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# Early events in herpes simplex virus type 1 infection: Photosensitivity of fluorescein isothiocyanate-treated virions

(glycoprotein crosslinking/virus entry/virus photosensitization)

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ABSTRACT **Herpes simplex virus type 1 is photosensitized by treatment with fluorescein isothiocyanate (FITC). The inactivation of FITC-treated virions upon subsequent exposure to light is inhibited by the presence of sodium azide, suggesting the involvement of singlet oxygen in the process. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis revealed that treatment with FITC plus light induces crosslinks in viral envelope glycoproteins. Treatment of virions with high concentrations of FITC (50 μg/ml) plus light causes a reduction in the adsorption of the virus to monolayers of human embryonic lung cells. For lower concentrations of FITC (10 μg/ml) plus light, treated virions adsorb to the host cells, but remain sensitive to light until entry occurs. The loss of light sensitivity coincides with the development of resistance to antibodies. These results are most consistent with a mechanism of entry for herpes simplex virus involving fusion of the viral membrane with the plasma membrane of the host cell.**

Nearly 50 years ago, Clifton (1), Perdrau and Todd (2), and Burnet (3) observed that viruses are sensitized to the effects of visible light by the presence of heterocyclic dyes. Since then, numerous studies have appeared on the photosensitizing effects of various dyes on bacterial and animal viruses. Melnick and his colleagues (4, 5) have advanced photodynamic therapy as a means for treating cutaneous infections caused by herpes simplex virus (HSV). The effectiveness of such treatment has not been firmly established, however, and conflicting results have emerged (5–9). Furthermore, the reports (10, 11) that cultured cells are transformed by photodynamically inactivated HSV have raised serious concern over the use of dyes and light to treat virus infections.

In most of the research on the photosensitized inactivation of viruses, the dyes that have been used are positively charged, planar molecules that intercalate into viral DNA. For some viruses the dye must be added to virus-infected cells in order to obtain photosensitized virions because certain dyes cannot gain access to the DNA in mature virions. Subsequent exposure of the virus to visible light in the presence of molecular oxygen results in DNA damage, often mediated by the reactive singlet oxygen species. The comparatively short lifetime of singlet oxygen (2 μsec) (12) and its diffusion coefficient of $2.5 \times 10^{-5}$ cm$^2$/sec (13) ensure that most of the photodynamic damage will be localized within several hundred angstroms of the photosensitizing dye molecule. We have presented evidence that the hydrophobic photosensitizer acridine plus near-ultraviolet light inactivate lipid-containing viruses via damage to the viral membrane (14).

Fluorescein isothiocyanate (FITC) (see Fig. 1 *Inset*) is a photosensitizer with novel and potentially useful characteristics. When added to biological systems, the isothiocyanate group reacts rapidly with free amines and sulfhydryl groups, thereby localizing the dye through covalent linkage. Exposure to light in the presence of oxygen can thus initiate photodynamic reactions that are likely to involve the FITC-labeled molecules. Lepock *et al.* (15) reported that the membrane proteins in human erythrocyte ghosts and cultured baby hamster kidney cells are extensively crosslinked upon treatment with FITC plus light. A probable mechanism suggested by these authors is that crosslinks form between the protein initially labeled with FITC and another protein at the time of light exposure. The potential usefulness of FITC plus light in investigating biological phenomena is thus due to the ability to separate, in time and space, the initial chemical reaction and the secondary light reaction leading to crosslinking or other biological damage. In the work presented here, we have used FITC plus light to investigate the early events in HSV infections *in vitro*.

## MATERIALS AND METHODS

**Virus, Cells, and Plaquing Procedures.** HSV type 1, strain KOS, was used for all the experiments. Virus stocks were prepared on monolayer cultures of human embryonic lung (HEL) cells grown in modified Ham's F12 medium supplemented with 10% (vol/vol) fetal bovine serum. Plaque assays were done with HEL cells in plastic petri dishes, with 0.5% methylcellulose in the overlay medium. Details of these procedures have been reported (16).

**Virus Purification.** HSV was purified by a procedure similar to that of Heine *et al.* (17). A crude virus extract from $1 \times 10^8$ infected cells was layered onto a 10–30% dextran T-10 gradient prepared in culture medium and centrifuged for 80 min at 20,000 rpm in an SW-41 Ti rotor. A visible band approximately halfway down the tube contained nearly all of the infectious virus. Fractions collected by puncturing the bottom of the centrifuge tube were assayed for plaque-forming units (PFU) and, when appropriate, for radioactivity. Selected fractions were pooled, and the virus was pelleted by centrifugation for 80 min at 20,000 rpm in the SW-41 Ti rotor. The supernatant was discarded and the virus was allowed to resuspend slowly in 0.3 ml of [*N*-[tris(hydroxymethyl)methyl]glycine (Tricine)/saline (16). All purification procedures were at 4°C.

Radioactively labeled virus was prepared by growth in the presence of the desired isotope (1–2 μCi/ml for [$^{35}$S]methionine and [$^{14}$C]glucosamine and 4–8 μCi/ml for [$^3$H]thymidine; 1 Ci = $3.7 \times 10^{10}$ becquerels). Cells were infected at a multiplicity of infection of 10 PFU/cell, label was added 6 hr after infection, and virus was harvested 24 hr after infection.

**Treatment with FITC and Light.** FITC was dissolved in dimethyl sulfoxide at 100 times the desired concentration, di-

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Abbreviations: HSV, herpes simplex virus; FITC, fluorescein isothiocyanate; HEL, human embryonic lung; PFU, plaque-forming units; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

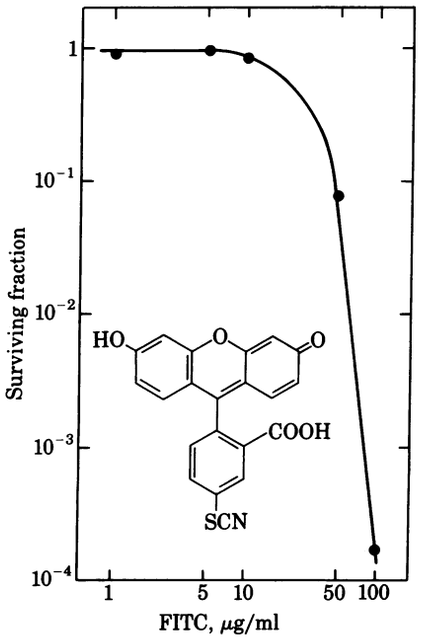


FIG. 1. Survival of HSV exposed to varying concentrations of FITC (*Inset*) in the dark. FITC was dissolved in dimethyl sulfoxide at 100 times the desired final concentration and diluted 1:100 into a virus suspension containing $\approx 10^8$ PFU/ml in Tricine/saline. Thirty minutes later, appropriate dilutions were assayed for surviving PFU on monolayers of HEL cells.

luted 1:100 into virus suspensions in Tricine/saline, and maintained in the dark at room temperature for 30 min. Two procedures were used for exposure to light. For irradiation of virus suspensions, the sample was placed in the geometric center of four 15-W cool-white fluorescent bulbs mounted vertically. The distance from the sample tube to each bulb was 6.5 cm. For irradiation of virus-infected cells on petri dishes, the dish was placed 2.0 cm from the lens of a vertically oriented slide projector containing a 500-W incandescent lamp. Both sources provided rapid, reproducible inactivation of FITC-treated virus. Sample temperatures increased by no more than 3°C above ambient temperature (≈22°C) for the exposure times used.

**Virus Adsorption Assay.** Confluent monolayers of HEL cells in 60-mm petri dishes were washed twice with Tricine/saline. The final wash was removed, and 0.2 ml of the purified, labeled virus sample to be tested was added to the cells. At various times thereafter, the amount of unadsorbed radioactivity was determined by removing 10 μl of the inoculum and assaying by liquid scintillation spectrometry. Multiplicities were always kept below 50 PFU/cell to avoid approaching the predetermined saturation limit of HEL cells (data not shown) and to maintain pseudo-first-order kinetics. Control experiments showed that, by this procedure, the radioactivity from labeled virus is quantitatively recovered as unadsorbed virus plus label recovered in cells scraped from the petri dishes.

**NaDodSO$_4$/Polyacrylamide Gel Electrophoresis.** Electrophoresis was done by the procedure of Laemmli (18) as modified by Manservigi *et al.* (19). Labeled virions were prepared for electrophoresis by resuspending pelleted virus in 0.1 ml of NaDodSO$_4$ sample solution. After electrophoresis the gels were impregnated with the commercial scintillant EN$^3$HANCE prior to gel drying and exposure to x-ray film. The film was fogged before use (20).

**Sources of Materials.** Dextran T-10 was from Pharmacia. [*methyl*-$^3$H]Thymidine (55 Ci/mmol), L-[$^{35}$S]methionine (955 Ci/mmol), D-[$^{14}$C]glucosamine·HCl (54 Ci/mol), and EN$^3$HANCE were from New England Nuclear. X-OMat x-ray film was supplied by Eastman–Kodak. Immune serum globulin (Gamastan) was obtained from Cutter Laboratories (Berkeley, CA).

## RESULTS

**Inactivation of HSV by FITC and Light.** Initial experiments were carried out to determine the sensitivity of HSV to FITC alone. Virus suspensions were exposed to various concentrations of FITC in the dark, diluted appropriately, and assayed for PFU. The data of Fig. 1 show that concentrations of FITC up to ≈10 μg/ml have no significant effect on HSV infectivity. At 50 and 100 μg of FITC per ml there is a precipitous drop in infectious titer.

Pretreatment of virus suspensions in the dark with 10 μg of FITC per ml rendered the virus irreversibly photosensitive. Fig. 2 shows survival curves for treated and control virus exposed for various lengths of time to fluorescent light. Samples exposed to light in the presence of FITC showed sensitivity comparable to that of samples that were diluted 1:$10^5$ to remove excess unreacted FITC prior to exposure. This result indicates that inactivation is primarily due to the effects of light absorbed by FITC molecules that have reacted with the virions.

We studied the effects of sodium azide on the light inactivation of FITC-treated virus and found that its presence completely inhibited the process (Fig. 2). Sodium azide quenches the reactive singlet oxygen species produced by many dyes and light (21, 22). These data suggest that the inactivation observed here is due to a photodynamic process mediated through singlet oxygen.

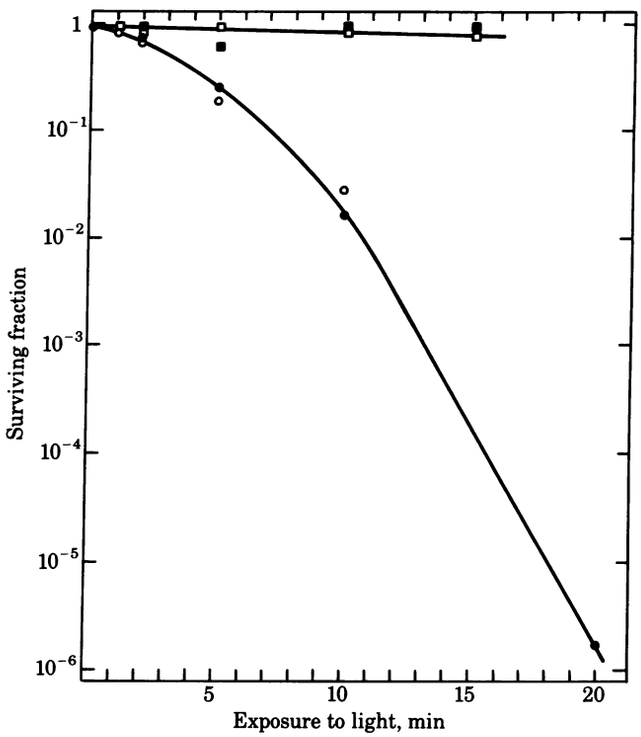


FIG. 2. Survival of FITC-treated HSV in light. A virus suspension containing $\approx 10^8$ PFU/ml in Tricine/saline was exposed to 10 μg of FITC per ml as described in the legend to Fig. 1 and then divided into three portions. ●, One sample was exposed directly for varying lengths of time to four fluorescent lamps. ○, A second sample was diluted 1:2 × $10^5$ in culture medium and then exposed to light. ■, A third sample was exposed to light after addition of 50 mM sodium azide. □, An untreated virus sample was exposed to light as a control.

**Crosslinking of HSV Glycoproteins.** $NaDodSO_4$/polyacrylamide gel electrophoresis was done on treated and control virus samples to ascertain whether virion proteins are crosslinked upon treatment with FITC plus light. HSV was grown in the presence of [$^{35}$S]methionine to label all virion proteins. A sample was also prepared from HSV-infected cells labeled with [$^{14}$C]glucosamine 6–12 hr after infection to infer the identity of the major HSV-specified glycoproteins. Fig. 3 shows gel patterns with [$^{35}$S]methionine labeling for untreated purified virions, FITC (50 $\mu$g/ml) treatment alone, and FITC plus a 30-min exposure to light. It also gives the profile of [$^{14}$C]glucosamine-labeled infected cells. The species labeled C, B, and D correspond to the three major, fully glycosylated glycoproteins, designated $C_2$, $B_2$, and $D_2$ by Spear (ref. 23; see also refs. 19 and 24). Comparison of the patterns for the [$^{35}$S]methionine-labeled virions shows that all three major glycoprotein bands are sharply diminished for the sample treated with FITC plus light (lane c). The small amount of radioactivity remaining as a sharp band of slightly lower mobility than B (lane c) probably corresponds to protein VP7.5. A similar result was observed by Sarmiento *et al.* (24) using conditions in which B existed as a dimer. Furthermore, there is a general haze in the low-mobility region of the gel (lane c), culminating with new material at the top of the gel, indicative of high molecular weight polypeptide. The slightly lower mobility of B after FITC treatment may be due to addition of a large number of FITC molecules. We interpret these results to mean that FITC plus light induces crosslinks in HSV proteins, primarily in the envelope glycoproteins C, B, and D. The presence of sodium azide during exposure to light inhibited the crosslinking reaction (data not shown).

Additional experiments were carried out with purified virions labeled by growth in the presence of [$^{14}$C]glucosamine (Fig. 4). One sample was treated with 10 $\mu$g of FITC per ml plus light and another with 50 $\mu$g of FITC per ml plus light. Untreated samples and samples treated with 50 $\mu$g of FITC per ml alone are also shown in Fig. 4. The gel was overexposed in order to show new, discrete bands that migrated more slowly than the HSV glycoproteins. Very high molecular weight material was also observed at the top of the gel. Careful inspection of these and other gels indicated that there is more material in the bands marked 1 and 2 for samples that were treated with 10 $\mu$g of FITC per ml plus light than for those treated with 50 $\mu$g of FITC per ml plus light. These bands probably correspond to dimers and trimers of the viral glycoproteins. The origin of the material in band 3 is not clear. It may be derived from glycoprotein D, but its migration is greater than expected for a linear polypeptide having a molecular weight twice that of D. One possibility is that two molecules of D are crosslinked near their centers, giving rise to a nonlinear species that migrates as band 3. The behavior of such branched polypeptides on $NaDodSO_4$/polyacrylamide gels has not been characterized.

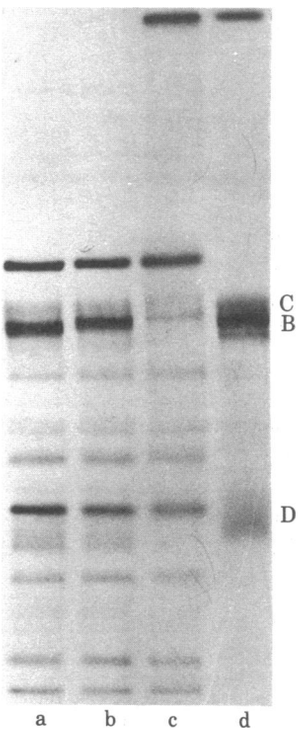


FIG. 3. $NaDodSO_4$/polyacrylamide gel profiles showing the effects of FITC and light on the proteins of purified HSV particles. The samples in lanes a–c were labeled with [$^{35}$S]methionine, purified, and treated as follows: lane a, untreated control; lane b, FITC (50 $\mu$g/ml) in the dark; lane c, FITC (50 $\mu$g/ml) followed by a 30-min exposure to light. Lane d gives the pattern for [$^{14}$C]glucosamine-labeled infected cells.

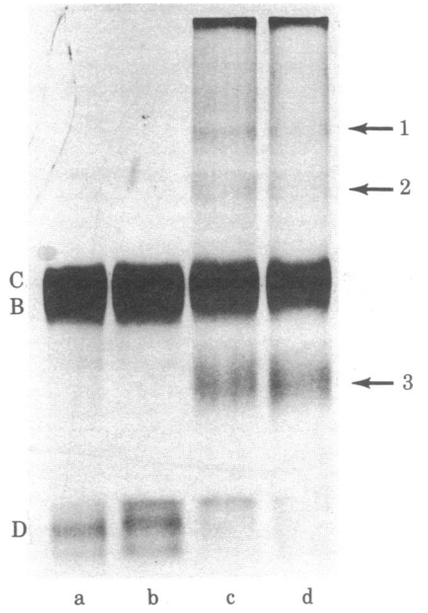


FIG. 4. $NaDodSO_4$/polyacrylamide gel profiles of [$^{14}$C]glucosamine-labeled virus, showing the appearance of new discrete bands of glycoproteins. The labeled, purified virions were treated as follows: lane a, untreated control; lane b, FITC (50 $\mu$g/ml) in the dark; lane c, FITC (10 $\mu$g/ml) plus 30 min of light; lane d, FITC (50 $\mu$g/ml) plus 30 min of light. The amount of material remaining in the glycoprotein B and glycoprotein C regions of the gel cannot be estimated from this radioautogram because of the long exposure used to demonstrate the existence of new, discrete bands designated 1, 2, and 3.

**Effect of FITC and Light on HSV Adsorption.** In order to determine if virus adsorption was photosensitive, purified virions were prepared and treated with FITC (50 $\mu$g /ml) with or without a 30-min exposure to light. Untreated samples were maintained as controls. The virions had been doubly labeled during growth—with [$^{3}$H]thymidine to label DNA and [$^{14}$C]glucosamine to label envelope glycoproteins. Adsorption kinetics were determined by assaying the amount of radioactivity remaining unadsorbed to monolayers of cells at various times after addition of virus. The data (Fig. 5) are normalized to the initial amount of virus at time zero. Control and FITC-treated virions adsorbed with the same kinetics and gave kinetics very similar to those obtained by measurement of unadsorbed PFU (data not shown). Although FITC treatment in

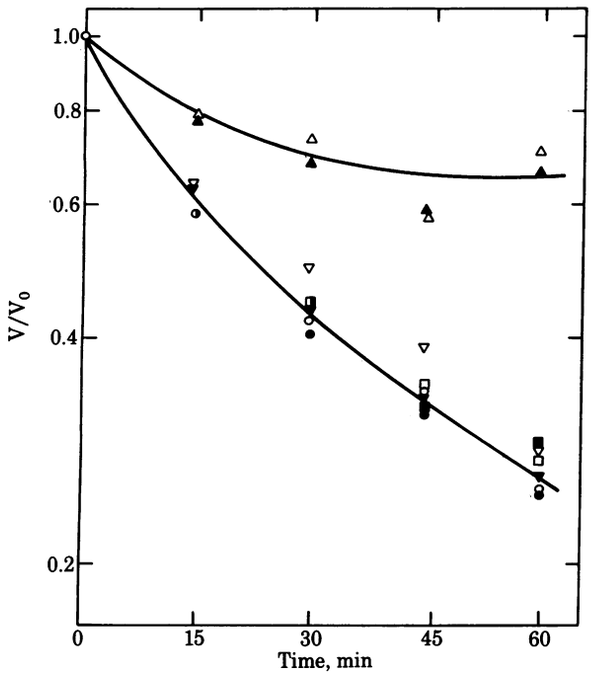


FIG. 5. Effect of FITC plus light on adsorption of HSV to HEL cells. Virus was doubly labeled with [$^3$H]thymidine and [$^{14}$C]glucosamine. After purification, samples were treated as follows (closed symbols $^3$H; open symbols, $^{14}$C): ● and ○, untreated control; ■ and □, FITC (50 μg/ml) in the dark; ▲ and △, FITC (50 μg/ml) followed by a 30-min exposure to light; ▼ and ▽, FITC (10 μg/ml) followed by a 30-min exposure to light. Data are presented as the amount of unadsorbed virus (V) normalized to the initial amount of unadsorbed virus ($V_0$) at time zero.

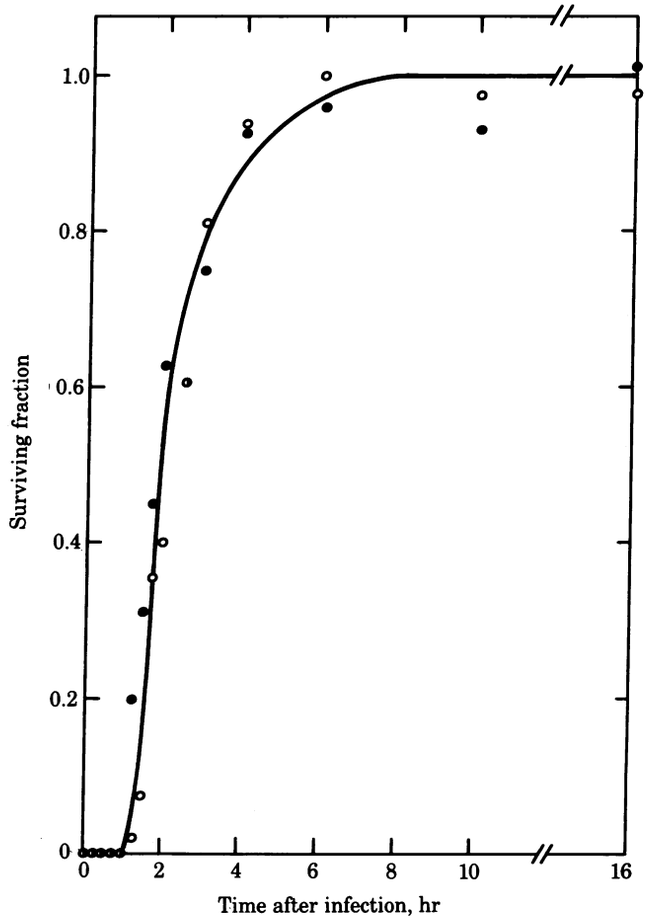


FIG. 6. Development of photoresistance and antibody resistance by FITC-treated HSV during entry. A virus suspension containing ≈$10^7$ PFU/ml in Tricine/saline was treated with 10 μg of FITC per ml in the dark for 30 min. The sample was diluted in Tricine/saline and plated (time 0) on monolayers of HEL cells. After adsorption for 1 hr at 4°C, the infected cells were incubated in medium at 37°C. At various times samples were treated either with light from a slide projector for 5 min (○) or with immune serum globulin for 30 min (●). In the latter case, medium was removed from the infected monolayer of cells, 5% (vol/vol) immune serum globulin in cold Tricine/saline was added for 30 min, the cells were washed, and warm medium containing methylcellulose was added. The number of surviving PFU has been normalized to the number of PFU for samples exposed to neither light nor immune serum globulin.

the dark caused about 90% decrease in infectivity (Fig. 1), virus adsorption was unaffected. However, adsorption was markedly reduced by exposure of treated virions to light (Fig. 5). This effect on adsorption was inhibited by the presence of sodium azide during exposure to light (data not shown). At low concentrations of FITC (10 μg/ml) plus light, no inhibition of attachment was observed (Fig. 5), even when virus inactivation was greater than 99%. These results indicate that the loss of HSV infectivity for treatment with FITC (10 μg/ml) plus light is associated with some stage in the infectious process after adsorption.

In all cases, adsorption kinetics for the DNA label and the glycoprotein label were the same, indicating that the viral envelope remains intact during treatment with FITC and light. This was confirmed by analysis on dextran gradients, which revealed that control and treated virions sedimented with equal velocities and contained the same ratio of $^3$H to $^{14}$C (data not shown).

**Transition from Photosensitivity to Photoresistance.** The following experiments were done to determine the stage in virus development at which virions treated with FITC (10 μg/ml) in the dark lose their photosensitivity. Approximately 80 PFU of treated virus were added (time 0) to each of several 60-mm diameter petri dishes containing monolayers of HEL cells. These were kept at 4°C in Tricine/saline for 1 hr for adsorption to occur. Warm medium was then added (time, 1 hr), the samples were placed at 37°C, and, at the indicated time intervals, one sample was exposed to intense light for 5 min (Fig. 6). During the adsorption period the virus remained completely photosensitive, with greater than 99% of the PFU being inactivated. Immediately after the samples were placed under growth conditions at 37°C there was a rapid rise in photoresistant PFU. By about 1 hr after warming to 37°C, ≈50% of the virus were no longer sensitive to exposure to light. These results suggest that the process of entry of HSV into the host cell is correlated with the loss of photosensitivity of FITC-treated virus.

In order to further establish this point, we compared the transition from photosensitivity to photoresistance for FITC (10 μg/ml)-treated virus with the kinetics for loss of antibody sensitivity. At various time intervals samples were exposed to human immune serum globulin to inactivate HSV virions that still remained outside the cell. These data (Fig. 6) show that the loss of antibody sensitivity is, within experimental error, coincident with the transition from photosensitivity to photoresistance.

To further examine the simultaneity of the development of antibody resistance and photoresistance, we performed the following experiment. A sample of HSV was treated with FITC, adsorbed to cells at 4°C for 1 hr in several petri dishes, and then incubated at 37°C in medium for 1 hr. One sample was then exposed to light alone, another to immune serum globulin, and

Table 1. Survival of FITC-treated virus upon exposure to light and immune serum globulin (ISG)

| Exposure | Survival, % |
|---|---|
| Light alone | 20 |
| ISG alone | 22 |
| ISG plus light | 26 |

a third to the globulin followed immediately by exposure to light. An untreated control was maintained in order to determine the survival level in each case. The results of this experiment (Table 1) gave essentially the same survival for all three treatments. This result shows that those virions that develop resistance to immune serum globulin also develop, either simultaneously or within a 5-min period, resistance to photosensitized inactivation by light.

## DISCUSSION

By three separate criteria, we have shown that FITC plus light is damaging to HSV. First, by biological assay for PFU, comparatively mild treatment with 10 $\mu$g of FITC per ml and a 10-min exposure to light reduces the infectivity by greater than 95%. Second, by analysis of virion proteins, treatment with 10–50 $\mu$g of FITC per ml plus a 30-min exposure to light results in observable crosslinking of viral glycoproteins. Third, treatment with 50 $\mu$g of FITC per ml plus a 30-min exposure to light significantly reduces the adsorption of virus to the host-cell surface. All three types of damage are greatly diminished when HSV are exposed to light in the presence of sodium azide, suggesting the involvement of singlet oxygen in each case. Although these three effects may be due to reactions that are similar in nature, there are quantitative differences in the degree of treatment, both in FITC concentration and length of exposure to light, required for each. Inactivation of PFU occurs before detectable crosslinks are observed, and crosslinking of envelope glycoproteins is quite extensive before adsorption is significantly inhibited. Thus, the inactivation by 10 $\mu$g of FITC per ml plus light is due to interference with some stage in virus development later than adsorption.

It is not certain that the damage caused by 10 $\mu$g of FITC per ml plus light inactivates the virus by photoinduced damage to envelope glycoproteins. It is possible that the inactivating event is damage to some virion component not associated with entry, such as viral DNA, and is mediated through the absorption of light by FITC molecules attached to the viral envelope. Singlet oxygen produced at the virus surface could diffuse a sufficient distance to react with some sensitive internal target. The transition from photosensitivity to photoresistance is a measure of the time at which the viral envelope is separated from the nucleocapsid.

Carefully controlled experiments (Fig. 6 and Table 1) revealed that virus treated with 10 $\mu$g of FITC per ml develops resistance to light and to antibody either simultaneously or within 5 min. This observation and its interpretation are relevant to the mechanism whereby HSV enters the host cell. The data of Figs. 3 and 4 indicate that, with regard to crosslinking, the major effect of FITC plus light is on viral envelope glycoproteins. This is to be expected for a highly reactive, charged moleule. It seems reasonable that the virus becomes photoresistant at the time it loses its envelope. If HSV enters the host by a process of fusion between the viral envelope and the cell plasma membrane, then entry and the development of photoresistance would coincide, in agreement with our observation. On the other hand, if entry involved the engulfment of HSV particles by an endocytotic process, there might be a period of time after the development of resistance to antibodies that the FITC-labeled virus remains sensitive to light. If such a period exists, our data limit its duration to 5 min or less. Thus, although these considerations are not conclusive, they support fusion rather than endocytosis as the mode of entry for HSV, in agreement with some existing electron microscopic (25) and biochemical (24) data.

The use of FITC plus light provides a convenient and useful method for monitoring the entry and variables that modify entry of HSV and other enveloped viruses. Two major advantages of this approach to studying viral entry are that the experiments yield information regarding *infectious* virions only and that cells can be infected at a low multiplicity of infection.

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