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# Transfer of Scrapie Prion Infectivity by Cell Contact in Culture

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## Summary

**Background:** When a cell is infected with scrapie prions, newly synthesized molecules of the prion protein $PrP^C$ are expressed at the cell surface and may subsequently be converted to the abnormal form $PrP^{Sc}$. In an experimental scrapie infection of an animal, the initial innoculum of $PrP^{Sc}$ is cleared relatively rapidly, and the subsequent propagation of the infection depends on the ability of infected cells to convert uninfected target cells to stable production of $PrP^{Sc}$. The mechanism of such cell-based infection is not understood.

**Results:** We have established a system in dissociated cell culture in which scrapie-infected mouse SMB cells are able to stably convert genetically marked target cells by coculture. After coculture and rigorous removal of SMB cells, the target cells express $PrP^{Sc}$ and also incorporate [$^{35}$S]methionine into $PrP^{Sc}$. The extent of conversion was sensitive to the ratio of the two cell types, and conversion by live SMB required 2500-fold less $PrP^{Sc}$ than conversion by a cell-free prion preparation. The conversion activity of SMB cells is not detectable in conditioned medium and apparently depends on close proximity or contact, as evidenced by culturing the SMB and target cells on neighboring but separate surfaces. SMB cells were killed by fixation in aldehydes, followed by washing, and were found to retain significant activity at conversion of target cells.

**Conclusions:** Cell-mediated infection of target cells in this culture system is effective and requires significantly less $PrP^{Sc}$ than infection by a prion preparation. Several lines of evidence indicate that it depends on cell contact, in particular, the activity of aldehyde-fixed infected cells.

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## Background

A key aspect of the prion hypothesis is the cellular conversion of the normal form of the prion protein ($PrP^C$) to the abnormal form ($PrP^{Sc}$) [1]. In a cell infected with scrapie prions, PrP is synthesized normally and inserted into the membrane via its C-terminal glycosylphosphatidylinositol (GPI) anchor. Molecules of $PrP^C$ may encounter $PrP^{Sc}$ in the plane of the plasma membrane in the vicinity of caveolae, or after endocytosis, and become converted to $PrP^{Sc}$ [2–4]. If a cell is treated with phosphatidylinositol-specific phosholipase C (PIPLC) to cleave the GPI anchor and release $PrP^C$, the synthesis of $PrP^{Sc}$ is abrogated, both in short-term pulse labeling experiments [3, 4] and after chronic treatment of infected cells in culture [5]. An uninfected target cell may be converted to stably produce $PrP^{Sc}$ by exposure to a subcellular preparation of $PrP^{Sc}$, such as a homogenate or fraction of a prion-infected brain. It has been difficult to obtain extensive infection of cells in culture; for example, an early report analyzed an infected culture on a clonal basis and found that only 1% of the cells were infected [6]. More recently, several laboratories have derived subclones of N2a neuroblastoma cells that are readily infected by brain homogenates, and these may provide important opportunities for approaches to diagnostics and therapeutics based in cell culture [5, 7, 8]. The mechanism of infection, however, remains unclear; for example, to what extent does the interconversion occur at the surface, as opposed to after internalization [1], and what role, if any, do accessory molecules play in the process?

Presumably, a critical aspect of the progression of prion infections in vivo is the ability of infected cells in the CNS, for example, to convert their neighbors to stably express $PrP^{Sc}$. The mechanisms underlying such cell-to-cell infection have not been studied directly, but the most obvious possibility is that infected cells produce extracellular forms of $PrP^{Sc}$ that then act on target cells as discussed above. Since some $PrP^{Sc}$ is expressed at the cell surface and is accessible to biotinylation [2, 9], it is also possible that cell-to-cell contact might play a role in intercellular conversion. In order to evaluate these possibilities, we have set up a system in dissociated culture in which genetically marked target cells are infected by coculture with scrapie-infected counterparts. In this model, intercellular prion transmission appears to be dependent on direct cell-cell contact.

## Results

The properties of the various cell lines used in this study are summarized in Table 1. Scrapie mouse brain (SMB)

Table 1. Characteristics of Cell Lines

| Cell Line | Mouse $PrP^C$ | Mouse $PrP^{Sc}$ | HMH-PrP | $Neo^R$ |
|---|---|---|---|---|
| SMB | + | + | − | − |
| PS | + | − | − | − |
| HMH1 | + | − | − | + |
| HMH8 | + | − | + | + |

The scrapie-infected SMB cell and its cured derivative, the PS cell, were as described [12]. PS cells were transfected with the pEE6 plasmid [31] expressing the chimeric mouse hamster PrP SP66 construct [14], referred to here as HMH, as well as the neo selectable marker. HMH8 transfectants were selected with G418 and expressed HMH-PrP (as detected both by immunofluorescence on live cells and by Western blotting with antibody 3F4 [13]) and *neo*$^R$, as detected by in situ hybridization. HMH1 cells expressed *neo*$^R$ but no detectable HMH protein. The column headed "Mouse $PrP^{Sc}$" refers to the status of PS, HMH1, and HMH8 before infection.

cells were originally derived from culture of mouse brain infected with the Chandler strain of mouse scrapie [10, 11] and have subsequently been cloned [12]. They divide rapidly in culture without indications of cytopathology, stably express $PrP^C$ and $PrP^{Sc}$, and maintain an invariant level of scrapie infectivity over many passages, as determined by bioassay of cell extracts after inoculation into mice [10, 11]. The SMB cells have been cured of infectivity and $PrP^{Sc}$ expression by growth in the presence of pentosan sulfate, and the resulting pentosan sulfate (PS) cell line was readily reinfected by exposure to homogenates of scrapie-infected mouse brain [12].

The target cells used in the present study were derived by transfecting PS cells under neomycin selection to obtain two clonal cell lines, HMH1 and HMH8 (Table 1). The HMH8 line expressed the selectable *neo* gene, as well as a cotransfected hamster/mouse PrP construct (referred to here as HMH), which carries the hamster 3F4 epitope [13] and, in earlier work, was converted to $PrP^{Sc}$ after transient transfection into scrapie-infected N2a cells [14]. Although the HMH protein in HMH8 was not stably converted to $PrP^{Sc}$ in the experiments described here, either by brain homogenates or by live SMB cells, it was converted after transient transfection of the construct into SMB cells and analysis after 7 days (data not shown). In our experiments, we assayed for the stable conversion of the endogenous mouse $PrP^C$ in the target cells (Table 1).

### Infection of Target Cell Lines with Scrapie Prions

The target cells were readily converted to stably express mouse $PrP^{Sc}$ by incubation with a homogenate of mouse brain infected with the 79A strain, which was originally derived from Chandler scrapie [15]. After washing to remove residual homogenate, the cells were passaged for 8–12 generations, lysed in detergent, and analyzed by Western blotting for the presence of protease-resistant and detergent-insoluble $PrP^{Sc}$. The $PrP^{Sc}$ fraction of SMB cells [12] migrates predominantly as the lower unglycosylated and upper monoglycosylated bands (depicted by arrows in Figure 1A, see legend), and infection of PS cells (Figure 1A, PS lanes) or HMH1 cells (H1 lanes) gave a comparable profile. The brain homogenate was titered on both HMH1 and HMH8, and an example with HMH1 is shown in Figure 1B, where stable expression

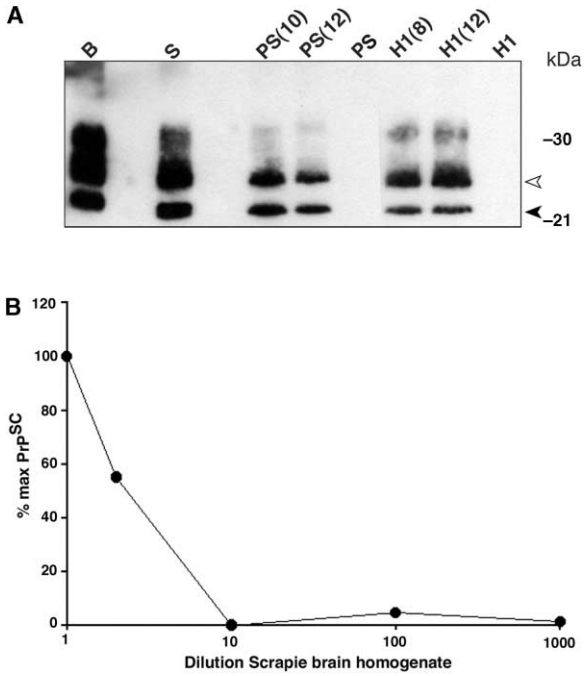


Figure 1. Infection of Target Cells with Scrapie-Infected Mouse Brain Homogenate Gives Stable Production of $PrP^{Sc}$

(A) Cells were infected with homogenates of infected mouse brain as described in the Experimental Procedures and were expanded by multiple passages prior to the preparation of $PrP^{Sc}$ fractions (see the Experimental Procedures) from equivalent numbers of cells and analysis by Western blotting. An aliquot of each cell lysate was analyzed without proteinase K treatment to detect $PrP^C$ expression, and this was comparable for each sample. The mobilities of the unglycosylated and monoglycosylated bands in the $PrP^{Sc}$ fractions are indicated with a solid or empty arrowhead, respectively. B, aliquot of the infected brain homogenate; S, SMB cells; PS(10), PS cells at 10 generations and 5 passages after infection; PS(12), the cells used for PS(10) at 12 generations and 6 passages; PS, PS cells; H1(8), HMH1 cells at 8 generations and 4 passages after infection; H1(12), HMH1 cells at 12 generations and 6 passages after infection; H1, HMH1 cells. The HMH1 samples are taken from a separate gel. Production of $PrP^{Sc}$ does not reflect the persistence of the original innoculum, because it does not decrease with passage number, and, furthermore, the position of the unglycosylated protease-resistant band in the infected brain homogenate runs characteristically above the corresponding band for SMB and infected cell samples (see lane 1).

(B) Titration of scrapie-infected mouse brain homogenate on HMH1 indicator cells. HMH1 cells were exposed to varying dilutions of 2% brain homogenate and expanded by passaging for 17 days, prior to the preparation of $PrP^{Sc}$ fractions and Western blotting after electrophoresis of 75 μg protein/lane. The expression of $PrP^{Sc}$ was quantitated by imager analysis. Control infections of PS cells with normal mouse brain homogenates gave no detectable appearance of $PrP^{Sc}$.

of $PrP^{Sc}$ was detected after exposure to the two lowest dilutions.

The maximum level of expression of $PrP^{Sc}$ in Figure 1B corresponds to approximately 10% of the level of expression observed in extracts of an equivalent number of SMB cells. In the absence of a single cell assay to identify $PrP^{Sc}$-positive cells, it is not clear if this represents conversion of 10% of the cells to the SMB level or conversion of a higher percentage to a level below that of SMB. Nonetheless, it is clear that PS cells, in

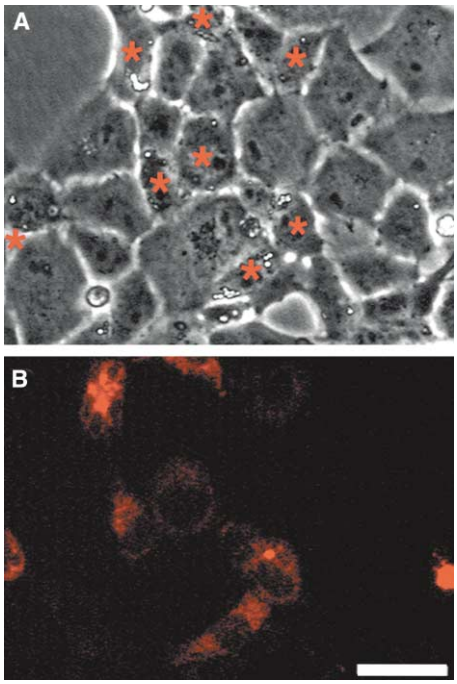


Figure 2. Coculture of SMB and HMH8 Cells and Selective Reisolation of Target Cells

(A) Phase contrast micrograph of SMB and HMH8 cells after 6 days of coculture, with tracker-labeled SMB cells identified by an asterisk. Note the close apposition of the two cell types.

(B) Fluorescence micrograph of (A) showing the SMB cells labeled by prior incubation with the Syto 64 cell tracker dye. The scale bar represents 50 μm.

agreement with earlier work [12], as well as their stably transfected derivatives (Table 1) are readily reinfected by mouse scrapie prions in suspension.

### Infection by Live SMB Cells

In order to assay for cell-based infection, in this case, the ability of SMB to stably convert its cured derivatives, the target cells, either HMH1 or HMH8, were plated out with an equal number of SMB cells. The two cell types divided and associated to form closely packed monolayers without sorting out, as evidenced by labeling one population with a fluorescent cell tracker dye (Figures 2A and 2B). After 7 days of coculture, the cells were passaged and propagated in medium containing G418. All of the SMB cells were killed after 10 days of exposure to the antibiotic, as shown by in situ hybridization of populations with an antisense probe to the *neo* transcript, which selectively labeled the target cells (Figures 3A–3C; see Figure 3 legend). The selected populations were generally passaged at least three more times (8–10 generations) in medium containing G418 prior to analysis for $PrP^{Sc}$. The coculture resulted in effective conversion of HMH1 or HMH8 to expression of mouse $PrP^{Sc}$ (Figure 4A), and this was maintained after prolonged drug selection. In parallel experiments with a *neo*-resistant sheep cell line expressing $PrP^{C}$, no conversion was observed after coculture with SMB. This provides further evidence that all SMB cells are killed after G418 treatment.

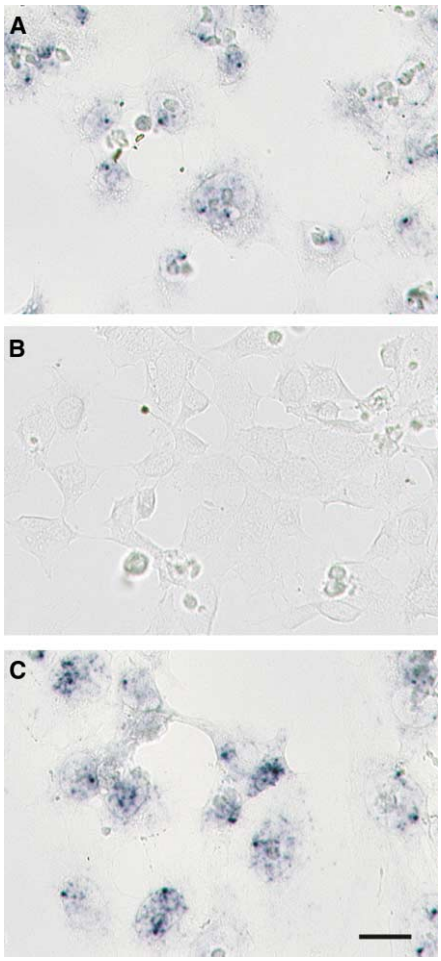


Figure 3. Labeling of $Neo^{R}$ Cells by In Situ Hybridization

(A) Hybridization of HMH8 with an antisense probe to the *neo* gene showing that all cells are labeled.

(B) Hybridization of SMB cells showing that no cells are labeled.

(C) All cells are labeled after hybridization to HMH8 cells isolated by *neo* selection after coculture with SMB. The hybridizations of (A)–(C) were performed in parallel. Analysis of such populations indicates that $<1$ cell in 1000 is an SMB cell after *neo* selection. Furthermore, when SMB cells were labeled with the PKH26 cell tracker dye (see the Experimental Procedures), no positive cells remained after 10 days of G418 selection. The scale bar represents 50 μm.

One important index of conversion is the biosynthetic incorporation of a radioactive amino acid into $PrP^{Sc}$ [3, 4]. The target cells were selected after coculture and pulse labeled in medium containing [$^{35}$S]methionine, followed by a chase in normal medium. The $PrP^{Sc}$ was isolated from detergent extracts by proteinase K digestion, followed by ultracentrifugation, and was denatured with acidic guanidine isothiocyanate (see the Experimental Procedures). Radiolabeled PrP was renatured and immunoprecipitated, followed by deglycosylation and analysis by gel electrophoresis. A radiolabeled band at 21 kDa was specifically immunoprecipitated from SMB (Figure 4B, lanes 1 and 2) and HMH8 cells reisolated by G418 selection from a coculture (lanes 3 and 4), but not from control PS cells (lanes 5 and 6). We conclude from

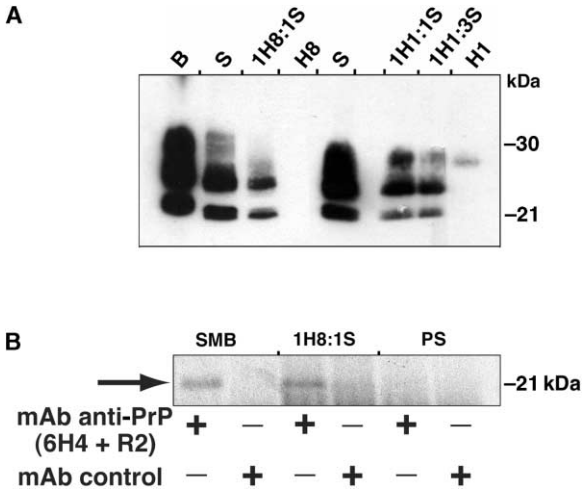


Figure 4. Production and Synthesis of $PrP^{Sc}$ in HMH Target Cells after Coculture with SMB Cells

(A) Production as analyzed by Western blotting. B, Scrapie-infected mouse brain standard; S, SMB cells used for coculture; 1H8:1S, HMH8 cells reisolated by *neo* selection from coculture at a 1:1 (HMH8/SMB) ratio; H8, HMH8 cells used for coculture; S, SMB cells used for coculture, the loading is higher than in the other S lane; 1H1:1S, HMH1 reisolated from a 1:1 coculture, 1H1:3S, HMH1 reisolated from a 1:3 coculture; H1, HMH1 input cells.

(B) Synthesis of radiolabeled $PrP^{Sc}$ after metabolic labeling of cultured cells with [$^{35}$S]methionine. Cultures were pulsed with medium containing [$^{35}$S]methionine and chased as detailed in the Experimental Procedures. Lysates were processed for $PrP^{Sc}$, denatured and renatured, immunoprecipitated, and deglycosylated as detailed in the Experimental Procedures. One half of each sample was precipitated, either with specific anti-PrP monoclonal antibodies or with control antibody as indicated, followed by gel electrophoresis and autoradiography. Lanes 1 and 2 are derived from SMB cells; lanes 3 and 4 are derived from HMH8 cells reisolated from a SMB coculture at a 1:1 ratio; and lanes 5 and 6 are derived from control PS cells. Note the 21 kDa deglycosylated band present in SMB cells and cocultured HMH8, but not in control PS cells. Comparable results have been obtained with independent isolates of cocultured HMH1 cells.

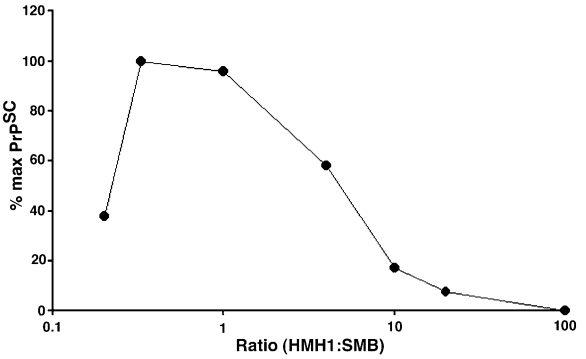


Figure 5. Dependence of $PrP^{Sc}$ Production on the Cell Ratio in the Coculture

HMH1 cells ($1.4 \times 10^5$/dish) were cocultured with SMB so as to vary the ratio over a range of 500-fold. After coculture, the HMH1 cells were G418 selected as before, extracted, processed for $PrP^{Sc}$, and analyzed by quantitative Western blotting at 60 μg protein/well. The decrease in the $PrP^{Sc}$ level seen at the lowest ratio of HMH1:SMB was reproducible and did not apparently reflect any toxic effect of higher cell density. It might be due to some sorting out of the two populations.

these studies that target cells were converted to stably express and synthesize $PrP^{Sc}$ by coculture with SMB.

### Efficiency of Infection by Live SMB Cells

The quantitative dependence of the interaction was analyzed by varying the input ratio of SMB to target cells, while keeping the number of target cells constant. After 1 week of coculture, the SMB cells were removed by antibiotic selection for 2 weeks as before, and the level of $PrP^{Sc}$ in the target cells was determined by Western blotting. The maximum expression of $PrP^{Sc}$ was achieved at around a unitary ratio, and increasing the number of SMB cells to a ratio of 0.2 (target/SMB) led to a decrease in expression (Figure 5). Conversion of the target cells was detected at a ratio of 20, but not at 100 (Figure 5).

In comparing infection of the HMH1 target cell line by a diluted homogenate of scrapie-infected brain (Figure 1B) to infection by the live SMB cell (Figure 5), the maximum level of target cell $PrP^{Sc}$ expression was similar, approximately 10% and 30%, respectively, to the level in SMB cells. Although infection by the two methods is not strictly comparable, it is noteworthy that conversion of a dish of target cells by SMB cells required 2,500-fold less $PrP^{Sc}$ than conversion by a brain homogenate, as determined by comparative Western blotting.

### Evidence for Contact Dependence of SMB Cell Infection

The SMB cells could conceivably convert the target cells by releasing or generating $PrP^{Sc}$ in the medium, and a previous report on GT1 cells is consistent with this possibility [16]. In order to investigate this possibility, medium was conditioned for 2 days by SMB cells at high density and then incubated with the two target cell lines for 7 days; but, in repeated attempts, it did not induce any detectable conversion to $PrP^{Sc}$. The activity of live SMB cells is therefore most unlikely to depend on the presence of stable infective components in the medium, either prion rods or some other species.

Other possibilities include the release of either an unstable activity or $PrP^{Sc}$ in membrane exovesicles, which might not be recovered adequately in conditioned medium. In order to bring the cells into close proximity but not into contact, we cocultured SMB or HMH cells as separate populations, one on an insert membrane with high-density pores of 0.4-μm diameter, the other on the bottom surface of the dish in medium shared by cells on the insert (Figure 6). Although such pores would not permit transfer of larger prion rods, they should permit transfer of exovesicles, as well as most soluble molecules. When cells were either cocultured in contact followed by G418 selection (Figure 6v) or were cocultured separately and assayed after a comparable time (Figures 6i and 6iii), the separated configuration did not give detectable conversion, that is less than 10% of the level of $PrP^{Sc}$ observed for the contact case. An alternative hypothesis is that it is the products of dying SMB cells that convert the target cells during the period of G418 selection of cocultures. When G418 was introduced into insert cultures in either configuration so as to expose

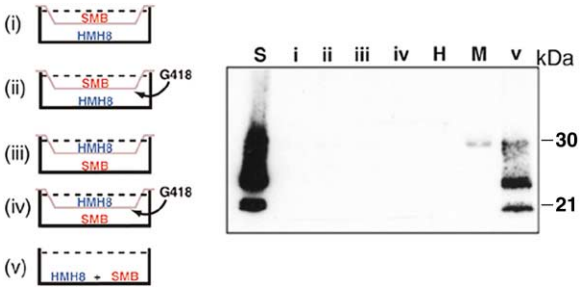


Figure 6. Appearance of $PrP^{Sc}$ Is Prevented by Separation of Infected and Target Cells

In (i)–(iv), the SMB and HMH8 cells were separated during coculture by growth in shared medium on different surfaces of a culture dish with an insert of pore size 0.4 μm. Note that one population grows on the insert, and the other on the bottom of the dish. In (ii) and (iv), G418 was added to the medium in order to kill SMB cells while separated from HMH8. In (v), HMH8 and SMB were cocultured as before. After coculture, the HMH8 cells were expanded in G418 ([i]–[iv]) or selected from the coculture [(v)] so as to undergo the same number of passages. The five populations were extracted, processed for $PrP^{Sc}$, and analyzed by Western blotting as before. S, SMB; H, HMH8 control; M, marker lane showing artifactual band. Titration of the signal in (v) indicated that separation of the populations gave <10% of the level of $PrP^{Sc}$ obtained for cells in contact.

target cells to the local death of SMB, no detectable conversion was observed, arguing against this possibility (Figures 6ii and 6iv). While the possibility of a short-range diffusible signal is not ruled out, the results support the importance of close apposition (as seen in Figures 2A and 2B) in cell-based infection.

### Infection by Aldehyde-Fixed SMB Cells

Direct evidence for the role of cell contact has come from the activity of aldehyde-fixed infected cells. SMB cells were fixed in a mixture of buffered paraformaldehyde and glutaraldehyde and then washed for 48 hr to remove the fixatives. The cells were dead, as assayed by staining with vital dyes, and did not incorporate [$^3$H]leucine into protein, as detected by autoradiography. The state of $PrP^{Sc}$ was investigated by fractionation of fixed cells. It was not detected in the high-speed pellet from fixed SMB cells after detergent lysis and proteolysis (Figure 7, lane F, compare live SMB, lane S), but was found as an aggregate in the initial low-speed pellet (lane P), presumably because of the crosslinking activity of the fixatives.

Target cells were plated onto the fixed and washed SMB cells for varying times and came into close apposition or partial overlap with them (Figure 7A). After trypsinization (see Figure 7 legend), the cells were propagated in medium containing G418 for 2 weeks. If any conversion occurred during the period of contact with fixed SMB cells, this was presumably amplified in the target cells during the period of subsequent culture. When this period was reduced to 48 hr, $PrP^{Sc}$ was detected only at low levels for the 7-day samples, and not at 3 or 5 days (not shown). This is consistent with a requirement for amplification and also shows that the $PrP^{Sc}$ signal cannot be accounted for by carry over from the fixed cells, although this would be inconsistent with the result in Figure 7, lane F anyway. After coculture for 3, 5, or 7 days with an identical number of either live or fixed SMB cells (Figure 7B, lanes 4, 6, and 8 or 5, 7, and 9, respectively), an increase in conversion with time was observed for both contexts. The level of conversion observed for fixed cells was about 10%–25% of the level for live cells. No detectable conversion was observed after 1 day of coculture, and no conversion was observed in parallel experiments with fixed, washed PS cells in place of SMB cells. Thus, the fixed SMB cells remain substantially active in converting target cells to stably produce $PrP^{Sc}$.

### Discussion

A principal conclusion from this study is that a scrapie prion-infected cell is able to convert a neighboring uninfected cell by a process that is dependent upon cell contact. Although it is plausible that the SMB cell might generate infectious forms of PrP in the medium, which then act on the target cells, this extracellular pathway appears not to operate in the particular circumstances analyzed here, since conditioned medium has no activ-

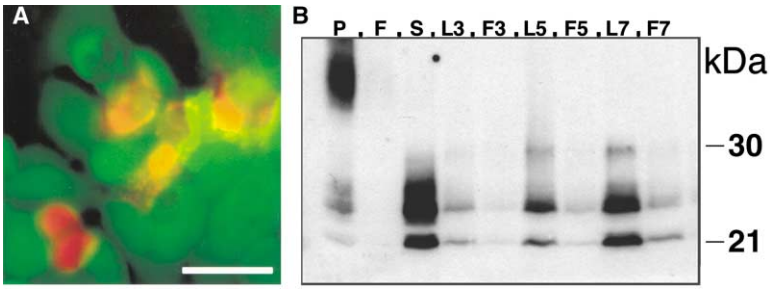


Figure 7. Aldehyde-Fixed SMB Cells Are Able to Convert Target Cells in Coculture

(A) Association of fixed SMB and live target cells labeled with cell tracker dyes. SMB cells were stained in suspension with Syto 64 cell tracker dye (red), plated, fixed, and washed for 48 hr as described in the Experimental Procedures. HMH1 cells were labeled in suspension with Cell Tracker Green and plated onto the fixed SMB cells for 3 days. The cells partially overlap on the upper surface of three of the SMB cells, giving a yellow zone of cell apposition. Note that the red cells are smaller, in part because SMB cells are smaller, and in part because the fixation induced some shrinkage.

(B) Infection of target cells by fixed SMB cells. Lanes P, F, and S: fixation leads to crosslinking of $PrP^{Sc}$ in SMB, as analyzed by Western blotting. P, low-speed pellet fraction from fixed SMB showing aggregates; F, $PrP^{Sc}$ fraction from fixed cells after protease K digestion and ultracentrifugation, note that no $PrP^{Sc}$ is visible; S, $PrP^{Sc}$ fraction from control live SMB cells processed in parallel. Lanes L3–F7: live and fixed SMB were cocultured with HMH8 target cells for 3, 5, or 7 days, followed by trypsinization, passaging, and growth for 14 days in medium with G418. Cells were extracted and processed for $PrP^{Sc}$, and 80 μg protein was loaded per lane prior to Western blotting as before. L3, 3 days with live SMB; F3, 3 days with fixed SMB; L5, 5 days with live SMB; F5, 5 days with fixed SMB; L7, 7 days with live SMB; F7, 7 days with fixed SMB.

ity. The target cells are sensitive to conversion by $PrP^{Sc}$ in homogenates of infected brain within a certain concentration range, the 10-fold dilution of Figure 1B corresponds to approximately 0.2% of an infected mouse brain in 0.5 ml, and it is possible that more sensitive target cells, such as one of the recently described subclones of N2a cells [5], would be converted. The activity of fixed and washed SMB cells argues directly for a central role for cell-cell contact, albeit one that is substantially retained under conditions of aldehyde fixation. It is a classical property of scrapie infectivity that it is resistant to the conditions of aldehyde fixation, which kill both cells and conventional infectious agents [17]. Given the expectation that $PrP^{Sc}$ on the cell surface should be functional, it is perhaps not surprising that fixed cells are active, but the present analysis has shown that this can account for at least 10%–25% of the activity of live cells assayed in parallel. There are several activities of a live cell that could initiate infection after close apposition. One example is the facilitated release of $PrP^{Sc}$ with its GPI anchor and its subsequent insertion into the target cell membrane [18, 19]. Another is the active release of membrane exovesicles (reviewed by Denzer [20]). Although it remains possible that these mechanisms could play a role, the results with fixed and dead cells essentially rule out that either is a necessary event.

Earlier studies of prion liposome complexes may help to explain the differences we observe in the specific activity of $PrP^{Sc}$ in subcellular aggregates and intact cells [21]. When prion rods are solubilized and incorporated into lipid vesicles, there is an associated increase in infectivity of approximately 100-fold, as measured by end point titration [22]. The presentation of monomeric or oligomeric $PrP^{Sc}$ embedded or anchored in a lipid membrane is apparently more effective for converting cells than the presentation of aggregates in suspension. The activity of fixed SMB cells is indicative of a templating mechanism, whereby $PrP^{Sc}$ on the infected cells is able to act in *trans* to convert $PrP^{C}$ on the surface of the target cell. This could occur directly, or by cleavage of $PrP^{Sc}$ on the infected cell by an activity of the target cell, followed by subsequent transfer to the target cell. The latter possibility is less likely because of the evidence for effective crosslinking of $PrP^{Sc}$. The conversion could then be amplified by the *cis* interaction between $PrP^{Sc}$ in the target and other $PrP^{C}$ molecules in the plane of the membrane. Such a templating mechanism might also be relevant to the high infectivity of $PrP^{Sc}$ after adsorption to metal surfaces [23].

A mechanism that is dependent on cell contact would, in general, be expected to propagate at a much slower rate than a mechanism involving diffusible extracellular forms of PrP. It is interesting to consider the potential relevance of this mechanism for the time course of prion infection in vivo. After peripheral administration of scrapie prions, the lymphatic organs show early accumulation of infectivity, but access to the CNS occurs via certain peripheral nerves [24–26]. In experimental studies after injection into a peripheral nerve, scrapie infectivity moves back along the nerve at a rate of approximately 1 mm per day [27, 28]. It is possible that this could reflect the propagation of the PrP conformational change within the axolemma, some form of slow retrograde axoplasmic transport, or the progressive conversion of Schwann cells or fibroblasts along the sheath by the type of mechanism described here. The culture system described in this report offers a favorable opportunity to analyze the mechanisms underlying contact-dependent infection as well as an opportunity to study any changes in the target cell that are acutely associated with infection.

## Conclusions

Cell-mediated infection of target cells in this culture system is effective and requires significantly less $PrP^{Sc}$ than infection by a prion preparation. Several lines of evidence indicate that it depends on cell contact, particularly the activity of aldehyde-fixed, infected cells.

### Experimental Procedures

#### Cell Culture, Labeling, and In Situ Hybridization

Cells were propagated in a 7.5% $CO_2$ incubator at 37°C in medium 199 with serum as described elsewhere [12]. For labeling of cells, we used either Syto 64 (Molecular Probes), PKH 26 (Sigma), or Cell Tracker Green (Molecular Probes) according to the manufacturer's instructions. In situ hybridization to fixed cultured cells was performed by standard methods with a 0.76-kb antisense *neo* riboprobe substituted with digoxygenin (DIG RNA labeling kit; Roche). The hybridized probe was detected with alkaline phosphatase-labeled antibody and was developed with the standard BCIP/NBT substrate.

#### Scrapie Infection in Culture

Scrapie (strain 79A)-infected mouse brains were homogenized in saline at 10% (w/v), stored in aliquots at −80°C, and dispersed by sonication prior to dilution in culture medium. Target cells were grown to approximately 75% confluence in wells of 35-mm diameter and exposed to 0.55 ml of 2% brain homogenate, diluted in growth medium, for 5 hr at 34°C in a 5% $CO_2$ incubator. After the addition of medium for an additional 18 hr, cells were washed, grown to confluence, and passaged at least twice at a 1:3 split ratio.

For infection by coculture, SMB and HMH cells (140,000 each) were mixed in an 80-$cm^2$ flask and cultured for 7 days. The confluent cultures were passaged at 1:2 into medium with 0.35 mg/ml G418. Cells were expanded in G418 medium and passaged at confluence. For insert cocultures, cells were plated at appropriate densities onto the two growth surfaces of 9.6-$cm^2$ dishes with translucent polyethylene terephthalate inserts (0.4 μm high pore density; Becton Dickenson). In some cases, G418 medium was introduced when the SMB cells had attained high density so that the HMH cells were exposed to the products of G418-induced cell death.

For fixation of SMB cells prior to coculture, 140,000 cells were plated into an 80-$cm^2$ flask, washed twice with phosphate-buffered saline, fixed for 5 min at room temperature with 1% paraformaldehyde, 0.05% glutaraldehyde in phosphate-buffered saline, washed again, and left for 48 hr in the incubator with growth medium to remove any residual fixative. Cocultures with fixed cells and target cells were generally compared to parallel cocultures with live SMB cells. Cocultures were trypsinized and replated in medium with G418 for 14 days prior to assay for $PrP^{Sc}$. Fixed cells were observed to rarely detach after trypsin treatment and not to reattach.

#### PrP Isolation from Cultured Cells

Cells were lysed on the dish in buffer containing 10 mM Tris (pH 8.0), 100 mM NaCl, 10 mM EDTA with 0.5% NP40 and 0.5% sodium deoxycholate, and aliquots were removed for methanol precipitation and $PrP^{C}$ analysis without protease digestion. The remainder was incubated with proteinase K (1 hr, 37°C, 30 μg/ml), adjusted to 0.2 mM PMSF, and centrifuged at 356,000 × g for 15 min at 4°C in a Beckman TLA 120.1 rotor. The pellet ($PrP^{Sc}$ fraction) was solubilized in sample buffer prior to electrophoresis on 12% SDS gels and Western blotting onto nitrocellulose. The blots were reacted with

6H4 monoclonal anti-PrP [29] (Prionics), followed by peroxidase-labeled anti-mouse antibody (Dako) and development by ECL (Amersham). For quantitation of $PrP^{Sc}$, blots were imaged with a Fujifilm Luminescent Image Analyzer and analyzed with Image Reader software.

### Metabolic Labeling of $PrP^{Sc}$

Cells were grown to near confluence in two 80-$cm^2$ flasks, changed to methionine-free medium 199 for 1–2 hr, and then to methionine-free 199 with 1 mCi of [$^{35}$S]methionine (Amersham). After 6–8 hr, the medium was changed to normal 199 growth medium for 24–48 hr, and the cells were washed, lysed, and processed to give $PrP^{Sc}$ pellets as above. Each pellet was solubilized in 0.1 ml 4M guanidine isothiocyanate, 0.1M acetic acid for 15 min at room temperature, prior to precipitation with 4 volumes cold methanol. The methanol pellet was dissoved in 0.5 ml DLPC buffer (20 mM Tris [pH 7.8], 0.15 M NaCl, 2% sarcosyl, 0.4% phosphatidyl choline) with PMSF and divided into two parts for reaction (1 hr, room temperature) with either control monoclonal antibody to digoxygenin or a mixture of 6H4 and R2 [30] monoclonal anti-PrP antibodies. Immune complexes were collected by rotating overnight at 4°C with Eupergit beads (Fluka) derivatized with sheep anti-mouse Ig (Sigma) according to the manufacturer's instructions. The beads were washed in DLPC, then boiled in N-glycanase incubation buffer containing 0.5% SDS, 5% mercaptoethanol prior to the addition of NP40 to 2% and 5 mU N-glycanase (Glyko). After overnight incubation at 37°C and the addition of SDS sample buffer, the samples were electrophoresed on a 12% gel that was analyzed by autoradiography and imaging. The recovery of [$^{35}$S] radioactivity was monitored throughout the preparation and was comparable for the different cell types (approximately 1% in the DLPC fraction prior to immunoprecipitation, relative to cell lysate). The N-glycanase digestion was included because it increased the sensitivity of detection of radioactive PrP after SDS gel electrophoresis.

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### References

1. Prusiner, S.B., Peters, P., Kaneko, K., Taraboulos, A., Lingappa, V., Cohen, F., and DeArmond, S.J. (1999). Cell biology of prions. In Prion Biology and Diseases, S.B. Prusiner, ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 349–391.
2. Vey, M., Pilkuhn, S., Wille, H., Nixon, R., DeArmond, S.J., Smart, E.J., Anderson, R.G., Taraboulos, A., and Prusiner, S.B. (1996). Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. Proc. Natl. Acad. Sci. USA *93*, 14945–14949.
3. Borchelt, D.R., Taraboulos, A., and Prusiner, S.B. (1992). Evidence for synthesis of scrapie prion proteins in the endocytic pathway. J. Biol. Chem. *267*, 16188–16199.
4. Caughey, B., and Raymond, G.J. (1991). The scrapie-associated form of PrP is made from a cell surface precursor that is both protease- and phospholipase-sensitive. J. Biol. Chem. *266*, 18217–18223.
5. Enari, M., Flechsig, E., and Weissmann, C. (2001). Scrapie prion protein accumulation by scrapie-infected neuroblastoma cells abrogated by exposure to a prion protein antibody. Proc. Natl. Acad. Sci. USA *98*, 9295–9299.
6. Race, R.E., Caughey, B., Graham, K., Ernst, D., and Chesebro, B. (1988). Analyses of frequency of infection, specific infectivity, and prion protein biosynthesis in scrapie-infected neuroblastoma cell clones. J. Virol. *62*, 2845–2849.
7. Bosque, P.J., and Prusiner, S.B. (2000). Cultured cell sublines highly susceptible to prion infection. J. Virol. *74*, 4377–4386.
8. Nishida, N., Harris, D.A., Vilette, D., Laude, H., Frobert, Y., Grassi, J., Casanova, D., Milhavet, O., and Lehmann, S. (2000). Successful transmission of three mouse-adapted scrapie strains to murine neuroblastoma cell lines overexpressing wild-type mouse prion protein. J. Virol. *74*, 320–325.
9. Lehmann, S., and Harris, D.A. (1996). Mutant and infectious prion proteins display common biochemical properties in cultured cells. J. Biol. Chem. *271*, 1633–1637.
10. Clarke, M.C., and Haig, D.A. (1970). Evidence for the multiplication of scrapie agent in cell culture. Nature *225*, 100–101.
11. Haig, D.A., and Clarke, M.C. (1971). Multiplication of the scrapie agent. Nature *234*, 106–107.
12. Birkett, C.R., Hennion, R.M., Bembridge, D.A., Clarke, M.C., Chree, A., Bruce, M.E., and Bostock, C.J. (2001). Scrapie strains maintain biological phenotypes on propagation in a cell line in culture. EMBO J. *20*, 3351–3358.
13. Kascsak, R.J., Rubenstein, R., Merz, P.A., Tonna-DeMasi, M., Fersko, R., Carp, R.I., Wisniewski, H.M., and Diringer, H. (1987). Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. J. Virol. *61*, 3688–3693.
14. Priola, S.A., and Chesebro, B. (1995). A single hamster PrP amino acid blocks conversion to protease-resistant PrP in scrapie-infected mouse neuroblastoma cells. J. Virol. *69*, 7754–7758.
15. Bruce, M.E., and Fraser, H. (1991). Scrapie strain variation and its implications. Curr. Top. Microbiol. Immunol. *172*, 125–138.
16. Schatzl, H.M., Laszlo, L., Holtzman, D.M., Tatzelt, J., DeArmond, S.J., Weiner, R.I., Mobley, W.C., and Prusiner, S.B. (1997). A hypothalamic neuronal cell line persistently infected with scrapie prions exhibits apoptosis. J. Virol. *71*, 8821–8831.
17. Pattison, I.H. (1965). Resistance of the scrapie agent to formalin. J. Comp. Pathol. *75*, 159–164.
18. Kooyman, D.L., Byrne, G.W., and Logan, J.S. (1998). Glycosyl phosphatidylinositol anchor. Exp. Nephrol. *6*, 148–151.
19. Anderson, S.M., Yu, G., Giattina, M., and Miller, J.L. (1996). Intercellular transfer of a glycosylphosphatidylinositol (GPI)-linked protein: release and uptake of CD4-GPI from recombinant adeno-associated virus-transduced HeLa cells. Proc. Natl. Acad. Sci. USA *93*, 5894–5898.
20. Denzer, K., Kleijmeer, M.J., Heijnen, H.F., Stoorvogel, W., and Geuze, H.J. (2000). Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. J. Cell Sci. *113*, 3365–3374.
21. Gabizon, R., McKinley, M.P., and Prusiner, S.B. (1987). Purified prion proteins and scrapie infectivity copartition into liposomes. Proc. Natl. Acad. Sci. USA *84*, 4017–4021.
22. Gabizon, R., McKinley, M.P., Groth, D.F., Kenaga, L., and Prusiner, S.B. (1988). Properties of scrapie prion protein liposomes. J. Biol. Chem. *263*, 4950–4955.
23. Zobeley, E., Flechsig, E., Cozzio, A., Enari, M., and Weissmann, C. (1999). Infectivity of scrapie prions bound to a stainless steel surface. Mol. Med. *5*, 240–243.
24. Kimberlin, R.H., and Walker, C.A. (1988). Pathogenesis of experimental scrapie. Ciba Symp. *135*, 37–62.
25. Race, R., Oldstone, M., and Chesebro, B. (2000). Entry versus blockade of brain infection following oral or intraperitoneal scrapie administration: role of prion protein expression in peripheral nerves and spleen. J. Virol. *74*, 828–833.
26. Glatzel, M., Heppner, F.L., Albers, K.M., and Aguzzi, A. (2001). Sympathetic innervation of lymphoreticular organs is rate limiting for prion neuroinvasion. Neuron *31*, 25–34.
27. Kimberlin, R.H., Hall, S.M., and Walker, C.A. (1983). Pathogenesis of mouse scrapie. Evidence for direct neural spread of infection to the CNS after injection of sciatic nerve. J. Neurol. Sci. *61*, 315–325.
28. Glatzel, M., and Aguzzi, A. (2000). PrP(C) expression in the peripheral nervous system is a determinant of prion neuroinvasion. J. Gen. Virol. *81*, 2813–2821.

29. Korth, C., Stierli, B., Streit, P., Moser, M., Schaller, O., Fischer, R., Schulz-Schaeffer, W., Kretzschmar, H., Raeber, A., Braun, U., et al. (1997). Prion (PrPSc)-specific epitope defined by a monoclonal antibody. Nature *390*, 74–77.
30. Williamson, R.A., Peretz, D., Pinilla, C., Ball, H., Bastidas, R.B., Rozenshteyn, R., Houghten, R.A., Prusiner, S.B., and Burton, D.R. (1998). Mapping the prion protein using recombinant antibodies. J. Virol. *72*, 9413–9418.
31. Stephens, P.E., and Cockett, M.I. (1989). The construction of a highly efficient and versatile set of mammalian expression vectors. Nucleic Acids Res. *17*, 7110.