Inactivation of the Scrapie Agent by Ultraviolet Irradiation in the Presence of Chlorpromazine

By C. DEES, W. F. WADE, T. L. GERMAN AND R. F. MARSH*

Department of Veterinary Science, 1655 Linden Drive, University of Wisconsin–Madison, Madison, Wisconsin 53706, U.S.A.

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SUMMARY

The sensitivity of the scrapie agent to u.v. inactivation was found to be related to the purity of the tissue preparation. Scrapie infectivity associated with membrane vesicles was unaffected when irradiated with $10^4 \text{ J/m}^2$. Irradiation of more highly purified preparations from detergent-extracted CsCl gradient fractions reduced scrapie infectivity from $10^{7.8} \log_{10} \text{LD}_{50} \text{ per ml}$ to as low as $10^{4.5}$. Sensitivity of membrane-associated scrapie infectivity to inactivation by u.v. irradiation could be increased by addition of chlorpromazine, a phenthiazine antipsychotic which penetrates lipid bilayers and induces single-strand breaks in nucleic acids under irradiation. Chlorpromazine without irradiation, and a semiquinone protein-binding radical of chlorpromazine, failed to decrease scrapie infectivity by themselves. A closely related phenthiazine antipsychotic, trifluoperazine, which does not bind to nucleic acids, did not reduce scrapie infectivity. These findings suggest that the target of u.v. radiation for inactivation of scrapie infectivity in the presence of chlorpromazine is an essential nucleic acid.

INTRODUCTION

Scrapie infectivity is substantially resistant to inactivation by u.v. and ionizing radiations (Alper et al., 1967; Latarjet, 1979). It has also been shown that the scrapie agent exhibits unusual resistance to u.v. inactivation in the presence of psoralens (McKinley et al., 1983), which form adducts with nucleic acids under irradiation. Three explanations have been offered to explain the high resistance to psoralen–u.v. inactivation: (i) psoralens failed to penetrate the protein coat of the agent, (ii) the nucleic acid of the agent is unreactive to psoralens, and (iii) the agent has no nucleic acid (McKinley et al., 1983). While the latter two possibilities await the detection of a scrapie-specific nucleic acid, protection of the agent from u.v. inactivation may result from its close association with cellular membranes (Millson et al., 1971; Semancik et al., 1976). Thus, lipid, a principal component of biological membranes, may be in part responsible for protecting a nucleic acid essential for scrapie infectivity from u.v. and u.v.–psoralen inactivation (Latarjet, 1979). This study investigates the sensitivity of the scrapie agent in various membrane preparations to u.v. inactivation. The results indicate that u.v. sensitivity is increased after removal of some lipids and proteins by detergent extraction, and also by treatment with chlorpromazine, a phenthiazine antipsychotic which binds to nucleic acids.

METHODS

Agent and bioassay. These studies used the Chandler strain of mouse scrapie after adaption to outbred hamsters as previously described (Kimberlin & Marsh, 1975). Infectivity was determined by the method of incubation interval assay (Prusiner et al., 1981) using outbred male weanling hamsters purchased from Harlan Sprague Dawley (Indianapolis, Ind., U.S.A.).

Preparation of membrane vesicles and CsCl fractions. Membrane vesicles were prepared from scrapie-infected and uninfected brain tissue on iodinated gradients as previously described (Marsh et al., 1984). Briefly, plasma membrane-enriched homogenates from scrapie-infected or age-matched healthy control hamsters were sonicated and separated on Nycodenz® using rate zonal centrifugation. Fractions enriched for large membrane vesicles

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were isolated and used for irradiation studies. In some instances, vesicle fractions were extracted with 0.5% Triton X-100 then re-fractionated by equilibrium density centrifugation in CsCl (Marsh et al., 1984). A CsCl fraction with high scrapie infectivity was used for further studies.

**U.v. irradiation.** Gradient fractions enriched for membrane vesicles were irradiated before and after detergent extraction and with or without various concentrations of chlorpromazine or trifluoperazine (TFP). Protein levels in all samples were approximately 50 µg/ml as determined by the Bradford dye binding assay (Bradford, 1976). Two ml samples in 100 mM-NaCl and 10 mM-Tris-acetate pH 7.8, were irradiated in 60 x 15 mm plastic dishes at room temperature with a Chromatovue Transiluminator Model C-63 (Ultraviolet Products Inc., Walnut Grove, Ca., U.S.A.) at a distance of 0.5 cm. Samples were irradiated at a dose rate of 84 J/m^2 sec until a total dose of 10^4 J/m^2 had been delivered. U.v. output and dosage was monitored with a Blak Ray u.v. meter (Blak Ray, San Gabriel, Ca., U.S.A.). Irradiated samples were sonicated for 1 min with a model W-10 Sonicator® (Heat Systems Ultrasonics, Plainview, N.Y., U.S.A.), then inoculated into hamsters.

**Formation of semiquinone radicals of chlorpromazine.** A semiquinone free radical of chlorpromazine that has previously been shown to bind to proteins of the Na^+,K^+ ATPase was prepared using the methods of Akera & Brody (1969). Various concentrations of the free radical, as determined by absorption spectroscopy at 527 nm, were added to membrane vesicles from scrapie-infected hamsters. Samples were incubated with the semiquinone for 1 h at 37 °C without u.v. irradiation, sonicated, then inoculated into hamsters to test for infectivity.

**Binding of [3H]TFP to membrane vesicles.** To measure TFP binding to membrane vesicles prepared from infected or uninfected hamster brain, [3H]TFP (Amersham) was used to monitor binding activity. 2.5 gCi of [3H]TFP (sp. act. 72 Ci/mmol) was added to a 1 mM solution of unlabelled TFP (Sigma). Unlabelled TFP was added along with the tritiated tracer to give a final concentration of 30 µM-TFP. Samples containing approximately 50 µg of protein per ml were incubated with and without Ca^2+ (150 µM-CaCl_2) for 10 min at 37 °C, then the unbound TFP was removed by dialysis against distilled water for 48 h at 4 °C. After dialysis, protein concentrations of the membrane vesicles were measured (Bradford, 1976) and the activity of [3H]TFP determined by scintillation counting.

**Statistical methods.** Results of the [3H]TFP binding studies were converted by data log transformation to obtain homoscedastic treatment group variances, then examined by analysis of variance (Barlett & Kendall, 1946). Significant differences between treatment group means were determined using Student’s t-test and pooled treatment group variance (Fisher, 1925).

**RESULTS**

**Effects of u.v. irradiation**

Table 1 shows that the effects of u.v. irradiation were more pronounced on the scrapie agent after detergent extraction and fractionation on CsCl gradients than on the agent in membrane vesicles. The infectivity in one detergent-extracted, u.v.-irradiated sample was reduced from 10^7.8 log_10 LD^50 per ml to 10^4.5. The infectivity of the scrapie agent in unextracted membrane vesicles was changed very little by u.v. irradiation.

**Effects of chlorpromazine, TFP and u.v. irradiation on infectivity**

U.v. irradiation of the scrapie agent in the presence of chlorpromazine resulted in losses of infectivity which were dependent on chlorpromazine concentration (Table 2). Irradiation of the scrapie agent in the presence of TFP failed to reduce scrapie infectivity at any concentration tested. Experiments examining higher concentrations of TFP (2 mM) were found to reduce scrapie infectivity without u.v. irradiation (data not shown), probably due to the extreme acidity of TFP at high concentrations.

**Effect of protein-binding radicals on scrapie infectivity**

Chlorpromazine semiquinone radicals that bind protein targets in brain tissue failed to inactivate scrapie infectivity at concentrations up to 0.5 mM.

**Binding of [3H]TFP to membrane vesicles**

To determine if the inability of TFP and u.v. irradiation to inactivate infectivity was due to failure to bind to protein or lipid targets in membrane vesicles, we examined the binding of TFP to vesicles prepared from infected and uninfected hamster brain. The results indicated that [3H]TFP binds equally to targets in vesicles from infected and uninfected samples without added Ca^2+ where α = 0.001 (P = 1.377). However, binding of [3H]TFP in the presence of 150 mM-Ca^2+ was significantly higher to membrane vesicles from infected tissue where α = 0.001 (P = 8.58).
Table 1. The effects of partial purification of the scrapie agent on sensitivity to u.v. inactivation

<table>
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<tr>
<th>Trial no.</th>
<th>Membrane vesicles</th>
<th>CsCl fractions</th>
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<tr>
<td>2</td>
<td>8 7</td>
<td>7.8 6.5</td>
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<tr>
<td>3</td>
<td>7 6</td>
<td>8.2 6.8</td>
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* All samples were irradiated with a total of 10^4 J/m^2.
† Titres are reported as log_{10} LD_{50} per ml as measured by the method of incubation interval assay (Prusiner et al., 1981).

Table 2. Effects of u.v. irradiation on scrapie infectivity associated with membrane vesicles in the presence of chlorpromazine or trifluoperazine

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Chlorpromazine</th>
<th></th>
<th>Trifluoperazine</th>
<th></th>
<th>log_{10} LD_{50}/ml*</th>
<th></th>
<th>Unirradiated</th>
<th>Irradiated†</th>
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<td>7.0 7.0</td>
<td></td>
<td>-------------</td>
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</tr>
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</table>

* As measured by the method of incubation interval assay (Prusiner et al., 1981).
† All samples were irradiated with a total of 10^4 J/m^2.

DISCUSSION

All currently known viral pathogens have a nucleic acid component that is essential to viral replication. However, there is speculation that the unusually high resistance of scrapie infectivity to u.v. inactivation may indicate that the agent has no nucleic acid (Alper et al., 1967; Prusiner, 1982; Lewin, 1982). It has also been reported that the scrapie agent has unusually high resistance to psoralen–nucleic acid adduct formation and one possible explanation for this finding is that the agent may have no nucleic acid (McKinley et al., 1983). Thus, one view of the physicochemical nature of the scrapie agent is that it is a pathogen with no nucleic acid.

One possible explanation for the unusual resistance of the scrapie agent to u.v. irradiation, and its high resistance to u.v.–psoralen adduct formation, might be that the nucleic acid is small and in close association with cellular components that provide protection (Marsh et al., 1974; Kimberlin, 1982: Latarjet, 1979). Highly u.v.-resistant small viral nucleic acids with pronounced biological effects are not unknown (Wagner et al., 1983). It is also possible for small viral nucleic acids to be in close association with cellular components (Gallinaro et al., 1980; Wagner et al., 1983).

In this study, we provide evidence that the scrapie agent may contain an essential nucleic acid that is protected from u.v. radiation by its small size and close association with cellular components, especially lipids. Scrapie infectivity was less sensitive to u.v. inactivation when associated with untreated membrane vesicles compared to irradiation after the vesicles had had all of their polar lipid and much of their neutral lipid removed by detergent extraction (Dees et al., 1985). Other studies on the u.v. sensitivity of purified preparations of scrapie infectivity have also found an increased susceptibility to u.v. irradiation (Marsh et al., 1978), while one study on fluorocarbon-extracted tissues was unable to demonstrate any differences in u.v. sensitivity (Haig et al., 1977). In this latter report, no information is given on purification relative to lipid content.
The insensitivity of the scrapie agent to psoralen photoadduct formation with u.v. irradiation can be explained in terms of properties exhibited by a small nucleoprotein complex. For example, psoralens form adducts between opposed nucleic acids, i.e. between double strands (Calvet & Pederson, 1979, 1981; Isaacs et al., 1977). An essential nucleic acid with little double-strand character would resist inactivation by psoralen crosslinking and would require high levels of psoralen for inactivation (Calvet & Pederson, 1979). High levels (concentration unreported) have in fact been reported to inactivate the scrapie agent (McKinley et al., 1983). A single-stranded essential nucleic acid in close association with cellular components could account for the observed resistance of the agent to psoralen inactivation.

These studies examined the effects of chlorpromazine, a phenthiazine antipsychotic which binds to RNA and DNA inducing single-strand breaks (Fujita et al., 1980) capable of inactivating viruses in the presence of u.v. radiation (Day & Diamattina, 1977; Fujita et al., 1981). Chlorpromazine reacts faster with single-stranded than with double-stranded nucleic acids (Kahn & Davis, 1970), and is able to alter the permeability of lipid bilayers by increased penetration through membrane lipids and proteins (Maoi et al., 1979; Ogido et al., 1981). Thus, chlorpromazine may penetrate cellular components more effectively than psoralens, inducing breaks in single-stranded nucleic acids. The results indicated that u.v. irradiation in the presence of relatively low levels of chlorpromazine (25 μM) was able to reduce scrapie infectivity even when associated with intact membrane vesicles, while chlorpromazine alone (without u.v. radiation) had no effect. Chemical derivatization of chlorpromazine to semiquinone radicals that bind to protein targets (Akera & Brody, 1969) failed to inactivate the scrapie agent. Trifluoperazine, a closely related phenthiazine antipsychotic capable of irreversibly binding to brain proteins after u.v. irradiation (Speaker et al., 1980), did not inhibit scrapie infectivity. We can find no reports that TFP causes photosensitization in patients treated with this drug due to binding of TFP to nucleic acids, or any reports of studies that show nucleic acid binding in vitro as demonstrated for chlorpromazine (Kahn & Davis, 1970; Rosenthal et al., 1978).

We suggest that these findings indicate that the target within the scrapie agent that is inactivated by u.v. irradiation in the presence of chlorpromazine is an essential nucleic acid. We also speculate that these data, and previous studies using psoralens, indicate that the essential nucleic acid has little double-strand character and is protected by close association with cellular components as previously proposed (Marsh et al., 1974; Kimberlin, 1982; Latarjet, 1979).

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REFERENCES


U.v. inactivation of the scrapie agent


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