells which express Forsmann antigen, are not lysed by the Forsmann antibody and complement, although they were lysed by a specific anti-L10 antibody and complement. A line of human lymphoid cells, RPMI 8866 was shown to vary in susceptibility to complement-mediated lysis, being least sensitive in the G1 phase (Pellegrino et al., 1974). Finally, a line of radiation-induced murine leukemia cells, RADA-1, are resistant to lysis by antibody specific for thymus leukemia antigens and guinea pig complement, although antigens are demonstratable by indirect immunofluorescence (Yu, Liang and Cohen, 1975).

The problem of resistance to complement-mediated antibody lysis assumes an even more interesting aspect when one considers the following studies, in which the enzyme neuraminidase was used to induce a greater antigenicity in the target cell. These experiments, which were conducted both in vivo and in vitro, were the theoretical basis of the decision to treat the immunoresistant HSV-infected cell lines with neuraminidase, in the hope that the enzyme would be able to reverse this resistance to complement-mediated antibody lysis. A brief summary of these experiments follows.

Neuraminidase treatment of oncogenic TA3 cells prior to injection into syngeneic mice reduced the mortality rate from 56 ± 5%

to 6 ± 4% (Sanford, 1967). It was suggested that the removal of sialic acids by neuraminidase increased the immunogenicity of the tumour cells by unmasking histocompatability antigens. Similar studies using neuraminidase-treated L1210 cells injected into mice confirmed the previous findings; again an increased survival rate was seen (Bagshaw and Currie, 1968). When the recipient mice were irradiated before the injection of neuraminidase-treated L1210 cells, the mice died. This implies that a functional immune system was vital in repressing tumour growth, and that neuraminidase had no effect on the oncogenicity of the tumour cells. This conclusion is in agreement with studies in which dimethylbenzdithionapthene-induced fibrosarcomas after treatment with neuraminidase showed a reduced transplantability when injected into mice. When immunosuppressed animals were used as recipients, however, the tumour cells grew as quickly as did untreated cells in normal animals (Ray, Thakur and Sundaram, 1976).

Neuraminidase treatment of bone marrow or spleen cells renders the cells less tolerigenic than normal, so that the hosts (mice) do not become tolerant and therefore no graft-versus-host disease occurs (Im and Simmons, 1971). Fibrosarcomas which have been induced by 3-methylcholanthrene ordinarily grow rapidly in syngeneic mice. Treatment with neuraminidase before injection results in markedly less growth; in immunosuppressed mice, however, these treated cells grew as rapidly as untreated cells (Simmons, Rios and Ray, 1971). Again, then, neuraminidase treatment seemed to increase

the immunogenicity of the tumour cells, but does not reduce the growth potential of the cells. This effect may be duplicated by direct injection of neuraminidase into tumours (Simmons and Rios; 1972). Direct injections have also been performed with some success in mice which had been spontaneously infected with mammary tumour virus (Simmons, Rios and Kersey, 1972).

Analagous studies in vitro have also been carried out. Treatment of TA3 cells with neuraminidase in vitro renders them susceptible to the cytotoxicity of normal guinea pig serum, which probably resides in a naturally occurring 19s antibody and complement (Hughes, Palmer and Sanford, 1973). C3H cells in the presence of specific anti-C3H antibody and rabbit complement are lysed only after treatment with neuraminidase (Ray and Simmons, 1972). Normal mouse serum is cytotoxic for autologous and allogeneic neuraminidase-treated cells (Rosenberg and Schwarz, 1974). Similarily human serum is cytotoxic for allogeneic human lymphocytes after treatment with neuraminidase (Reisner and Amos, 1972).

Neuraminidase is an enzyme which cleaves the α-ketosidic linkage joining sialic acids such as N-acetyl neuraminic acid to a glycoprotein substrate. These linkages may be 2→3', 2→4', 2→6' or 2→8'; all four are susceptible to cleavage by neuraminidase derived from Vibrio cholerae (comma) or Clostridium perfringins, while influenza virus neuraminidase is more specific, and will cleave only 2→3' and 2→8' linkages (Ray and Simmons, 1972).

The mechanism by which neuraminidase treatment results in the regression or cessation of growth of tumour cells *in vivo* or reverses resistance to lysis by antibody and complement *in vitro* is unknown. As already mentioned, unmasking of antigens on the plasma membrane (Sanford, 1967) or a non-specific increase in immunogenicity of the cell under study (Ray, Thakur and Sundaram, 1976; Bagshaw and Currie, 1968; Simmons, Rios and Ray, 1971) are hypotheses which have been favourably received.

An understanding of the events which occur when complement attacks a cell and lyses it, or fails to lyse it, and what happens to a cell when it is treated with neuraminidase, has important consequences both scientifically and clinicially. Knowledge of these phenomena may help elucidate features of the plasma membrane - a subject which increasingly attracts interest because of the vital role it appears to play at almost all levels of a cell's activities. Clinically, complement may be important in the pathogenesis of a disease, as in the case of glomerulonephritis. Alternatively, the failure of complement to lyse cells *in vivo*, if complement in fact has such a role, may have immunological sequellae. Finally, the effects seen with neuraminidase treatment *in vivo* and *in vitro* are sufficiently promising to encourage studies on its future use in treating human diseases.

C. Cells Infected with Herpes Simplex Virus

A system in which complement-mediated antibody lysis may be thoroughly studied, in conjunction with neuraminidase treatment,

is therefore very desirable. Such an *in vitro* system occurs when herpes simplex virus infects a cell. A great deal of information has accumulated on the topic of herpes simplex virus in recent years, but pertinent to these studies is the following.

Herpes simplex virus is a double-stranded DNA virus. Its nucleic acid has a molecular weight of 1×10^8 daltons. Two types of HSV exist: type 1, which is the causative agent of cold sores, and type 2, which results in a very common venereal disease and has been linked to cervical cancer (Rawls *et al.*, 1968; Aurelian, 1973). The virus has a particularly interesting clinical aspect; it is a latent virus. The disease is recurrent, and the virus intermittently goes into hiding - in the case of HSV-1, the trigeminal ganglion (Paine, 1964) and for HSV-2, the third sacral ganglion (Baringer, 1974). How the virus escapes detection by the body's immune system during latency is not understood.

Infection of a susceptible cell with HSV-1 or HSV-2 may lead to either a productive infection, in which case the host cell is killed, or an abortive infection. In the latter case, the virus either fails to produce viable progeny, or the host cell becomes transformed. The attachment of the virion to the host plasma membrane is very rapid, and does not require energy; it is electrostatic in nature (Hochberg and Becker, 1968; Morgan, Rose and Mednis, 1968). There is some controversy as to whether unenveloped virions can initiate an infection (Spring and Roizman, 1968; Hochberg and Becker, 1968; Abodeely, Lawson and Randall, 1970), since it is

believed that virions enter the host cell either by phagocytosis (Morgan, Rose and Mednis, 1968; Abodeely, Lawson and Randal., 1970) or by fusion of the viral envelope with the host cell's plasma membrane (Miyamoto and Morgan, 1971).

Once the capsid is within the cell's cytoplasm, uncoating of the icosohedral capsid occurs very rapidly, perhaps with the aid of the host cell's lysosomes. The viral coat proteins stay within the cytoplasm, and the double-stranded DNA is transported rapidly into the nucleus by an unknown mechanism (Hochberg and Becker, 1968).

Transcription now commences, but it does not proceed in the classical early and late pattern seen with other viral infections. Control of transcription in HSV appears to be at two levels. First, two classes of RNA are present which differ in their relative abundance, and second, although both classes are present throughout infection, different proteins may be involved. Thus, an on-off control exists in conjunction with this abundance control (Roizman and Frenkel, 1973). The scarce class of RNA is believed to code for enzymes involved with replication and synthesis of macromolecules (Frenkel and Roizman, 1972). The abundant class, which is polyadenylated (Harris and Wildy, 1975; Silverstein et al., 1973), is thought to code mainly for structural proteins; between 0.5 and 2 hrs post-infection, nineteen of the twenty-four known structural proteins may be detected. This accounts for 68% of the coding capacity of the genome (Frenkel and Roizman, 1972). Thus, HSV differs from most viruses in that other

viruses do not synthesize the bulk of their structural proteins until after the viral nucleic acid has been replicated.

It is probable that at least some of these RNA species are polymessengers that must be cleaved before translation (Wagner and Roizman, 1969). HSV-2 patterns of synthesis have not been studied as extensively, but the basic mechanism appears to be similar (Frenkel et al., 1973).

Early messenger RNA apparently codes for a thymidine kinose, which is necessary to commence DNA synthesis (Roizman, 1963). Viral DNA synthesis proceeds by a semi-conservative mechanism, within the nucleus. Structural proteins are transferred from the cytoplasm into the nucleus, a step which may involve arginine (Courtenay, McCombs and Benyesh-Melnick, 1970 and 1971). This could explain the requirement of arginine for HSV growth (Becker, Olshevsky and Levitt, 1967). Capsids are assembled in the nucleus, in an intranuclear crystal (Nii, Morgan and Rose, 1968). An envelope is acquired by budding of the capsid through the nuclear membrane into a cytoplasmic vacuole (Asher, Heller and Becker, 1969; Darlington and Moss, 1968). A number of the virus-specific proteins are glycosylated prior to insertion in the nucelar membrane (Spear and Roizman, 1970); the virus apparently buds through regions of the membrane which contain viral-specific antigens but no cellular antigens.

The effects of the virus upon its host are manifold. The killing of the host cell is due to the cessation of cellular DNA, RNA

and protein synthesis (Kaplan, 1973). Viral-specific antigens appear upon the plasma membrane, rendering the cell susceptible to neutralization by antibody, lymphocyte attack or complement-mediated antibody lysis. A visible cytopathic effect occurs, manifested as any one or a combination of the following: rounding up of cells, aggregation, and syncitia formation (Ejercito, Kieff and Roizman, 1968).

Because certain cells which have been infected with HSV-1 or 2 are susceptible to lysis by antibody and complement, it seems worthwhile to review briefly the information concerning HSV-specific antigens. Human epidermoid carcinoma cells which have been infected with HSV-1 synthesize forty-nine new polypeptides, as detected by high resolution polyacrylamide gel electrophoresis. Of these, twenty-four are known to be structural, and fifteen are non-structural; the remaining nine have an unknown function, but are probably also non-structural. These polypeptides account for 75% of the genetic information in HSV-DNA (Honess and Roizman, 1973). Using an identical system, fifty-one polypeptides are demonstratable when HSV-2 is the infecting agent (Powell and Courtenay, 1975).

In HSV-infected cells, at least twelve new proteins, of which nine are glycosylated, appear on the plasma membrane (Heine, Spear and Roizman, 1972). HSV-1 is closely related antigenically to HSV-2; hybridization studies show a base sequence homology of 50%. Now, of these twelve proteins, at least two are known to be type-specific (Sim and Watson, 1973). The bulk of the cross-reacting

antigen resides in a precipitin band detectable by polyacrylamide gel electrophoresis, called Band II (Watson and Wildy, 1969). Anti-serum to Band II will neutralize both HSV-1 and HSV-2; adsorption of this serum with the heterologous virus produces a type-specific antibody activity (Nahmias et al., 1971).

The extent of cross-reaction between HSV-1 and HSV-2 causes great difficulties in distinguishing by immunological means the infecting type. Many tests have been devised; these have been reviewed by Plummer (Plummer, 1973). Included among the more successful are: neutralization kinetics (Ashe and Scherp, 1963); microneutralization tests (Pauls and Dowdle, 1967); microindirect hemagglutination (Fucillo et al., 1970); indirect fluorescence (Geder and Skinner, 1971); direct fluorescence (Nahmias et al., 1969); and the ^{51}Cr release test (Smith et al., 1972). Various biological markers, such as thermolability, density of DNA, and plaque size may be used if one isolates the virus (Figueroa and Rawls, 1969). Of these procedures, the ^{51}Cr release test has been found to be an especially useful tool; the test is flexible, quantitative, reproducible and can distiniguish cross-reacting and type-specific HSV antigens (McClung, Seth and Rawls, 1976).

In these studies, various cell lines were examined for susceptibility to complement-mediated antibody lysis after infection with HSV-1 or HSV-2. The ^{51}Cr release test, in which infected cells are labelled with radioactive sodium chromate and subsequently treated with antibody and complement was used to quantitate the response of

these cell lines. Growth curves of HSV were performed in each cell line to determine whether viral progeny was being produced; the production of viral specific antigens was demonstrated by the use of indirect immunofluorescence assays. Because certain cell lines were resistant to lysis even with very high concentrations of antibody and complement, neuraminidase treatment was carried out. Reversal of resistance to lysis was seen in all four HSV-1-infected cell lines tested, and in two of four HSV-2-infected lines. Studies were then performed in an attempt to determine the mechanism of resistance to lysis and the effect of neuraminidase treatment on these cells.

MATERIALS AND METHODS

A. Tissue Culture and Virological Techniques

1. Cell Lines Used

Studies of complement-mediated cytotoxicity in herpes simplex virus-infected cells were performed on the following cell lines: HCT-8, a human adenocarcinoma of the colon (Tompkins et al., 1974); HT-29, another human adenocarcinoma of the colon (obtained from J. Fogh at the Sloan-Kettering Institute, New York, N.Y.): HEI-407, human embryonic intestine (GIBCO); HEL, human embryonic lung (obtained from S. Goldstein, Department of Medicine, McMaster University, Hamilton, Ontario); VERO, a line of African green monkey kidney cells (Flow Laboratories); BHK-21, baby hamster kidney cells (Ann Arbor); MB-1, MB-2, MB-3, clones of BHK-21 (isolated by M. Buchmeier, McMaster University, Hamilton, Ontario).

2. Medium

HCT-8, HT-29 and HEI cells were propagated in 75 cm^2 tissue culture plastic flasks (Corning) containing RPMI 1640 medium supplemented with 20% v/v heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.03% w/v glutamine, 0.75 g/l $NaHCO_3$, 0.01 M Hepes buffer and 0.01 M Tricine buffer. These reagents were obtained from GIBCO. HEL, VERO, BHK-21, MB-1, MB-2 and MB-3 cells were grown in Minimal Essential Medium, F-15 (MEM, GIBCO).

plus 10% v/v fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.03% w/v glutamine, and 0.75 g/l $NaHCO_3$.

3. Virus Strains

KOS was a herpes simplex virus type 1 isolate obtained from a lip lesion on a patient in Houston, Texas. Herpes simplex virus strain 219 was a type 2 isolate derived from a patient with chronic cervicitis in India.

Stocks of these two strains were prepared in VERO cells and assayed by the plaque-overlay method. Monolayers of VERO cells were incubated with 10-fold dilutions of virus for 1.5 h. at 37°C and then overlaid with 2x MEM containing 10% w/v fetal calf serum, 400 µg/ml protamine sulfate (Sigma), 1.5 g/l $NaHCO_3$, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 U/ml mycostatin (Squibb) and 1% w/v Bactoagar (DIFCO Laboratories). After incubation for 3-4 days at 37°C, the agar overlay was removed. Cells were then fixed and stained with 2.5% v/v buffered formalin (BDH Chemicals) plus 1.0% crystal violet (Fischer Scientific Company), and the plaques were enumerated.

4. Growth Curves

A multiplicity of infection (MOI) of 1 PFU/cell was used to infect monolayers of cells. After adsorption at 37°C for 1 hr., cultures were refed with the appropriate medium and incubated at 37°C for various times. Duplicate samples were withdrawn and frozen at -45°C; prior to assay, the infected cells were frozen and

thawed twice. The duplicate samples were then pooled and 10-fold dilutions were made. A volume of 0.2 mls of each dilution was then plated out in triplicate on monolayers of VERO cells in 60 x 15 mm standard plastic dishes (LUX Scientific) and assayed by the agar overlay technique already described. Results were expressed as log PFU/cell.

B. Immunological Techniques

1. Preparation of Antisera and Complement

Ultra-violet irradiated HSV, at a concentration of 1×10^8 PFU was injected intra-peritoneally into New Zealand white rabbits. Beginning 2 weeks later, the rabbits received a series of 3 injections, given 10 days apart. The inoculum consisted of 1×10^8 PFU live HSV in complete Freund's adjuvant injected intramuscularly. The rabbits were bled 10 days after the final injection and serum was collected.

Pooled guinea pig serum was used as a source of complement. Both antisera and complement was stored at -70°C until used.

2. Indirect Immunofluorescence Assays

Monolayers of cells were infected with 1-2 PFU/cell of HSV-1 or 2 and incubated at 37°C for various times. Monolayers were then washed twice in phosphate buffered saline without Ca^{++} or Mg^{++} (PBS) and trypsinized. These cells were then suspended in TBS + 2% FCS and counted via trypan blue exclusion; the concentration

was adjusted to 5 x 10^5 viable cells/ml. One ml of cells was centrifuged for 3' at 1500 rpm in an IEC PR-J centrifuge (Damon/IEC Division), and the pellet of cells was incubated with 0.1 ml of rabbit anti- HSV antisera diluted 1:20 or 1:40 for 30' at 37°C. Cells were then washed twice in 2.0 mls of TBS + 2% FCS. If cells were to be fixed, at this point 3% buffered formalin was added for 4' at 4°C. Subsequently, cells were washed once with TBS, and the pelleted cells were incubated with 0.10 ml of 1:10 fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories) - for 30' at 37°C. Again, cells were washed twice and then resuspended in TBS - glycerol (1:9) and mounted on slides. Fluorescence was examined using a Leitz OrthroPlan fluorescent microscope.

C. ^{51}Cr Release Test

The method of Smith et al. (1972) was used, with some modifications. Confluent monolayers of cells were infected with HSV-1 or 2 at an MOI of 2-5 PFU/cell. After adsorption for 1 hr. at 37°C, the cells were refed with 10.0 mls of medium and incubated at 37°C. At about 8 hrs. post-infection, 200 µCi of ^{51}Cr, obtained as sodium chromate (New England Nuclear), was added. Following further incubation at 37°C for 10-12 hrs., cells were harvested by trypsinization. Single-cell suspensions were washed 5 times in 10.0 ml volumes of cold TBS + 2% FCS. Cells were then counted by trypan blue exclusion, and the concentration was adjusted to 5 x 10^5

viable cells/ml. After heat-inactivation at 56°C for 30', doubling dilutions of the test antiserum in a volume of 0.1 ml were then mixed with 0.1 ml of the labelled cell suspension and incubated for 1 hr. at 37°C. An appropriate dilution of guinea pig complement, in a volume of 0.2 mls, was added, and this mixture was incubated for a further 1.5 hrs at 37°C. Two mls of cold TBS + 2% FCS were added to stop the reaction. Cells were then pelleted by centrifugation at 1500 rpm for 3'. One ml of the supernatant was carefully removed, transferred to a fresh test-tube, and counted for radioactivity in a Beckman Gamma 300 Radiation Counter (Beckman Instruments, Inc., Fullerton, Ca.).

Maximum ^{51}Cr release was determined by lysing 0.1 ml of labelled cells with 0.3 mls of distilled water and freeze-thawing once. Spontaneous ^{51}Cr release was determined in two ways. As a complement control, 0.1 ml cells was mixed with 0.2 ml guinea pig complement and 0.1 ml diluent (TBS + 2% FCS). For antibody controls, 0.1 mls cells received 0.2 ml diluent plus 0.1 ml dilution of antisera. All dilutions and controls were performed in duplicate and counts per minute were averaged.

The percent specific ^{51}Cr release was calculated according to the formula of Brunner et al. (1968):

$$\% \text{ specific } {}^{51}Cr \text{ release} = \frac{{}^{51}Cr \text{ release with Ab and complement} - \text{spontaneous release} \times 100\%}{\text{maximum } {}^{51}Cr \text{ release} - \text{spontaneous release}}$$

D. Treatment of Cells with Neuraminidase

As described above, labelled infected cells were washed and counted. Dilutions of neuraminidase (GIBCO, stated activity = 500 U/ml)were made in a total volume of 1.0 ml of TBS containing calcium and magnesium ions, pH 7.2 and added to 1 x 10^6 pelleted cells. The enzyme was allowed to react at 37°C for 1 hr with occasional shaking. Ten mls of TBS + 2% FCS were used to wash the treated cells, and the concentration was again adjusted to 5 x 10^5 cells/ml. Analagous controls for maximum and spontaneous release were performed. Alternately, neuraminidase treatment was carried out after antibody incubation or after complement had reacted. In these latter experiments, treated cells were washed with 2.0 mls of TBS + 2% FCS, and controls were done for each dilution of antibody and complement.

E. Adsorption of Antisera with HSV - Infected Cells

Monolayers of cells to be tested were inoculated with HSV type 1 or 2 at MOI = 2-5 PFU/cell. After adsorption for 1 hr at 37°C and refeeding, cells were incubated at 37°C until a complete cytopathic effect was observed (usually 18 hrs). Infected cells were then washed twice with PBS, trypsinized, and counted. Uninfected cells were used as controls and were treated identically to the infected cells.

If neuraminidase treatment was carried out, at this point dilutions of cells were incubated with 10.0 U of neuraminidase in a total volume of 1.0 ml for 1.0 hr at 37°C with occasional shaking. Then cells were washed once with 10.0 mls of TBS.

Treated, untreated, and control cells were then diluted into concentrations varying between 5×10^5 cells/ml and 1×10^7 cells/ml. These were centrifuged and the pelleted cells were incubated with 2, 5, or 10 units of antibody. One unit of antibody was defined as the dilution which produced 50% specific ^{51}Cr release in a standard cytotoxicity test, using HSV - 1 or 2 - infected BHK-21 cells. Adsorption of antisera was carried out for 1 hr at 37°C with occasional shaking and then overnight at 4°C with continual shaking. The serum was clarified by centrifuging at 4000 rpm for 2 hrs. Residual antibody activity was assayed in the standard ^{51}Cr release test using HSV - infected BHK - 21 cells.

F. Complement Consumption Test

The procedure for the complement consumption test as described by Kagan and Norman (1970) was followed, with some modifications.

1. Colour Standards

Sheep red blood cells, obtained as a 50% suspension in Alsever's solution (Connaught Laboratories, Limited, Willowdale, Ontario) were washed three times in Veronal Buffered Diluent

(VBD; 1 volume of 5 x Veronal Buffered Saline plus 4 volumes of 0.125% w/v gelatin water). Packed red blood cells were resuspended in VBD at a final concentration of 2.8% v/v.

The washed cells were diluted 10-fold to give a 0.28% cell suspension. Aliquots of these cells were then mixed with a haemoglobin solution to produce a series of tubes corresponding to 0% to 100% haemolysis, in 10% increments. The haemoglobin solution was prepared by lysing 1.0 ml of the 2.8% v/v washed sheep red blood cells with 7.0 mls of distilled water; 2.0 mls of 5 x VBS was added to stabilize the solution. The test proper was read against the colour standards. Extrapolation to the nearest 5% was made when necessary.

2. Titration of Complement

Sheep red blood cells were sensitized by incubating 1 volume of the washed 2.8% v/v cell suspension with 1 volume of haemolysin (GIBCO), diluted 1:2000, for 15' at room temperature. This dilution of haemolysin was previously determined as optimal, by plotting percent haemolysis versus haemolysin dilution. The optimal haemolysin dilution was that dilution on the plateau of the curve.

A series of test-tubes containing aliquots of VBD was set up. Then aliquots of guinea pig complement (obtained in a lyophilized form from GIBCO) diluted 1:200 in VBD were added so that the total volume was equal to 0.8 mls. Thus a concentration bracket of complement dilutions varying from 1:200 to 1:1600 was

arranged. This was incubated with 0.2 ml of sensitized sheep red blood cells for 30' at 37°C in a water bath. After centrifugation at 2000 rpm for 10 minutes to pack unlysed cells, the tubes were read against the colour standards. The graphing method was then employed to determine the volume of this dilution of complement which contained one 50% unit of complement (1 C'H50). The $\log_{10}$ of the ratio of the percentage of lysed cells, y, to the percentage of non-lysed cells, 100-y, was plotted against the $\log_{10}$ of the volume of the complement dilution in each test-tube. The volume which contained 1 C'H50 was estimated by extrapolation. The dilution of complement required to produce 5 C'H50 in a volume of 0.4 ml was then calculated.

3. Antigen Titration

A checkerboard titration of antigen was carried out. All cell lines to be tested were infected with HSV under identical conditions, as described previously, and then harvested and counted on the next day. Each cell line was divided into 2 equal portions. Ten units of neuraminidase per 1 x 10^6 pelleted cells was added to one portion. The enzyme was incubated with the infected cells for 1 hr at 37°C with occasional shaking. After washing, treated and untreated cells were diluted in serial 2-fold dilutions. A volume of 0.2 ml of each dilution was mixed with 0.2 mls of each antibody dilution. For antibody, heat-inactivated rabbit anti-HSV antisera was diluted in 2-fold increments. After incubation with antigen at room temperature for 20 minutes, 0.4 ml cold complement was added

(5 C'H50/tube). All tubes were incubated for 15-18 hrs at 4°C. Prewarmed tubes each then received 0.2 ml sensitized sheep red blood cells, and these were incubated for 30 minutes at 37°C in a water bath. After centrifugation at 2000 rpm for 10 minutes, the percent haemolysis was determined by comparison with the colour standards.

Each test always included antibody controls in which 0.2 mls VBD was substituted for antigen, and complement controls for each antigen dilution using 5 C'H50, 2.5 C'H50, 1.25 C'H50 and no complement. If the controls were not acceptable, the test was discarded.

G. Thiobarbituric Acid Assay of Neuraminidase Activity

A standard assay of neuraminidase activity was performed using human α-1 acid glycoprotein (gift of Dr. Mark Hatton, McMaster University, Hamilton, Ontario) as substrate. Released sialic acids were determined by the thiobarbituric acid procedure described by Warren (1959) and modified by Aminoff (1961) and Aymard-Henry et al. (1973).

Concentrations of human α-1 acid glycoprotein were reacted with different dilutions of neuraminidase for various time intervals. Then 0.2 ml of the sample was mixed with 0.1 ml periodate-phosphoric acid reagent (see Appendix A for preparations of the reagents used in this test) for 20' at room temperature. One ml of arsenite reagent was added, and the test-tubes were shaken vigorously twice. The reaction is stable at this point and may be stored at 4°C for several days

without loss of activity. Three mls of 2-thiobarbituric acid reagent was added and the test-tubes were immersed in a boiling water bath for 15'. After cooling with tap water for 5', 4.0 mls of cyclohexanone (Eastman Kodak) was added and the tubes vortexed twice. The extracted chromogen was separated from the aqueous phase by centrifugation for 3' at 1000 rpm, and the upper red organic layer was read spectrophotometrically (Beckman Acta III, Beckman Instruments, Inc., Fullerton, Ca.). The spectrophotometer was zeroed with distilled water and optical densities were read at 549 nm and 532 nm. The latter reading corrects for interfering non-sialic acid chromophores which absorb maximally at 532 nm.

μ moles of sialic acid present in the sample was calculated according to the formula:

$$\epsilon \text{ sialic acid} = 57{,}000$$

1) μ moles sialic acid = $0.070 \times O.D._{549}$ (no interfering biological material)

<u>or</u>

2) μ moles sialic acid = $(0.084 \times O.D._{549}) - (0.031 \times O.D._{532})$ (in the case of contaminating non-sialic acids)

Readings were performed within the first hour of completion of the test. Calibration curves, using N-acetyl neuraminic acid (Sigma) in a concentration range between 0.01 and 0.05 μ moles were performed for each separate experiment.

H. Assay for Protease Activity in the Neuraminidase Preparation

Preparations of neuraminidases frequently have been found to be contaminated with proteases. Therefore an assay was designed to determine whether any protease activity was demonstratable in the GIBCO neuraminidase. Ten λ of Iodine-125 - labelled immunoglobulin G, which had been labelled by the chloramine-T method (McConahey and Dixon, 1966) was precounted and used as substrate. A volume of 0.1 ml of bovine serum albumin (Sigma Chemical Co.), 1.0 mg/1.0 ml, was used as a carrier protein. All reagents were dissolved in TBS, and where necessary, TBS was used as a diluent. Proteases VI and X (Sigma Chemicals) were used as positive controls; the test proper involved incubation of ^{125}I-IgG + BSA with no additions, with neuraminidase or with these proteases in a total volume of 1.0 ml for 1 hour at 37°C, a condition chosen to emulate the procedure used in the ^{51}Cr release test. One ml of 10% w/v trichloroacetic acid was added to precipitate any protein, and this was incubated for 1 hr at room temperature. Tubes were then centrifuged for 30 minutes at 4000 rpm, and then the supernatant was removed by aspiration and transferred to a clean test-tube. Both supernatant and pellet were counted in a gamma counter, and the percent of total counts found in the supernatant was then calculated from duplicate aliquots.

RESULTS

A. Growth Curves

To ascertain that virus replication was occurring in all cell lines studied, growth curves of herpes simplex virus types 1 and 2 were carried out. Graphs of log PFU/cell versus time are shown in Figs. 1, 2 and 3 and the results are summarized in Table I.

An eclipse phase was clearly evident in all KOS-infected cells. An increase of 1 to 2 logs PFU/cell was visible in all cell lines by 24 hrs post-infection (Table I) except the BHK clone MB-1. Although no net synthesis was seen in this cell line, an eclipse was obvious, suggesting that replication of KOS was occurring but at greatly reduced levels. Values for the 48 hr time points did not differ markedly from the 24 hr levels (Table I).

Replication of type 2 (strain 219) could not be demonstrated in all cell lines. Production of progeny was lowered by 0.5 to 2 logs compared to KOS-infected cells (Table I). No replication occurred in MB-3 cells, and very low levels were apparent in MB-1 cells. Again, however, eclipse occurred. In addition, viral-specific antigens were detectable by immunofluorescence. These two facts suggested that viral adsorption, penetration and uncoating had taken place, and that low levels of viral progeny had been produced.

FIGURE 1

Growth Curves of HSV-1 and 2 in HT-29 Cells

Monolayers of cells were infected with MOI of 1 PFU/cell. After adsorption for 1 hr. at 37°C, monolayers were washed, refed and incubated at 37°C. Duplicate samples were withdrawn at the indicated times and assayed for total virus yield by the plaque overlay technique.

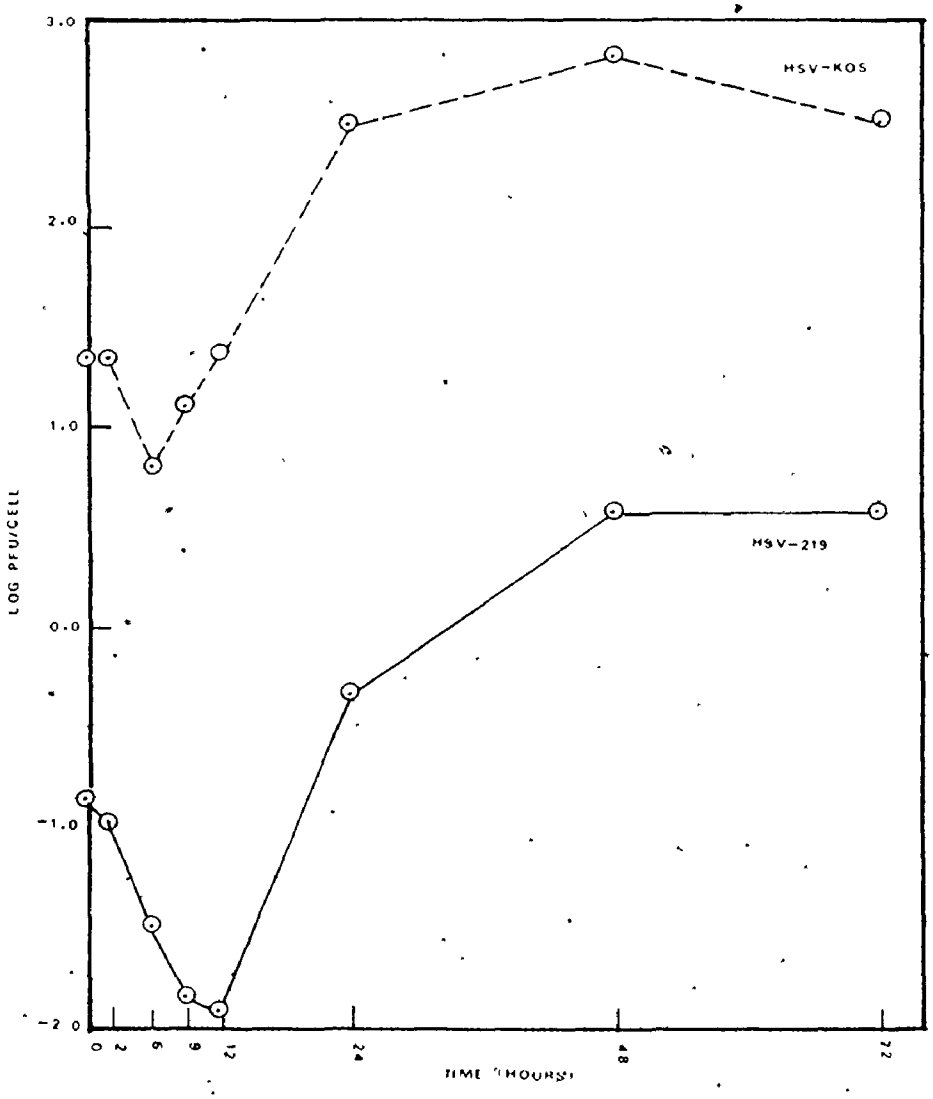

3.0
2.0
1.0
0.0
-1.0
-2.0
LOG PFU/CELL
HSV-KOS
HSV-219
0
2
6
9
12
24
48
72
TIME (HOURS)

FIGURE 2

Growth Curves of HSV-1 and 2

in Various Human Cell Lines and in VERO Cells

Monolayers of cells were infected with MOI of 1 PFU/cell. After adsorption for 1 hr. at 37°C, monolayers were washed, refed and incubated at 37°C. Duplicate samples were withdrawn at the indicated times and assayed for total virus yield by the plaque overlay technique.

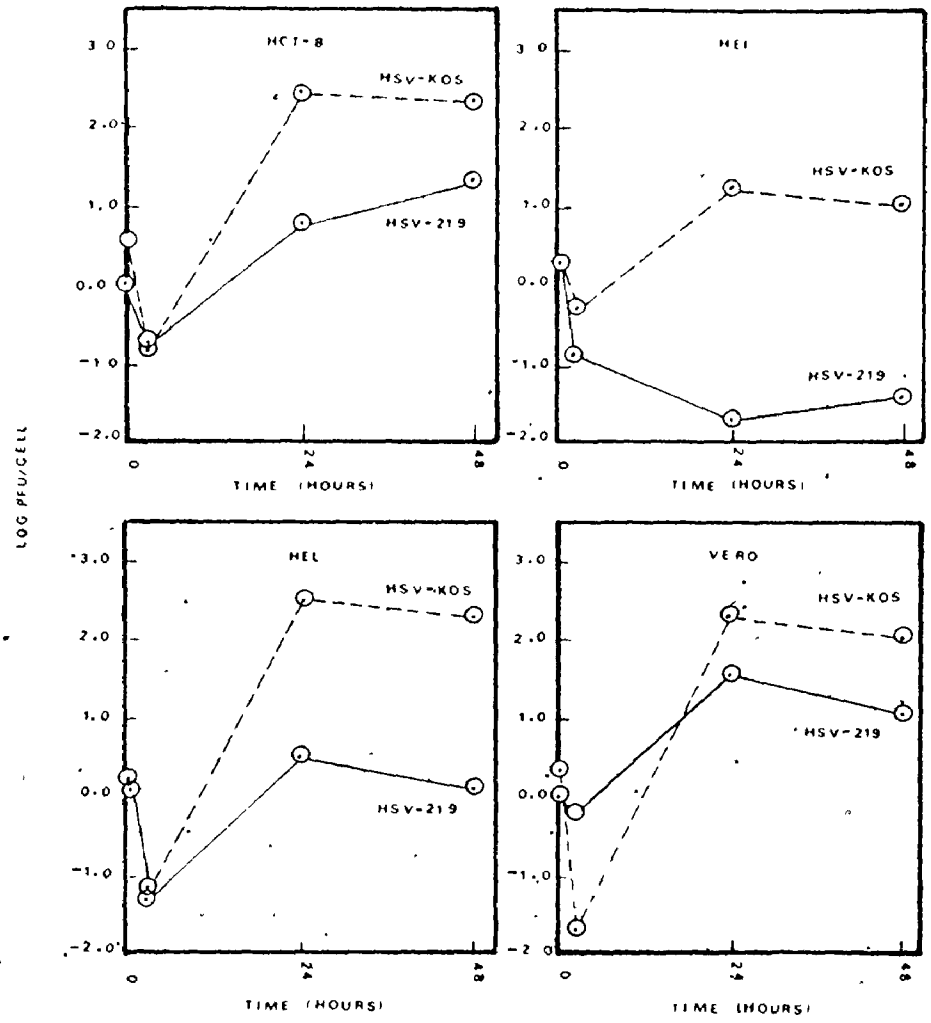

HCT-8
HSV-KOS
HSV-219
HEL
HSV-KOS
HSV-219
HEL
HSV-KOS
HSV-219
VERO
HSV-KOS
HSV-219
LOG PFU/CELL
TIME (HOURS)
TIME (HOURS)
TIME (HOURS)
TIME (HOURS)

FIGURE 3

Growth Curves of HSV-1 and 2

in Cells of Baby Hamster Kidney Origin

Monolayers of cells were infected with MOI of 1 PFU/cell. After adsorption for 1 hr. at 37°C, monolayers were washed, refed and incubated at 37°C. Duplicate samples were withdrawn at the indicated times and assayed for total virus yield by the plaque overlay technique.

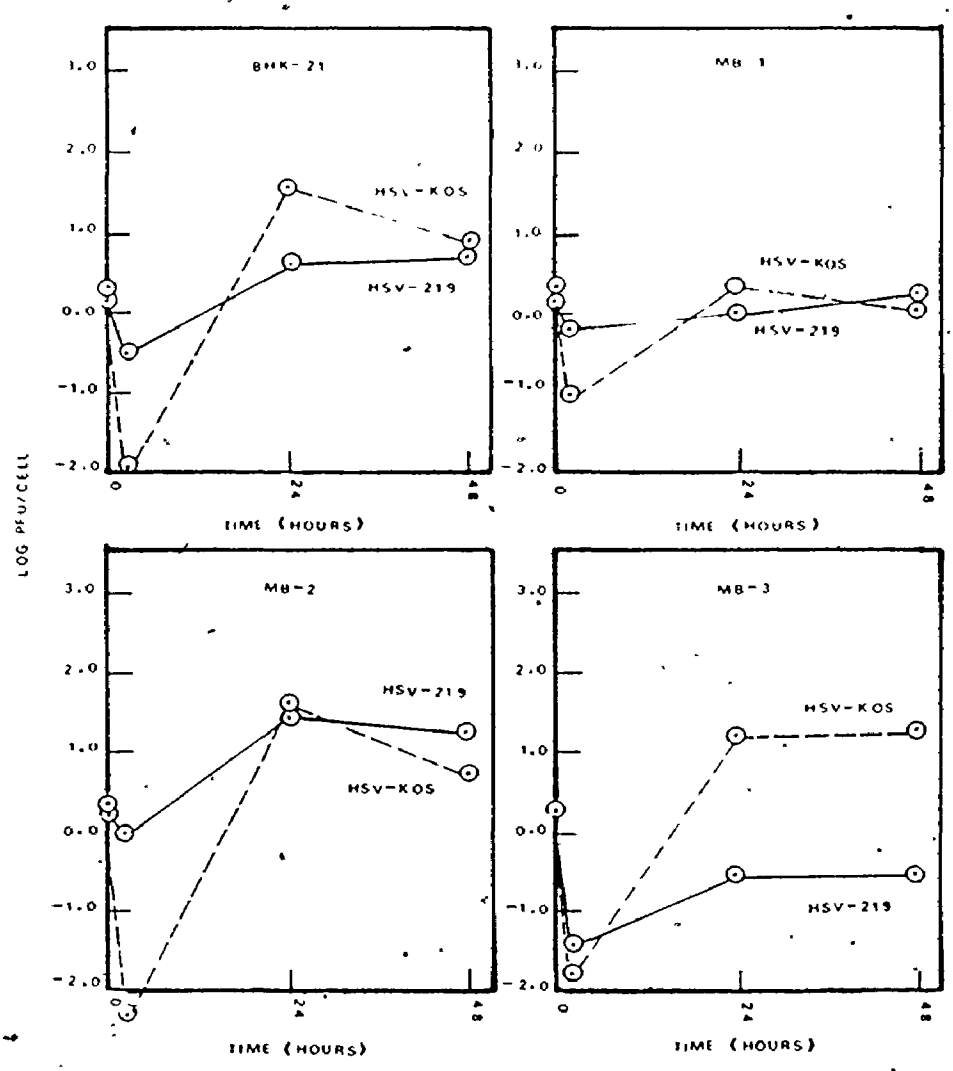

BHK-21
HSV-KOS
HSV-219
MB 1
HSV-KOS
HSV-219
MB-2
HSV-219
HSV-KOS
MB-3
HSV-KOS
HSV-219
LOG PFU/CELL
TIME (HOURS)
TIME (HOURS)
TIME (HOURS)
TIME (HOURS)

TABLE I

HSV REPLICATION IN VARIOUS CELL LINES

A. REPLICATION OF HSV-KOS (TYPE 1)

	Log_{10} Increase, PFU/cell, in HSV-KOS Titre			
	Experiment 1		Experiment 2	
Cell line	24 hr	48 hr	24 hr	48 hr
HT-29	1.17	54	2.08	1.90
HCT-8	2.01	2.06	1.79	1.57
HEI	0.91	0.96	0.78	0.70
HEL	2.49	2.00	2.36	2.18
VERO	2.01	1.69	not done	
BHK-21	1.27	0.85	not done	
MB-1	0.00	0.00	not done	
MB-2	1.32	0.44	not done	
MB-3	0.89	0.96	not done	

TABLE I (CONTINUED)

HSV REPLICATION IN VARIOUS CELL LINES

B. REPLICATION OF HSV-219 (TYPE 2)

	Log_{10} Increase, PFU/cell, in HSV-219 Titre			
	Experiment 1		Experiment 2	
Cell line	24 hr	48 hr	24 hr	48 hr
HT-29	0.53	1.45	0.86	
HCT-8	0.63	1.65	0.90	1.11
HEI	-1.64	-1.62	-1.53	-0.89
HEL	0.14	0.09	0.41	0.07
VERO	1.53	1.04	not done	
BHK-21	0.44	0.52	not done	
MB-1	-0.16	0.07	not done	
MB-2	1.30	1.07	not done	
MB-3	-0.82	-0.82	not done	

The case of HEI cells differed. Repeated attempts to observe virus replication or at least an eclipse were always negative. The results of immunofluorescent studies in this cell line suggested that the viral genome was expressed within the cell, since type 2-specific antigens were observed on the surface of infected cells. The precise step at which replication is blocked is unknown.

B. Indirect Immunofluorescence Assays

In all cell lines infected with herpes simplex virus type 1 or 2, the presence of viral-specific antigens was demonstratable by indirect immunofluorescence (Table II). Little difference could be seen in any of the cell lines, either quantitatively or qualitatively. Fluorescence was seen in 75 to 100% of all cell lines, and the intensity of the fluorescence was similar in all lines. No significant change in this pattern occurred by 18-20 hrs. post-infection, when ^{51}Cr release assays were performed. Surface antigens appeared within 5 hours after infection.

The time course of appearance of intracellular antigens, which were detected by fixing the cells in acetone and then performing the standard immunofluorescent technique, was followed in VERO, HCT-8 and HT-29 cells. Generally, perinuclear antigen was visible by 4 to 6 hrs post-infection, followed by the appearance of nuclear antigen at approximately 10 hrs. Cytoplasmic fluorescence occurred between 12 and 14 hrs post-infection.

TABLE II

EXPRESSION OF ANTIGENS ON THE SURFACE OF HSV-INFECTED CELLS AS DETECTED BY IMMUNOFLUORESCENCE[a]

	Intensity of Reaction[b]	
Cell line	HSV-KOS-infected cells	HSV-219-infected cells
HT-29[c]	3+	3+
HCT-8[c]	3+	3+
HEI	3+	3+
HEL	3+	3+
VERO	3+	3+
BHK-21	4+	3+
MB-1	3+	3+
MB-2	3+	3+
MB-3	3+	2+

[a] An MOI = 1-3 PFU/cell was used to infect cells. The immunofluorescence test was performed 10-12 hr post-infection.

[b] Rabbit anti-HSV antisera, diluted 1:20, was the source of antibody. All controls, using normal rabbit sera in place of specific antibody, were negative. Intensity was estimated on a scale from 0 to 4+, where 4+ was maximum.

[c] These two cell lines were observed to cap, with low frequency (estimated at approximately 10% of all cells).

Antibody-induced redistribution of the surface antigens was observed on about 10% of infected HCT-8 and HT-29 cells.

C. Cytotoxicity Studies in HSV-infected Cells

The cell lines studied were shown to differ in their susceptibility to complement-mediated antibody lysis as detected by the ^{51}Cr release test. Two patterns of response seemed evident. First, a difference between KOS- and 219-infected cells was apparent, and the other effect involved the response of infected cells to increasing complement concentration.

^{51}Cr release data for KOS-infected cells of baby hamster kidney origin are shown in Table III. At a high complement dilution of 1:64, the 50% endpoint titres of antibody were 126 for BHK-21 cells, < 10 for MB-1, 29 for MB-2, and 28 for MB-3 cells. These figures may be compared to 112, < 10, < 10, and < 10, respectively, for 219-infected cells (Table IV). In at least two of these clones, then, the 50% endpoint antibody titres were lower than in KOS-infected cells.

When complement concentration was increased to 1:4, percent specific ^{51}Cr release increased dramatically. KOS-infected cells showed 6 to 9-fold rises in titres, while 12 to 17-fold increases occurred in 219-infected cells. There was no significant difference observed in 50% endpoint antibody titres between KOS- and 219-infected cells at such high complement concentrations.

TABLE III

EFFECT OF COMPLEMENT DILUTION OF THE CYTOLYTIC TITRE OF ANTIBODY IN CELLS OF BABY HAMSTER KIDNEY ORIGIN INFECTED WITH HSV-KOS (TYPE 1)

Cell Line	Dilution of Guinea Pig Complement	Percent Specific ^{51}Cr Resease Reciprocal Antibody Dilution						50% Endpoint Titre of Antiserum[a]
		20	40	80	160	320	640	
BHK-21	1:64		104.0	75.1	37.5	8.6	<5.0	126
MB-1	1:64	12.6	20.3	5.1	<5.0	<5.0	<5.0	<10
	1:4	86.8	78.9	34.7	<5.0	<5.0	<5.0	63
MB-2	1:64	71.0	41.1	30.6	<5.0	<5.0	<5.0	29
	1:4	76.3	91.9	82.6	70.7	41.7	13.8	260
MB-3	1:64	72.9	75.3	42.3	17.0	8.8	<5.0	28
	1:4	78.8	85.9	74.5	57.8	24.8	<5.0	186

[a] The 50% endpoint is defined as the reciprocal of the dilution of antisera which produces 50% specific ^{51}Cr release.

TABLE IV

EFFECT OF COMPLEMENT DILUTION ON THE CYTOLYTIC TITRE OF ANTIBODY IN VERO CELLS AND CELLS OF BABY HAMSTER KIDNEY ORIGIN INFECTED WITH HSV-219 (TYPE 2)

Cell Line	Dilution of Guinea Pig Complement	Percent Specific ^{51}Cr Release — Reciprocal Antibody Dilution						50% Endpoint Titre of Antiserum[a]
		20	40	80	160	320	640	
BHK-21	1:64		56.5	51.2	31.2	16.8	13.3	112
MB-1	1:64		25.1	20.2	12.4	5.8	<5.0	<10
	1:4	90.9	77.7	69.7	40.8	17.8	<5.0	129
MB-2	1:64		19.0	18.3	13.3	5.9	<5.0	<10
	1:4	92.0	78.2	52.6	20.4	<5.0	<5.0	170
MB-3	1:64		20.0	13.5	7.0	<5.0	<5.0	<10
	1:4	86.3	81.0	69.3	40.6	15.7	<5.0	126
VERO	1:64	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<10
	1:4	79.2	63.3	51.6	31.6	8.6	<5.0	148

[a] See Table III.

The results for cell lines of human origin are shown in Table V. All cell lines remained insusceptible to lysis at complement dilutions of 1:64 and 1:16, regardless of whether KOS or 219 was the infecting agent. When complement was used at a dilution of 1:4, KOS-infected HCT-8 and HEL cells produced 50% endpoint antibody titres of 35 and 71 respectively. HT-29 cells and HEI cells were still completely immunoresistant. Infection with type 2 virus resulted in 50% endpoints of less than 10 in all four human cell lines. Again, the patterns of response differs between KOS- and 219-infected cells. With cells of human origin, however, increasing the concentration of complement seemed to have little or no effect, in contrast to VERO cells and cells of baby hamster kidney origin.

D. Neuraminidase Treatment of HSV-infected Human Cells

Because various authors (Bagshaw and Currie, 1968; Ray and Simmons, 1971) had suggested that treatment of cells with neuraminidase increased their immunogenicity, this enzyme was chosen to treat the relatively immunoresistant HSV-infected cells of human origin.

The first step was to determine which concentration of neuraminidase should be used to treat cells. A titration of HSV-specific antiserum in KOS-infected HT-29 cells which had been treated with various concentrations of neuraminidase gave the results shown in Table VI. (The activity of the preparation of neuraminidase was tested by the thiobarbituric acid assay for released sialic acids;

TABLE V

EFFECT OF DILUTION OF COMPLEMENT ON THE 50% ENDPOINT TITRE OF ANTISERUM IN HSV-INFECTED HUMAN CELLS

Cell Line	Infection with[a]	50% Endpoint Titre of Antiserum[b] Dilution of Guinea Pig Complement		
		1:64	1:16	1:4
HT-29	KOS	<10	<10	<10
	219	<10	<10	<10
HCT-8	KOS	<10	<10	35
	219	<10	<10	<10
HEI	KOS	<10	<10	<10
	219	<10	<10	<10
HEL	KOS	<10	<10	71
	219	<10	<10	<10 (3)

[a] Each cell line was infected with an MOI=2-5 PFU/cell. After adsorption for 1 hr at 37°C and refeeding, the infected cells were incubated for a further 18-20 hr before assay by the ^{51}Cr release test.

[b] See Table III.

TABLE VI

TITRATION OF HSV-SPECIFIC ANTISERA ON HSV-KOS-INFECTED HT-29 CELLS AFTER TREATMENT WITH VARIOUS CONCENTRATIONS OF NEURAMINIDASE

Concentration of Neuraminidase[b]	Percent Specific ^{51}Cr Release[a] Reciprocal Antibody Dilution					50% Endpoint Titre of Antisera
	10	20	40	80	160	
200u	100	100	100	78	37	126
150u	99	94	92	73	33	118
100u	100	99	93	74	36	123
50u	91	92	86	60	23	96
20u	100	100	91	54	21	87
10u	93	92	77	48	15	76
5u	100	83	72	37	10	62
1u	96	75	54	22	<5	44
.1u	49	37	25	10	<5	<10
.01u	26	19	10	<5	<5	<10
none	12	6	<5	<5	<5	<10

[a] Percent specific ^{51}Cr release in the presence of excess complement (1:4).

[b] Concentration of neuraminidase used to treat 2×10^6 cell before the addition of antibody or complement.

see Appendix 1. The results agreed with the stated activity of 500 U/ml). Fifty percent endpoint titres of antibody in a range between 44 and 126 were seen when 2 x 10^6 cells were treated with 1 to 200 U of neuraminidase. Lower concentrations of the enzyme had very little effect in removing resistance to lysis; antibody titres remained at less than 10. When complement was titred in the presence of excess antibody in the same system (Table VII), treatment with 1 to 200 U of neuraminidase resulted in 50% endpoint titres of complement of between 12 and 20, respectively. Therefore, 10 U of neuraminidase per 2 x 10^6 cells was chosen as a concentration able to produce the desired effect in KOS-infected cells.

The immunoresistant human cell lines were then treated with neuraminidase (Table VIII). In the presence of excess guinea pig complement, this treatment boosted the 50% endpoint antibody titre of KOS-infected HT-29 cells from < 10 to 72. HCT-8 cells were also susceptible to neuraminidase; the titre rose from 35 to 145. HEI cells underwent a similar effect, from < 10 to 78, while HEL cells showed only a 2-fold increase in the 50% endpoint antibody titre, from 71 to 135.

A similar pattern was seen when the cytolytic titre of complement was measured (Table IX). In all cases, this was seen to rise, from < 4 to 15 in the case of HT-29; 4.4 to 25 for HCT-8; and < 4 to 7.2 for HEI cells.

In the experiments just outlined, cells were treated with neuraminidase before the addition of antibody or complement. At

TABLE VII

TITRATION OF COMPLEMENT IN HSV-KOS-INFECTED HT-29 CELLS AFTER TREATMENT WITH VARIOUS CONCENTRATIONS OF NEURAMINIDASE

Concentration of Neuraminidase[b]	Percent Specific ^{51}Cr Release[a] Reciprocal Complement Dilution					50% Endpoint Titre of Complement
	4	8	16	32	64	
200u	100	100	57	7	<5	20
150u	99	93	64	11	<5	19
100u	100	81	73	12	<5	20
50u	91	86	42	7	<5	14
20u	100	99	46	<5	<5	15
10u	93	88	48	10	<5	15
5u	83	77	56	19	<5	18
1u	75	63	40	<5	<5	12
.1u	37	21	6	<5	<5	<4
.01u	19	7	<5	<5	<5	<4
none	12	<5	<5	<5	<5	<4

[a] Percent specific ^{51}Cr release in the presence of excess rabbit anti-HSV antiserum (1:10).

[b] Concentration of neuraminidase used to treat 2×10^6 cells before the addition of antibody or complement.

TABLE VIII

EFFECT OF NEURAMINIDASE TREATMENT ON THE LYSIS OF HSV-KOS-INFECTED HUMAN CELL LINES. TITRATION OF HSV-SPECIFIC ANTISERUM

Cell Line	Treatment	Percent Specific ^{51}Cr Release Reciprocal Antibody Dilution						50% Endpoint Titre of Antiserum[a]
		10	20	40	80	160	320	
HT-29	None	12	6	<5	<5	<5	<5	<10
	Neuraminidase[b]	100	80	79	46	11	<5	72
HCT-8	None	53	55	49	29	13	<5	35
	Neuraminidase	100	94	97	84	41	<5	145
HEI	None	10	8	.5	7	<5	<5	<10
	Neuraminidase	63	46	52	49	25	5	78
HEL	None	82	73	71	46	17	7	71
	Neuraminidase	88	89	89	78	40	9	135

[a] In the presence of excess guinea pig complement (1:4).

[b] 10u of neuraminidase was used to treat 2 X 10^6 cells for 1 hr. at 37°C before the addition of antibody or complement.

TABLE IX

EFFECT OF NEURAMINIDASE TREATMENT ON THE CYTOLYTIC TITRE OF COMPLEMENT IN HSV-KOS INFECTED HUMAN CELL LINES

Cell Line	Treatment	Percent. Specific ^{51}Cr Release Reciprocal Complement Dilution					50% Endpoint Titre of Complement[a]
		4	8	16	32	64	
HT-29	None	12	<5	<5	<5	<5	<4
	Neuraminidase[b]	85	79	48	10	<5	15
HCT-8	None	53	31	13	<5	<5	4.4
	Neuraminidase	100	93	90	31	<5	25
HEI	None	10	<5	<5	<5	<5	<4
	Neuraminidase	63	48	21	<5	<5	7.2
HEL	None	82	62	35	9	<5	12

[a] In the presence of excess rabbit anti-KOS antisera (1:10).

[b] 10u of neuraminidase was used to treat 2 X 10^6 cells for 1 hr. at 37°C before the addition of antibody or complement.

this point, it was impossible to determine whether neuraminidase exerted its effect on the step involving the reaction of antibody with antigen, or on the binding and activation of complement. Although not conclusive, the data shown in Fig. 4, suggested that the latter possibility was correct. When HT-29 cells were treated with 10 U of neuraminidase before the addition of antibody or before the addition of complement, lysis was enhanced to comparable extents. However, if neuraminidase treatment was delayed until after cells had been incubated with antibody and complement, there was no reversal of resistance to lysis.

Similar studies were carried out in 219-infected cells. Antibody was titred in the presence of excess complement in HCT-8 cells which had been treated with various concentrations of neuraminidase (Table X). Resistance to lysis was reversed by treatment with neuraminidase, but to a much smaller extent than in KOS-infected cells. Since 100 U of neuraminidase per 2×10^6 cells produced the highest percent specific ^{51}Cr release with all antibody dilutions tested, this concentration was used in all further experiments with 219-infected cells.

Failure of neuraminidase to reverse resistance to lysis was found in HT-29 and HEI cells (Table XI). Percent specific ^{51}Cr release remained less than 5 at all antibody dilutions, even in the presence of enzyme. In HCT-8 cells, neuraminidase treatment resulted in 32% specific ^{51}Cr release, compared to < 5% when cells were not treated. This is a significant increase, albeit low. HEL cells showed a marked increase in 50% endpoint antibody titre

FIGURE 4

The Effect of Neuraminidase Treatment on the Titration of Anti-HSV Antiserum in HSV-infected HT-29 Cells

KOS-infected HT-29 cells, which had or had not been treated with 10.0 U of neuraminidase, were reacted with dilutions of antibody and excess guinea pig complement.

● cells reacted with antibody and complement only

○ cells treated with neuraminidase and then reacted with antibody and complement

△ cells reacted with antibody, treated with neuraminidase, and then reacted with complement

▲ cells reacted with antibody and complement, and then treated with neuraminidase

□ cells treated with neuraminidase, and then reacted with normal rabbit serum and complement.

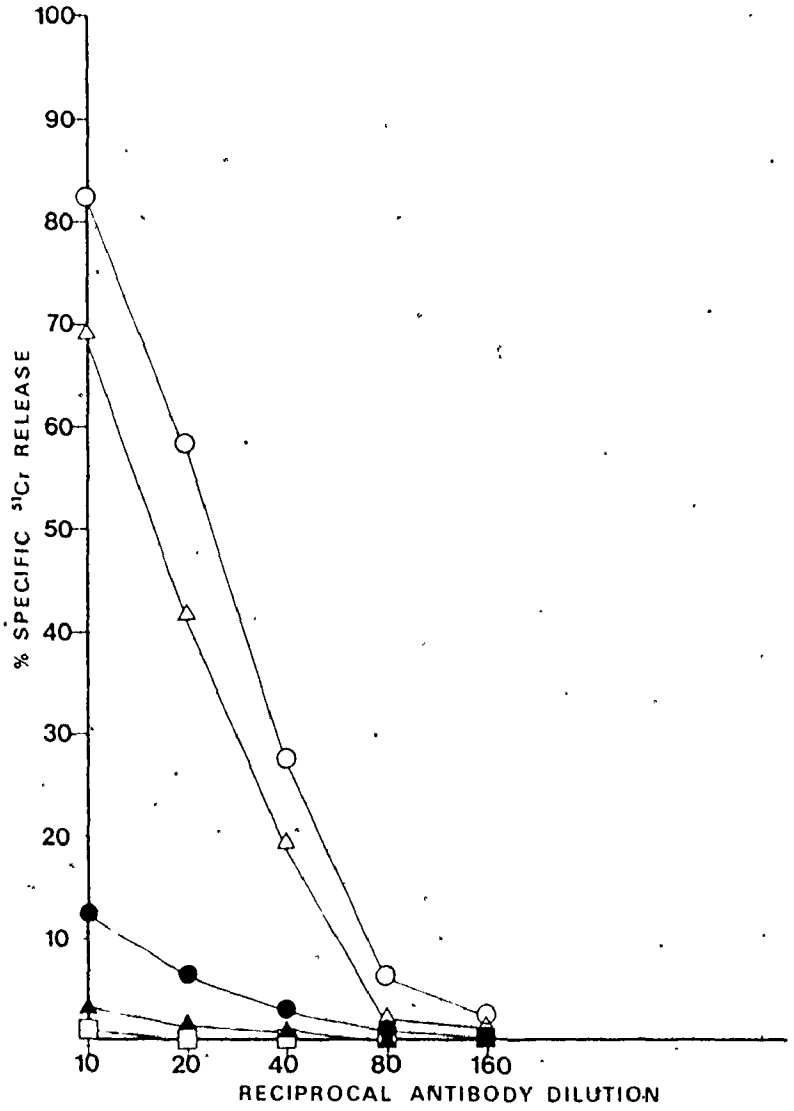

100
90
80
70
60
50
40
30
20
10
% SPECIFIC ^{51}Cr RELEASE
10
20
40
80
160
RECIPROCAL ANTIBODY DILUTION

TABLE X

TITRATION OF HSV-SPECIFIC ANTISERA ON HSV-219 INFECTED HCT-8 CELLS AFTER TREATMENT WITH VARIOUS CONCENTRATIONS OF NEURAMINIDASE

Concentration of Neuraminidase[b]	Percent Specific ^{51}Cr Release[a] Reciprocal Antibody Dilution						50% Endpoint Titre of Antisera
	10	20	40	80	160	320	
200u	8.2	7.6	<5.0	<5.0	<5.0	<5.0	<10
150u	15.1	10.1	6.1	5.2	<5.0	<5.0	<10
100u	20.1	14.4	12.4	8.7	5.9	5.8	<10
50u	18.2	10.8	10.8	8.6	8.3	<5.0	<10
20u	16.2	10.2	6.9	<5.0	6.6	5.3	<10
10u	5.8	<5.0	<5.0	<5.0	<5.0	<5.0	<10
5u	9.4	4.9	<5.0	<5.0	<5.0	<5.0	<10
1u	7.8	<5.0	<5.0	9.4	<5.0	<5.0	<10
none	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<10

[a] Percent specific ^{51}Cr release in the presence of excess guinea pig complement (1:4).

[b] Concentration of neuraminidase used to treat 2 X 10^6 cells before the addition of antibody or complement.

TABLE XI

EFFECT OF NEURAMINIDASE TREATMENT ON THE LYSIS OF HSV-219-INFECTED HUMAN CELL LINES. TITRATION OF HSV-SPECIFIC ANTISERUM

Cell Line	Treatment	Percent Specific ^{51}Cr Release Reciprocal Antibody Dilution						50% Endp i Titre of Antiserum
		10	20	40	80	160	320	
HT-29	None	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<10
	Neuraminidase	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<10
HCT-8	None	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<10
	Neuraminidase	31.6	17.8	10.0	<5.0	<5.0	<5.0	<10 (3)
HEI	None	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<10
	Neuraminidase	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<10
HEL	None	34.6	32.0	21.9	5.4	<5.0	<5.0	<10 (3)
	Neuraminidase	92.6	90.7	74.5	59.6	28.5	14.9	100

[a] In the presence of excess guinea pig complement (1:4).

[b] 100 u of neuraminidase was used to treat 2 X 10^6 cells before the addition of antibody or complement.

after neuraminidase treatment, from < 10 to 100.

An almost identical pattern occurred when complement was titred in the presence of excess antibody (Table XII). HT-29 and HEI cells showed no change; HCT-8 cells gave a response of 32% specific ^{51}Cr release at a complement dilution of 1:4, compared to < 5 without neuraminidase; and HEL cells showed an increase in the cytolytic titre of complement from < 4 to 19. This pattern does not rule out the possibility that enzyme treatment effects a change in complement binding and/or activation, as was suggested in KOS-infected cells. However, the specific experiment was not performed (as in Fig. 4).

The effect of neuraminidase treatment on the titration of antibody and complement in HSV-infected human cells is summarized in Tables XIII and XIV.

E. Adsorption Studies

Dilutions of HSV-specific antisera were adsorbed with various concentrations of infected cells and neuraminidase-treated infected cells in an effort to quantitate more precisely the expression of antigens on the surfaces of HSV-infected cells. Uninfected cells were used as controls. The results shown in Table XV clearly demonstrated that cells did not differ in the expression of antigens, since all cell lines adsorbed essentially identical quantities of antibody from the test serum. Neuraminidase treatment did not alter the expression of antigens reactive in this test; no "unmasking" had taken place.

TABLE XII

EFFECT OF NEURAMINIDASE TREATMENT ON THE CYTOLYTIC TITRE OF COMPLEMENT IN HSV-219-INFECTED HUMAN CELL LINES

Cell Line	Treatment	Percent Specific ^{51}Cr Release Reciprocal Complement Dilution					50% Endpoint Titre of Complement[a]
		4	8	16	32	64	
HT-29	None	<5.0	<5.0	<5.0	<5.0	<5.0	<4
	Neuraminidase[b]	<5.0	<5.0	<5.0	<5.0	<5.0	<4
HCT-8	None	<5.0	<5.0	<5.0	<5.0	<5.0	<4
	Neuraminidase	31.6	22.3	<5.0	<5.0	<5.0	<4
HEI	None	<5.0	<5.0	<5.0	<5.0	<5.0	<4
	Neuraminidase	<5.0	<5.0	<5.0	<5.0	<5.0	<4
HEL	None	34.6	49.2	8.3	9.6	<5.0	<4
	Neuraminidase	92.6	96.5	60.5	10.3	<5.0	19

[a] In the presence of excess rabbit anti-HSV antisera (1:10).

[b] 100u of neuraminidase was used to treat 2 X 10^6 cells for 1 hr at 37°C before the addition of antibody and complement.

TABLE XIII

SUMMARY: EFFECT OF NEURAMINIDASE TREATMENT ON THE TITRATION OF ANTISERUM IN HSV-INFECTED HUMAN CELL LINES

Cell Line	Infection With[a]	50% Endpoint Titre of Antiserum[b]	
		Treament	
		None	Neuraminidase[c]
HT-29	KOS	<10	72
	219	<10	<10
HCT-8	KOS	35	145
	219	<10	<10 (3)
HEI	KOS	<10	78
	219	<10	<10
HEL	KOS	71	135
	219	<10 (3)	100

[a] Conditions of infection as in Table V.

[b] In the presence of excess guinea pig complement (1:4).

[c] For KOS-infected cells, 10 u of neuraminidase was used to treat 2 X 10^6 cells for 1 hr at 37°C before the addition of antibody or complement.
In the case of 219-infected cells, 100 u of neuraminidase was used under identical conditions.

TABLE XIV

SUMMARY: EFFECT OF NEURAMINIDASE TREATMENT ON THE CYTOLYTIC TITRE OF COMPLEMENT IN HSV-INFECTED HUMAN CELLS

Cell Line	Infection With[a]	50% Endpoint Titre of Complement[b] Treatment	
		None	Neuraminidase[c]
HT-29	KOS	<4	15
	219	<4	<4
HCT-8	KOS	4.4	25
	219	<4	<4
HEI	KOS	<4	7.2
	219	<4	<4
HEL	KOS	12	
	219	<4	19

[a] Conditions of infection as in Table V.

[b] In the presence of excess rabbit anti-HSV antiserum (1:10) specific for the virus seed.

[c] Treatment was the same as in Table VIII.

TABLE XV

EFFECT OF NEURAMINIDASE TREATMENT ON THE ADSORPTION OF ANTI-KOS ANTISERUM BY HSV-KOS-INFECTED HUMAN CELLS

Cell Line	Treatment	Percent Specific ^{51}Cr Release[a] Adsorbed Anti-KOS Antiserum[b]	
		10 Units	2 Units
HT-29	Uninfected	95	93
	KOS-infected	9	<5
	KOS-neuraminidase[c]	12	<5
HCT-8	Uninfected	95	94
	KOS-infected	<5	<5
	KOS-neuraminidase	<5	<5
HEI	Uninfected	91	59
	KOS-infected	<5	<5
	KOS-neuraminidase	<5	<5
HEL	Uninfected	95	91
	KOS-infected	<5	<5
	KOS-neuraminidase	<5	<5

[a] 5×10^6 cells were adsorbed

[b] 1 unit of antibody activity is the dilution which produces 50% specific ^{51}Cr release in the standard assay.

[c] Cells were infected with HSV-KOS and then treated with 10 u of neuraminidase for 1 hr at 37°C prior to adsorption of antiserum

When various concentrations of HT-29 cells were used to adsorb a constant dilution of antiserum (Fig. 5), the results confirmed and extended the previous observations in that neuraminidase treatment did not alter the binding of antibody to HSV-specific antigens, and that increasing concentrations of infected cells removed progressively more antibody until all had been adsorbed.

Identical patterns were seen in all KOS-infected human cell lines. Differences in susceptibility to lysis, therefore, are not attributable to the expression of antigens or to the amount of antibody bound to the surface of infected cells.

F. Complement Consumption Tests

Resistance to complement-mediated antibody lysis in KOS-infected cell lines was removed by treatment with neuraminidase. In contrast, such treatment failed to remove entirely the resistance to lysis seen in 219-infected cells. To summarize previous findings (Tables XIII and XIV), HT-29 cells and HEI cells were totally resistant to lysis either before or after neuraminidase treatment. Only a small increase in percent specific ^{51}Cr release was seen in neuraminidase treated HCT-8 cells, from 0 to 3.2% specific ^{51}Cr release when both antibody and complement were present in great excess. HEL cells differed in that resistance to lysis was totally abrogated by neuraminidase treatment. Therefore, it was considered vital to determine whether or not complement was in fact being bound and activated, and whether differences in binding and

FIGURE 5

Adsorption of Anti-HSV Antiserum

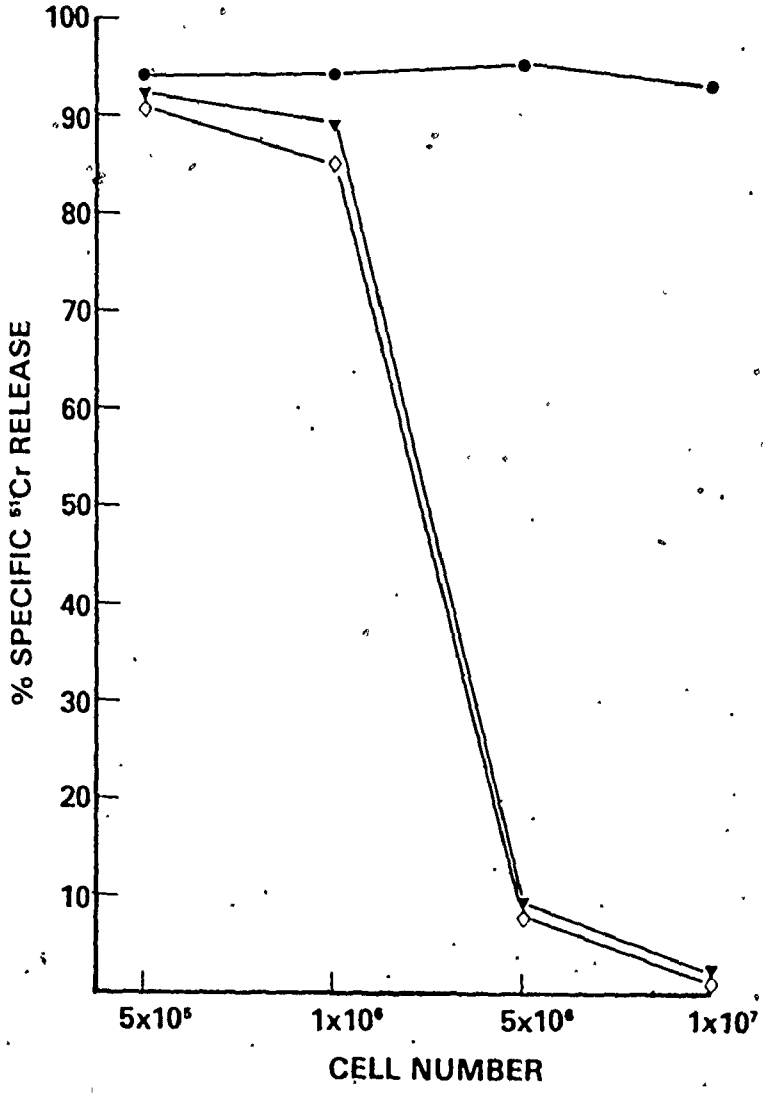
with HSV-1-infected HT-29 Cells

Ten U. of antibody, in a volume of 1.0 ml., were adsorbed with various concentrations of HT-29 cells. The adsorbed serum was then titred in KOS-infected BHK-21 cells.

● uninfected HT-29 cells

◇ KOS-infected HT-29 cells

▼ KOS-infected, neuraminidase-treated, HT-29 cells


100
90
80
70
60
50
40
30
20
10
% SPECIFIC ^{51}Cr RELEASE
5x10^5
1x10^6
5x10^6
1x10^7
CELL NUMBER

activation could be detected between these cell lines. Such questions were answered by means of complement consumption tests.

The complement consumption test utilizes sensitized sheep red blood cells as an indicator system. These are lysed by any complement remaining after reaction with antigen (the infected cells) and antibody. Because the concentration of complement used in this test is critical to comparison of test results, a titration of guinea pig complement was performed for each test.

The results of such a titration are shown in Table XVI, and are plotted in a graph in Fig. 6 according to the method of Kagan and Norman (1970). A complement dilution of 5 C'H50 in a volume of 0.4 mls is desired in the actual test, where 1 C'H50 is that dilution of complement which lyses 50% of the sensitized sheep red blood cells. From the graph (Fig. 6), it is seen that the log of the volume of guinea pig complement, diluted 1:200 in VBD, which contains 1 C'H50 is equal to -0.678. This corresponds to a volume of 0.21 mls. Since 0.21 mls of 1:200 complement contains 1 C'H50, 1.05 mls must contain 5 C'H50. Accordingly, then, when complement is diluted 1:76, the desired concentration of 5 C'H50/0.4 ml is obtained.

A preliminary screening of HT-29, HEI, and HEL cells infected with HSV-219 was first performed, using $\log_{10}$ dilutions of antigen and antibody (data not shown). This test indicated that HT-29 and HEI cells behaved almost identically, and provided the range of dilutions of antibody and antigen to be tested more precisely.

TABLE XVI

TITRATION OF COMPLEMENT

Volume of Complement Dilution[a]	Percent Haemolysis, y	$\frac{y}{100-y}$	Log_{10} of Volume	Log_{10} of $y/100-y$
0.10 ml	5	-	-	-
0.15 ml	10	0.111	-0.824	-0.955
0.20 ml	40	0.670	-0.699	-0.174
0.25 ml	75	3.000	-0.602	0.477
0.30 ml	95	19.000	-0.523	1.279
0.40 ml	100	-	-	-
None	0	-	-	-

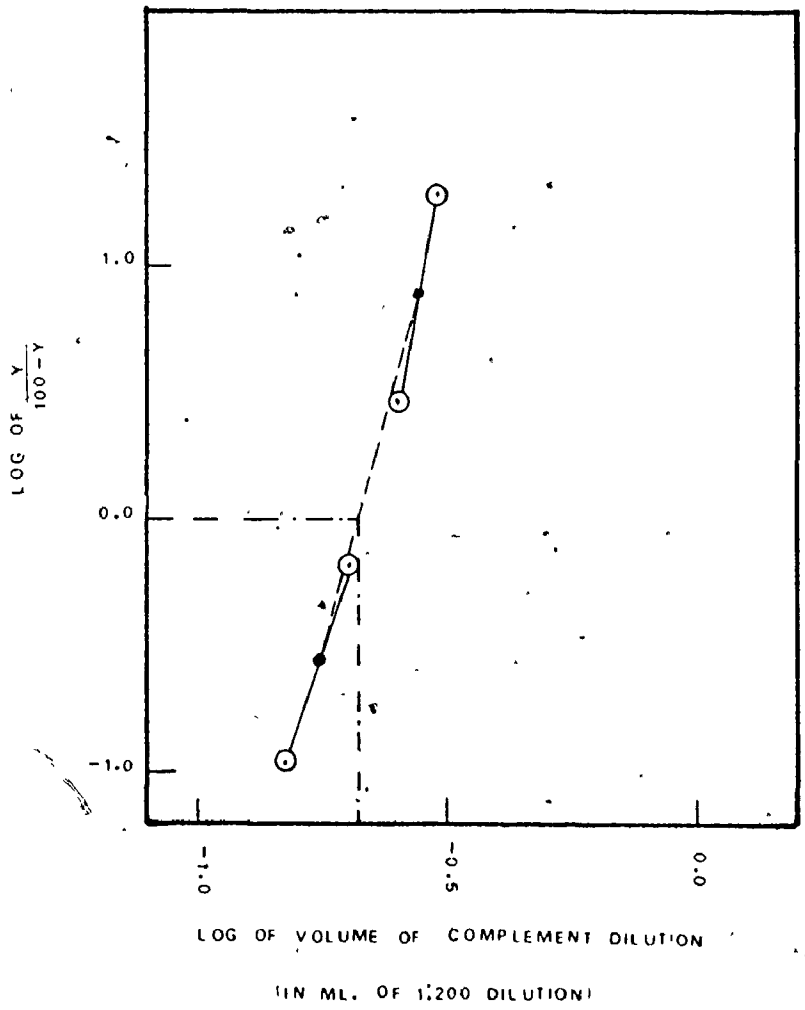
[a] Guinea pig complement, diluted 1:200 in VBD.

FIGURE 6

Titration of Guinea Pig Complement

for Complement Consumption Tests

The log of the volume of guinea pig complement, which had been diluted 1:200 in VBD, is plotted against the log of the ratio of the percent of lysed cells, y, to the percent of non-lysed cells, 100-y.


1.0
0.0
-1.0
LOG OF $\frac{Y}{100-Y}$
-1.0
-0.5
0.0
LOG OF VOLUME OF COMPLEMENT DILUTION
(IN ML. OF 1:200 DILUTION)

The results of complement consumption tests in HEI, HCT-8 and HEL cells infected with HSV-219 are shown in Table XVII. The results are expressed as the number of cells which consumed 50% of the available guinea pig complement, resulting in lysis of 50% of the sensitized sheep red blood cells. This facilitates comparison of complement consumption in the individual cell lines, before and after neuraminidase treatment. All complement controls were satisfactory, but at dilutions of antibody less than 1:80, evidence of anti-complementary action was seen. That is, antiserum controls without antigen resulted in less than 75% haemolysis. Data using antiserum dilutions of 1:80, 1:160 and 1:320 are therefore presented. These dilutions exhibited optimal percent haemolysis values for the range of antigen dilutions tested.

An example of the procedure used to calculate the 50% endpoint titre of antigen follows, using typical data obtained with HEI cells which had not been treated with neuraminidase:

Concentration of HEI cells (number of cells/tube)	Percent Haemolysis (Antiserum 1:160)
3.2×10^5	0
1.6×10^5	5
8.0×10^4	35
4.0×10^4	75
2.0×10^4	85
1.0×10^4	85
none	100

The concentration of cells which consumed 50% of the complement obviously was between 8×10^4 and 4×10^4 cells/tube.

TABLE XVII

COMPLEMENT CONSUMPTION IN CELLS INFECTED WITH HSV-219

Cell Line	Treatment	50% Endpoint Titre of Antigen (Cell number[a]) Reciprocol Antiserum Dilution		
		80	160	320
HEI	None	1.41×10^4	6.18×10^4	1.45×10^5
	Neuraminidase[b]	4.00×10^4	7.46×10^4	$(>1.60 \times 10^5)$[c]
HCT-8	None	3.53×10^3	1.52×10^4	5.44×10^4
	Neuraminidase	1.78×10^4	3.47×10^4	7.33×10^4
HEL	None	1.36×10^3	2.81×10^3	5.00×10^3
	Neuraminidase	2.10×10^3	4.36×10^3	1.08×10^4

[a] The 50% endpoint titre of antigen is defined as that number of cells which consumes 50% of the available guinea pig complement, resulting in 50% haemolysis of the sensitized sheep red blood cells.

[b] 10 U of neuraminidase was used to treat 1×10^6 cells for 1 hr at 37°C. Appropriate dilutions were then made, and cells were treated with antibody and then complement, 5 C'H50/tube.

[c] Highest concentration of cells tested. 1.60×10^5 cells removed only 25% of the complement, resulting in 75% haemolysis.

A mathematical adaptation of the graphing method was performed to calculate this critical concentration. When percent haemolysis is plotted against the log of the concentration of cells, a characteristic reverse S-shaped curve is observed. The central portion of this curve, which includes those values bracketing 50% haemolysis, may be considered to have a uniform slope, given by:

$$\text{slope} = \frac{a - b}{\log A - \log B}$$

where a = percent haemolysis given by the higher cell concentration

b = percent haemolysis given by the lower cell concentration

A = concentration of cells giving higher % haemolysis

B = concentration of cells giving lower % haemolysis

Note that since doubling dilutions were always used in the antigen dilutions, the lower term of this equation (log A - log B) must always be equal to log 2.00, or 0.301. To determine the log of the cell concentration which produced 50% haemolysis, use may be made of the fact that this slope is uniform over this range of cell concentrations. Therefore:

$$\frac{a - b}{\log A - \log B} = \frac{a - b}{0.301} = \frac{50\% - b}{\log A - \log X}$$

where X = cell concentration producing 50% haemolysis.

Thus,

$$\log X = \log A - \left(0.301 \times \frac{50\% - b}{a - b}\right)$$

Substituting for the values in the example used,

$$\log X = \log (8 \times 10^4) - (0.301 \times \frac{50 - 35}{75 - 35}) = 4.790$$

$$\text{and } X = 6.18 \times 10^4$$

The results presented in Table XVII confirmed that all cell lines tested were taking up complement. Moreover, the 50% endpoint titres of antigen were well within the concentration of cells used in the performance of the ^{51}Cr release test (5×10^4 cells/tube).

When HSV-219 infected HEI cells were incubated with antiserum diluted 1:80 and 5'C'H50, it was found that 1.41×10^4 cells consumed 50% of the complement. After neuraminidase treatment of the infected cells, more cells - 4.00×10^4 - were required to consume 50% of the complement. In other words, cells were fixing less complement after neuraminidase treatment. This trend was evident at all antiserum dilutions; at dilutions of 160 and 320, 6.18×10^4 and 1.45×10^5 cells respectively were required to fix 50% of the complement before neuraminidase treatment. After incubation with neuraminidase, 7.46×10^4 and $> 1.60 \times 10^5$ cells were required to consume sufficient complement so that 50% of the sensitized sheep red blood cells were lysed.

Identical trends were seen when HCT-8 and HEL cells were used as antigen. Neuraminidase treatment of these cells always resulted in an increase in the 50% endpoint titre of antigen, at all antiserum dilutions. It is interesting to compare complement consumption in these three cell lines. HEI cells, which represent

immunoresistant cells, were least efficient of all three lines in fixing complement, before or after neuraminidase treatment at all antiserum dilutions tested. With antiserum diluted 1:160, the 50% endpoint antigen titre increased from 6.18×10^4 to 7.4×10^4 cells after neuraminidase treatment. This should be contrasted next with HCT-8 cells, in which resistance to lysis may be partially reversed. In these cells, an increase from 1.52×10^4 cells to 3.47×10^4 cells was seen at an antiserum dilution of 1:160. It should be noted that fewer cells were required to consume 50% of the complement. In keeping with this pattern, HEL cells, which are markedly susceptible to neuraminidase action, were able to consume 50% of the complement at cell concentrations of 2.81×10^3 and 4.36×10^3 cells, before and after neuraminidase treatment, respectively.

DISCUSSION

The susceptibility of HSV-infected cells to complement-mediated antibody lysis is clearly a property of the cell. Under identical conditions of viral infection and assay, it was seen that the various cell lines differed in their response. Limiting concentrations of complement were responsible for the low levels of lysis seen in KOS and 219-infected BHK clones (MB-1, MB-2 and MB-3). This is evident from the fact that increasing the concentration of complement, in a method in which all other parameters remained constant, was sufficient to permit lysis in these cells. This explanation may partly hold for KOS-infected HCT-8 and HEL cells. However, no increase in percent specific ^{51}Cr release was seen in HT-29 or HEL cells, even at very high concentrations of complement. Similarily, all HSV-219 infected human cells were resistant to complement-mediated antibody lysis, even in the presence of excess antibody and complement. Resistance to lysis in these cells was therefore not attributable to limiting complement concentrations. Rather, this resistance appears to be an innate property of the cell, and could operate at a number of levels.

However, the failure of complement and antibody to lyse the resistant cell lines cannot be explained by a lack of antigenic expression on the surface of infected cells. Indirect immunofluorescence assays, which were performed on all cell lines,

showed no detectable difference in the intensity of fluorescence or in the proportion of cells expressing fluorescence. A more precise quantitation of antigen expression, obtained by adsorbing HSV-specific antisera with known concentrations of the various KOS-infected human cell lines, confirmed these findings; identical quantities of antibody were removed by all cell lines at each cell concentration. This is consistant with results reported for the immunoresistant L10 cells, a line of guinea pig hepatoma cells, which adsorb as much antibody as the sensitive L1 cells (Ohanian, Borsos and Rapp, 1973) and for RPMI 8866 cells, in which the density of HL-A antigens does not alter throughout the growth cycle although these cells are resistant to complement-mediated lysis in the G1 phase (Pellegrino et al., 1974).

In addition, no correlation between this resistance and the ability to produce viral progeny could be made. In KOS-infected cells, only MB-1 cells failed to produce a net increase in virus titre, yet these cells were susceptible to complement cytolysis. HT-29 and HEI cells, which were immunoresistant, produced quite respectable progeny yields. Again, in HSV-2 infected cells, virus growth could not be demonstrated in HEI or MB-3 cells, and occurred at very low levels in HEL and MB-1 cells. HEI cells were resistant to complement-mediated antibody lysis, but MB-3, MB-1 and HEL cells were susceptible; of the remaining cell lines, HT-29 and HCT-8 were not vulnerable to lysis. Thus, the lack of lysis in these cells can not be attributed to a failure to produce viral progeny, to the

lack of expression of viral-specific antigens, or to the inaccessibility of these antigens to antibody.

Other suggestions have been put forward in an attempt to explain resistance to complement-mediated antibody lysis. For example, the stage of the cell cycle is important in determining whether or not Moloney virus transformed lymphocytes (Cooper, Polley and Oldstone, 1974) and the human lymphoid cell line RPMI 8866 (Pellegrino et al., 1974) will be lysed by the appropriate antibody and complement. C8 is demonstratable on the plasma membrane of both cell lines, in an amount identical to that present when lysis is produced during the remainder of the cell cycle. In fact, the characteristic lesions may actually be seen with the electron microscope (Cooper, Polley and Oldstone, 1974). To add to the confusion, there is a contradiction here; RPMI cells are least sensitive in G1 phase, while the Moloney virus-transformed lymphocytes are lysed only in G1. However, it seems doubtful that there is a correlation between the resistance seen in certain HSV-infected cells with a particular growth phase, since these cells were not synchronized.

In this system, C3 could be demonstrated on the surface of the immunoresistant HT-29 cells (Dr. W.A.F. Tompkins, personal communication). Thus, inefficient activation or a complete failure to activate complement were considered unlikely. This is borne out by complement consumption tests on susceptible and immunoresistant cells which had been infected with HSV-219. All cell lines were shown to fix complement. A more efficient utilization was seen in HEL

than in HCT-8; HEI were comparable to HT-29 cells, and both of these lines consumed less complement per cell than did the other two. Note the interesting correlation with the fact that HEL cells are most susceptible to reversal resistance to lysis, HCT-8 are partially susceptible, and HEI and HT-29 cells are completely resistant. Although HEL cells are very large, a correlation between size and susceptibility to lysis was considered unlikely. When complement is not in excess, both IgG and IgM produce one hit rather than multiple lesions (Frank, Dourmashkin and Humphrey, 1970). This is supported by the observation that increasing the number of immunosensitive L1 cells to give a total surface area equal to that of the larger, resistant L10 cells did not change their susceptibility to complement-mediated cytolysis (Ohanian, Borsos, and Rapp, 1973). Still another cell line, RADA-1 cells which are resistant to complement cytotoxicity, have been shown to consume as much complement as the sensitive, parental cells from which they were derived (Yu, Liang and Cohen, 1975).

Capping has been observed in HCT-8 and HT-29 cells infected with either HSV-1 or HSV-2. Capping is defined as the antibody-induced redistribution of antigens on the cell surface. If capping were to proceed rapidly in these cells, it is possible that the antigen-antibody complexes would be cleared before reaction with complement. This possibility is considered unlikely, since the efficiency of capping in these cells is very low, involving only about 10% of the cells. Other cells, in which capping is known to occur but fail to lyse, have also been studied; examples are the

RADA-1 cell line (Yu, Liang and Cohen, 1975) and L10 cells (Ohanian, Borsos and Rapp, 1973).

The density of antigens on the cell surface may be correlated with the failure of complement to lyse cells (Rose, Milgrom and Van Oss, 1973). The bright fluorescence and extensive adsorption of antibody by HSV-infected cells suggest that this explanation does not apply in the present studies.

To summarize, the experimental findings indicate that resistance to complement-mediated antibody lysis in certain HSV-infected cell lines cannot be due to the failure to produce viral progeny or to a lack of antigenic expression on the cell surface. These antigens are probably in close enough proximity to bind to antibody molecules of the proper class. Complement is bound to the surface of these cells. Resistance to lysis appears to be an innate property of the cell itself, perhaps at the level of membrane composition, which could be altered as the result of viral antigen expression, or in the ability to repair the lesions produced by complement.

Treatment of the resistant cells with neuraminidase was then carried out. As anticipated from previous findings that neuraminidase increases the immunogenicity of cells, neuraminidase treatment was able to reverse the resistance to lysis by antibody and complement seen in KOS-infected HEl and HT-29 cells, and to increase the lysability of HCT-8 and HEL cells. No effect was seen in 219-infected HEI and HT-29 cells; a small increase in percent specific chromium release was seen with HCT-8, and a dramatic increase was seen with

HEL cells.

The mechanism by which neuraminidase is able to induce a change at the cell surface, and so affect biological behaviour is not understood. One of the most popular theories is that neuraminidase, by disruption of the sialomucin coat or by inducing conformational changes in blocking antigens, is able to unmask hidden antigens. Sanford postulated that neuraminidase treatment of TA3 tumour cells increased their immunogenicity in mice by exposure of previously hidden histocompatability antigens (Sanford, 1967). Reisner and Amos (1967) claimed that treatment of 5×10^6 human lymphocytes with only 2 units of neuraminidase for twenty minutes resulted in an increased ability to adsorb antibody activity in human alloantisera compared with untreated cells. This seemingly confirmed the unmasking hypothesis. These authors suggested that sialic acid residues could physically block antigens from reacting with antibodies, and that enzyme-treated cells used complement more efficiently.

Studies with human lymphoid cells showed that neuraminidase treatment caused a 3 to 4 fold increase in the amount of antibody activity adsorbed from normal rabbit serum. Complement levels were unaffected by this adsorption (Rosenberg, Plocinik and Rogentine, 1972). These authors postulated that a nonspecific unmasking occurred, that the newly exposed antigens reacted with previously existing hetero-antibodies in normal rabbit serum. This observation has recently been extended to a murine system. Again, neuraminidase-treated cells adsorbed a substance, probably an antibody, from normal mouse serum while

untreated cells did not. The newly exposed antigen was not a tumour or transplantation antigen, and could react with naturally occurring antibodies in normal mouse serum (Rosenberg and Schwarz, 1974).

In HSV-infected cells, it is obvious that neuraminidase did not act by specifically unmasking HSV antigens. This was shown by adsorption studies in which infected cells and infected, neuraminidase-treated human cells adsorbed essentially identical quantities of antibody activity from heat-inactivated anti-HSV antiserum. Nor did the cell lines studied differ greatly in the quantity of HSV antigens expressed on the plasma membrane. Rather, in this system, neuraminidase acted at the level of the reaction of complement with the antigen-antibody complex. This was suggested by the following. Treatment with neuraminidase increased both the 50% endpoint titre of antibody in the presence of excess complement, and the 50% endpoint titre of complement in the presence of excess antibody. In addition neuraminidase could be added either before the addition of antibody or before complement without a change in its effect; after complement had been bound, however, no effect was observed. These two facts rule out the possibility that neuraminidase non-specifically unmasks antigens that could react with heteroantibodies in the anti-HSV serum. Nor is it likely that such activity resides in the complement source; spontaneous controls, in which only labelled cells and complement were reacted, remained at low levels, although a small increase was seen.

The unmasking hypothesis has been rejected by many authors in favour of the concept of a non-specific increase in immunogenicity of neuraminidase-treated cells. This concept has been used for both

in vivo systems (Bagshaw and Currie, 1968; Im and Simmons, 1971) and for in vitro systems (Schlesinger and Gottesfield, 1971). It was suggested that the enhancement of the ability of the cell to be recognized as antigen may be due to the fact that perhaps antigens are sterically hindered from reacting with antibody, or that the sialic acid residues interfered with contact between antigen-bearing and antigen-processing cells (Simmons, Rios and Ray, 1971). In light of this possibility, it is interesting that neuraminidase-treated cells are more easily phagocytized than are normal cells. This would facilitate antigen processing (Ray and Simmons, 1971). These suggestions are not applicable to the HSV system since the present in vitro studies showed that treated and untreated cells adsorbed equal amounts of antibody.

Alternatively, neuraminidase may reduce the net negative charge on the cell surface by removal of the sialic acid residues. This could have two effects: a reduction in the rigidity of the cell membrane, or facilitation of the interaction between the cell membrane and complement, which also has a net negative change. The latter suggestion would promote an increased susceptibility to complement-mediated antibody lysis.

It is important that one realizes that the specific action of neuraminidase alone is responsible for the diverse effects seen in these studies and in others. The preparation of neuraminidase used in the present experiments contained no proteases (see Appendix). Heat-inactivated neuraminidase cannot duplicate any effect; nor does

neuraminidase alone release ^{51}Cr from labelled cells. The addition of N-acetyl neuraminic acid specifically inhibits the action of neuraminidase in in vitro systems (Ray, Gewurz and Simmons, 1972). Thus, observation made after treatment of cells with neuraminidase is due to a specific enzymatic effect of neuraminidase. Support of this conclusion also arises from the fact that trypsin, which removes sialoglycopeptides (under certain conditions, as much sialic acid is removed as by neuraminidase treatment), does not render TA3 cells susceptible to lysis by guinea pig serum, as does neuraminidase (Hughes, Palmer and Sanford, 1973). Also, incubation of mouse plasmocytoma cells with 1% v/v trypsin for 1 hr. resulted in very little damage to the relevant antigens (Fakri and Tan, 1976).

Finally, Weiss has suggested that the increased immunogenicity of neuraminidase-treated cells in vivo is attributable to the killing of these cells by neuraminidase, resulting in a dose less than an LD 100. This argument seems unlikely. Synthesis of RNA, DNA protein, and membrane components in TA3 cells was unaltered by neuraminidase treatment; nor was cell viability or cell growth affected (Hughes, Sanford and Jeanloz, 1972). In an independant study, no cell death after treatment of 5×10^6 cells with 1-250 U of neuraminidase for 1 hour could be detected by trypan blue exclusion or by an increase in the release of ^{51}Cr from labelled cells (Ray, Gewurz and Simmons, 1972) although other authors have claimed that trypan blue exclusion does not detect cell killing. Yuhas et al., claimed that the treatment of 1×10^6 cells with 200 U of neuraminidase

resulted in 73 - 84% cytotoxicity, which was only detectable by measuring colony formation or ^{3}H-thymidine incorporation (Yuhas, Toya and Pazmiño, 1974). This would of course, account for the increased immunogenicity in vivo.

Sialic acid residues are rapidly regenerated on the surface of cells after neuraminidase treatment; estimates as low as 8 hrs have been made for complete regeneration (Rosenberg and Schwarz, 1974). This would make it unlikely that a primary immune response is induced against any newly exposed weak antigens, but it may permit a preexisting cytotoxic factor in the serum to remove a large number of these cells. This would reduce the effective dose to a level which could be easily handled by the immune system.

In HSV-infected human cells, the effect of neuraminidase treatment appears to be at the level of complement interaction with antigen-antibody complexes. To summarize the evidence pointing towards this conclusion, neuraminidase treatment increases both the 50% endpoint titre of antibody and the 50% endpoint titre of complement; neuraminidase treatment may be carried out before or after the addition of antibody, but not during or after the addition of complement; finally, complement consumption tests on 219-infected human cells before and after neuraminidase treatment demonstrated that the neuraminidase-treated cells actually fixed less complement per cell, while at the same time were markedly more susceptible to lysis. Thus neuraminidase treatment permitted the cells to utilize complement more efficiently; this could result in an increased

susceptibility to lysis, as in the case of all KOS-infected human cells and for 219-infected HEL and HCT-8 cells.

The following working model may explain my results. The resistance to complement-mediated antibody lysis may be related to the ability of cells to repair lesions. Sensitive cells would be unable to repair the damage rapidly enough to prevent lysis. Neuraminidase treatment of these resistant cells may inhibit the repair of lesions, and lysis would result.

In the system under study here, it is not known why there is a difference in the response of KOS-infected cells compared to 219-infected cells, nor is it known why HT-29 and HEI cells are still resistant to lysis even after neuraminidase treatment. This could be a fundamental property of the cell membrane, altered by the insertion of virus-specific glycoproteins.

Conclusions

The susceptibility of various HSV-1 and HSV-2-infected cell lines to complement-mediated antibody cytolysis was investigated. A difference in response could be detected on two levels. First, HSV-1-infected cells were generally more susceptible to lysis than HSV-2 infected cells. Second, increasing the complement concentration markedly increased percent lysis in cells of BHK origin, but had little effect on cells of human origin. The resistance to complement-mediated antibody lysis in HSV-infected human cells could not be correlated with the ability to produce virus progency. All resistant cell lines

expressed viral-specific antigens at the cell surface, in essentially identical quantities as sensitive cells. All cells bound antibody efficiently. Capping could not account for failure to lyse. Complement was bound at least up until C3, and most likely proceeded to completion. Neuraminidase treatment of the immunoresistant cells resulted in the reversal of resistance to lysis in all four HSV-1 infected human cells, and in two of the four HSV-2 infected human cell lines. Neuraminidase treatment did not affect the expression of viral antigens or the binding of antibody. It appeared to act at the level of complement, by increasing the efficiency of utilization. The effect of neuraminidase was enzyme-specific.

APPENDIX I

A. Preparation of Reagents used in the Thiobarbituric Acid Assay

Periodate Reagent

To 38.0 mls of hot distilled water was added 4.28 g of sodium periodate (Sigma Chemicals). After cooling to room temperature, 62.0 mls of syrupy orthophosphoric acid (Fischer Scientific) was added. This reagent was stored at 4°C in a brown glass-stoppered bottle.

Arsenite Reagent

10.0 g of sodium arsenite (Fischer Scientific) and 7.1 g Na_2SO_4 (Fischer Scientific) was dissolved in 100.0 mls distilled water by heating. Following cooling to room temperature, 0.3 ml concentrated H_2SO_4 (Mallinckrodt) was added. The reagent is stable at room temperature.

Thiobarbituric Acid Reagent

7.1 g of Na_2SO_4 and 0.6 g of 2-thiobarbituric acid (Sigma) was dissolved in 100.0 mls of distilled water by boiling in a water bath. This may be stored at room temperature, but must be prepared weekly as the salts precipitate out.

B. Standard Assay of Neuraminidase Activity

To check that the thiobarbituric acid assay was being performed correctly, calibration curves using a pure preparation of N-acetyl neuraminic acid were run with each experiment. Typical results are presented in Table XVIII and Fig. 7. The theoretical concentrations, which were calculated by diluting a stock preparation of N-acetyl neuraminic acid, agreed closely with the experimentally determined concentrations in all experiments.

The following studies were done to define standard conditions for the assay of neuraminidase activity. All incubations of neuraminidase with substrate were carried out at 37°C, the optimum temperature, and reagents were always prewarmed to 37°C. The pH was 5.5, which was the stated pH of the neuraminidase preparation. The optimum pH is in a range between 4.5 and 7.0.

Various dilutions of the substrate, human α -1 acid glycoprotein, were then incubated with 78 U of neuraminidase for 15' at 37°C, so that an optimum substrate concentration could be chosen for the enzyme assay (Table XIX, Fig. 8). A concentration of 0.003 µmoles/.2 ml was the lowest amount of substrate from which a maximum amount of N-acetyl neuraminic acid was released under the conditions of assay. A plateau was seen at concentrations greater than this.

The effect of time on the reaction between enzyme and substrate was then investigated (Table XX; Fig. 9). A linear relationship between time of assay and the concentration of product

TABLE XVIII

CALIBRATION CURVE FOR THE THIOBARBITURIC ACID ASSAY OF N-ACETYL NEURAMINIC ACID

Theoretical Concentration of N'ANA, μ moles/.2 ml[a]	A		B			Corrected Concentration of N'ANA μ moles/.2 ml[b]
	O.D.549	O.D.532	O.D.549 X .084	O.D.532 X 0.31	A-B	
0.00	0.116	0.120	0.0097	0.0036	0.0041	0.000
0.01	0.237	0.145	0.0199	0.0047	0.0152	0.011
0.02	0.391	0.217	0.0328	0.0067	0.0261	0.022
0.03	0.488	0.231	0.0410	0.0072	0.0338	0.030
0.04	0.562	0.226	0.0472	0.0070	0.0402	0.036
0.05	0.769	0.318	0.0646	0.0076	0.0570	0.053
0.10	1.400	0.565	0.1176	0.0175	0.1001	0.096

[a] A stock solution of N-acetyl neuraminic acid, 0.5 mM, was prepared. Dilutions were made which gave the above range of concentrations for use in the standard assay.

[b] The zero concentration reading was subtracted from each value to give the corrected concentrations.

FIGURE 7

Calibration Curve for the Thiobarbituric Acid Assay of Released Sialic Acids

A stock preparation of N-acetyl neuraminic acid, 0.5 mM, was diluted and assayed by the standard thiobarbituric acid assay.

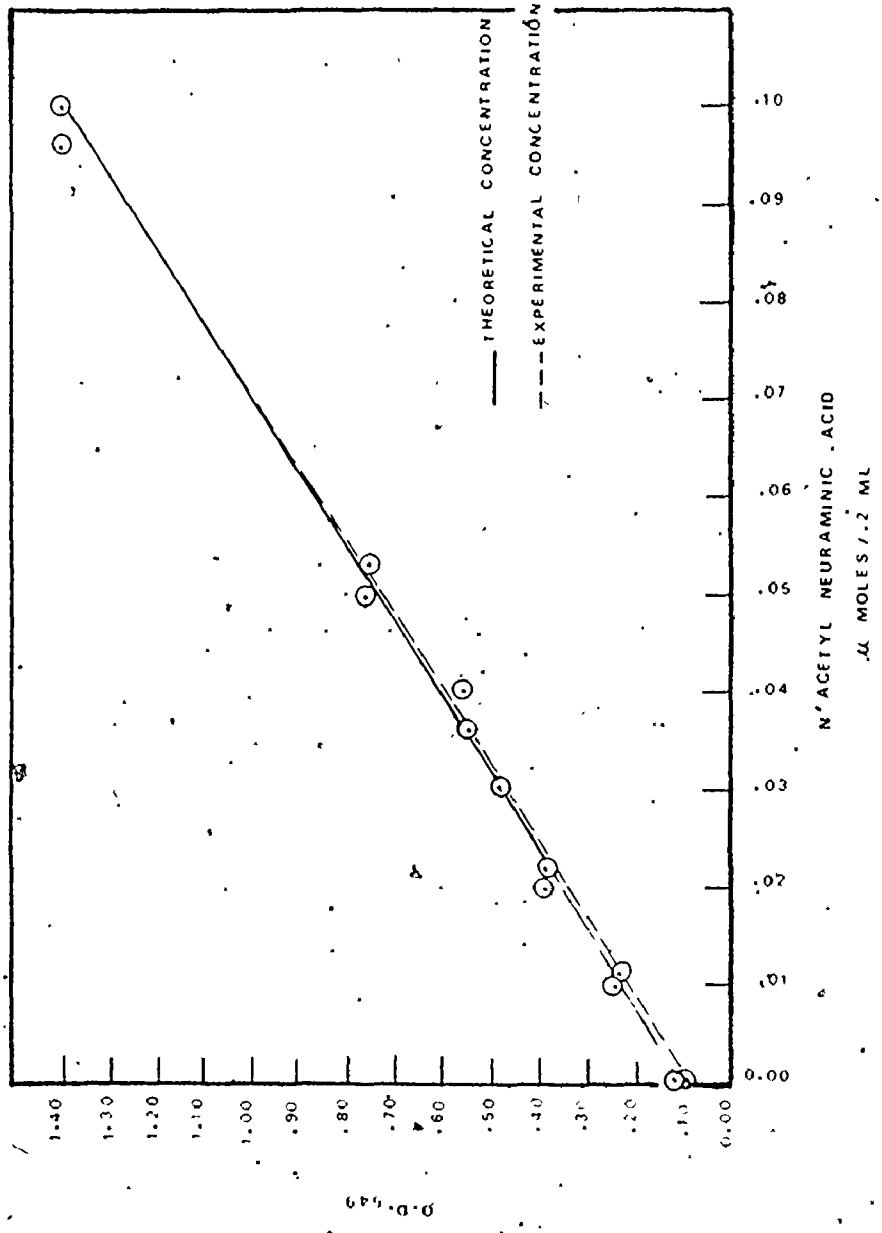

THEORETICAL CONCENTRATION
EXPERIMENTAL CONCENTRATION
N'ACETYL NEURAMINIC ACID
μ MOLES/.2 ML
O.D. 549
.10
.09
.08
.07
.06
.05
.04
.03
.02
.01
0.00
1.40
1.30
1.20
1.10
1.00
.90
.80
.70
.60
.50
.40
.30
.20
.10
0.00

TABLE XIX

KINETICS OF NEURAMINIDASE

INFLUENCE OF SUBSTRATE CONCENTRATION

Final Concentration of Substrate[a] μ moles/.2 ml	A		B		A-B (corrected)[b] Concentration of N'AHA μ moles/.2 ml
	$O.D._{549}$	$O.D._{532}$	$O.D._{549}$ X .084	$O.D._{532}$ X 0.31	
.006	0.506	0.176	0.0425	0.0055	0.032
.005	0.391	0.131	0.0328	0.0041	0.024
.004	0.514	0.180	0.0432	0.0056	0.033
.003	0.541	0.191	0.0454	0.0059	0.035
.002	0.306	0.098	0.0257	0.0030	0.018
.001	0.121	0.028	0.0102	0.0009	0.005
.000	0.000	0.000	0.0000	0.0000	0.000
No neuraminidase .006	0.057	0.005	0.0048	0.0002	0.000

[a] Dilutions were made from a stock solution of human α-1 acid glycoprotein, 2.6687 mg/ml or .06 μ moles/ml. 0.1 ml substrate was incubated with 0.1 ml neuraminidase solution, 78 U/ml for 15 min at 37°C. Reagents were prewarmed to 37°C.

[b] The value of the control containing no neuraminidase was first subtracted from each value to give the correctedconcentrations.

FIGURE 8

Influence of Substrate Concentration

on the Assay of Neuraminidase

Dilutions of human α-1 acid glycoprotein were incubated w. .78 U. neuraminidase for 15' at 37°C. Released sialic acids were determined by the thiobarbituric acid assay.

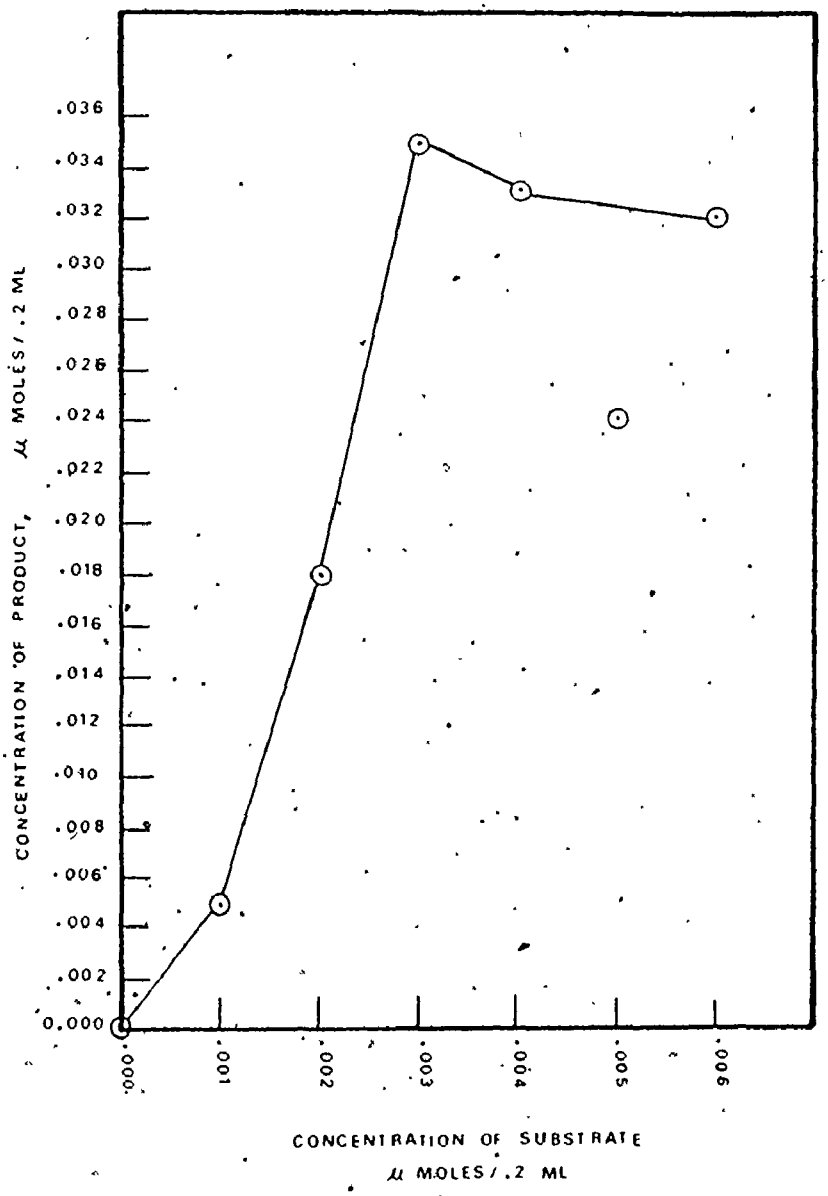

.036
.034
.032
.030
.028
.026
.024
.022
.020
.018
.016
.014
.012
.010
.008
.006
.004
.002
0.000
CONCENTRATION OF PRODUCT, μ MOLES/.2 ML
.000
.001
.002
.003
.004
.005
.006
CONCENTRATION OF SUBSTRATE
μ MOLES/.2 ML

TABLE XX:

KINETICS OF NEURAMINIDASE

EFFECT OF TIME ON THE ENZYME-SUBSTRATE REACTION

Time of Incubation[a]			A	B	A-B
	$O.D._{549}$	$O.D._{532}$	$O.D._{549}$ X .084	$O.D._{532}$ X .031	μ moles NANA/.2 ml
0 minutes	0.139	0.027	0.0117	0.0008	0.011
5	0.117	0.028	0.0098	0.0009	0.009
10	0.294	0.088	0.0247	0.0027	0.022
15	0.363	0.120	0.0305	0.0037	0.027
20	0.385	0.126	0.0323	0.0039	0.028
25	0.614	0.208	0.0516	0.0064	0.045
30	0.724	0.251	0.0608	0.0078	0.053
45	0.784	0.275	0.0659	0.0085	0.057
60	0.885	0.327	0.0743	0.0101	0.063

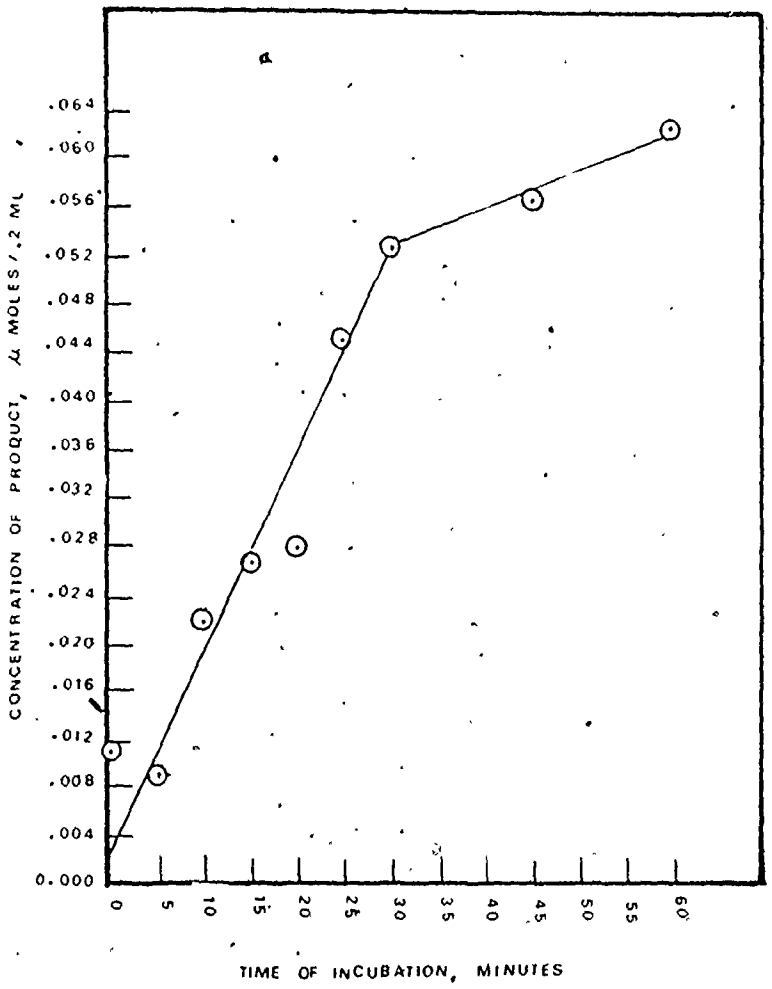
[a] 0.10 ml of neuraminidase, 78 U/ml, was incubated with 0.10 ml of human α-1 acid glycoprotein, 2.6687 mg/ml, at 37°C for various times. All reagents were prewarmed to 37°C.

FIGURE 9

The Effect of Time on the

Enzyme-substrate Reaction

Human α-1 acid glycoprotein, 0.06 μmoles/ml, in a volume of 0.1 ml was incubated with 0.1 ml of neuraminidase, 78 U/ml, for various times at 37°C. Released sialic acids were determined by the thiobarbituric acid assay.


CONCENTRATION OF PRODUCT, μ MOLES/.2 ML
.064
.060
.056
.052
.048
.044
.040
.036
.032
.028
.024
.020
.016
.012
.008
.004
0.000
0
5
10
15
20
25
30
35
40
45
50
55
60
TIME OF INCUBATION, MINUTES

was observed from 0' to 30', after which the slope began to decline. Thus, use of 15' incubation assured that the initial velocity of the reaction was being measured.

Finally, a standard assay for activity was performed on the neuraminidase preparation. Various concentrations of neuraminidase were incubated with excess substrate (0.06 μmoles/0.2 ml of human α -1 acid glycoprotein) for 15' at 37°C, pH 5.5 (Table XXI, Fig. 10). GIBCO defines 1 unit of neuraminidase as that required to release 1 μg of N-acetyl neuraminic acid from human α -1 acid glycoprotein in 15' at 37°C (pH 5.5). Therefore the amount of NANA released from the substrate by neuraminidase was calculated under these conditions. Concentrations of NANA within an $O.D._{549}$ range of 0.45 to 0.85 were chosen to make this determination, since this O.D. range was considered most accurate (Aymard-Henry, et al., 1973).

Calculated (neuraminidase)	$O.D._{549}$	μmoles/.2 ml NANA	μg/.2 ml	μg NANA released / U of N'ase
20	0.744	0.053	16.39	0.82
15	0.645	0.046	14.23	0.95
10	0.584	0.042	12.49	1.30
			Average:	1.02

a. Calculated concentration of neuraminidase was determined from dilutions of the standard preparation, stated activity = 500 U/ml.

In 15' at 37°C, 1.02 μg of NANA was released; this value agrees well with the expected value of 1.00 μg. Therefore the activity of the neuraminidase was accepted as stated to be 500 U/ml.

TABLE XXI

STANDARD ASSAY FOR NEURAMINIDASE ACTIVITY[a]

Concentration of Neuraminidase[b] .10 ml/tube			A	B	A-B μ moles NANA per .2 ml
	$O.D._{549}$	$O.D._{532}$	$O.D._{549}$ X 0.84	$O.D._{532}$ X .031	
200 U/ml	0.744	0.295	0.0625	0.0092	0.053
150	0.645	0.256	0.0542	0.0079	0.046
100	0.584	0.238	0.0491	0.0074	0.042
50	0.385	0.168	0.0323	0.0052	0.027
20	0.210	0.092	0.0176	0.0029	0.015
10	0.154	0.070	0.0129	0.0022	0.011
5	0.033	0.034	0.0028	0.0011	0.002
1	0.063	0.054	0.0053	0.0018	0.004
.1	0.036	0.039	0.0030	0.0012	0.002
0	0.000	0.000	0.0000	0.0000	0.000
No substrate 150 U/ml	0.021	0.024	0.0018	0.0007	0.001

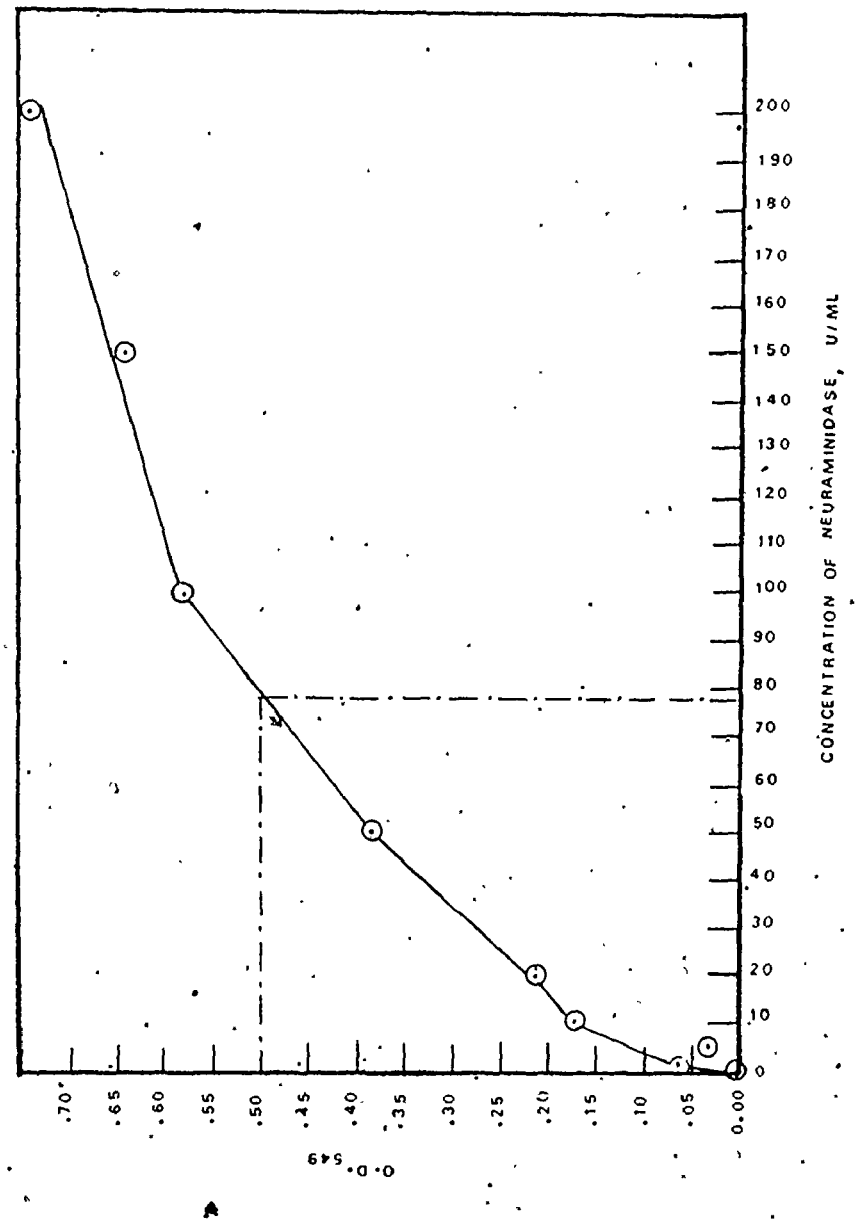
[a] 0.10 ml of neuraminidase dilution was mixed with 0.10 ml of substrate (human α-1 acid glycoprotein, 2.6687 mg/ml) and incubated for 15 min at 37°C. All reagents were prewarmed to 37°C.

[b] Concentrations of neuraminidase were carried out by diluting the stock preparation according to the stated activity of 500 U/ml.

FIGURE 10

Standard Assay for Neuraminidase Activity

Various dilutions of the original preparation of neuraminidase (stated activity = 500 U/ml) in a volume of 0.1 ml were incubated with 0.1 ml excess substrate (0.06 µmoles/ml) for 15' at 37°C. Released sialic acids were determined by the thiobarbituric acid assay.


200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
CONCENTRATION OF NEURAMINIDASE, U/ML
.70
.65
.60
.55
.50
.45
.40
.35
.30
.25
.20
.15
.10
.05
0.00
O.D.549

APPENDIX II

C. Assay for Protease Activity in the Neuraminidase Preparation

Incubation of ^{125}I-IgG with several concentrations of neuraminidase resulted in the release of approximately 5% of the total counts into the supernatant (Table XXII). This agrees very well with the value obtained when no enzyme was present, and therefore may be ascribed to free iodine. In contrast, positive controls using two concentrations of protease VI and protease X released approximately 65% and 33% respectively. These are pronases of a broad specificity.

Under the incubation conditions used for the treatment of cells, this preparation of neuraminidase exhibits no protease activity, even at higher concentrations of enzyme than were actually used in most routine studies.

TABLE XXII

ASSAY FOR PROTEASE ACTIVITY IN NEURAMINIDASE

Enzyme[a]	Average % $\frac{\text{CPM-Supernatant}}{\text{CPM-Total}}$[b]
None	4.89
Neuraminidase, 89 U/ml	4.97
Neuraminidase, 50 U/ml	4.47
Neuraminidase, 20 U/ml	5.38
Protease VI, 2.5 mg/ml	65.85
Protease VI, 1.0 mg/ml	64.81
Protease X, 2.5 mg/ml	34.43
Protease X, 1.0 mg/ml	32.04

[a] Enzyme was incubated with 10λ^{125} I-IgG and 0.1 mg/ml BSA in a total volume of 1.0 ml for 1.0 hr at 37°C with occasional shaking.

[b] Average of two trials. Supernatant and pellet were counted separately. The total number of counts agreed well with the values obtained by precounting each tube containing 10 λ ^{125}I-IgG before addition of enzyme.

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