



growth factors, stress, or other inducers of c-Jun activity. Such an effect might be compounded by a similar regulation of the Jun partner molecule, c-Fos, which also exhibits phosphorylation-dependent changes of its half-life (16).

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- 18 JNK1 and Cdc42<sup>L61</sup> expression vectors have been described (14). NIH 3T3 cells were transiently transfected by calcium phosphate coprecipitation (17). Purification of c-Jun-ubiquitin conjugates and protein immunoblot analysis were done as described (6). Ubiquitination assays were performed in HeLa or in NIH 3T3 cells with either His<sub>6</sub>-tagged or HA-tagged c-Jun expression vectors with essentially identical results (compare Figs. 1 and 2).
19. His<sub>6</sub>-tagged c-Jun expression vectors have been described (6). c-Jun<sup>HA</sup>-His<sub>6</sub> and c-Jun<sup>ASP</sup>-His<sub>6</sub> eukaryotic expression vectors were generated as described for His<sub>6</sub>-tagged wild-type c-Jun (11). The c-Jun<sup>HA</sup> mutant contains alanine residues in place of serines or threonines at position 58, 62, 63, 73, 89, 90, 91, 93, and 95. c-Jun<sup>ASP</sup> contains aspartic acid residues in place of the serines and threonines at position 58, 62, 63, 73, 91, and 93. The hemagglutinin (HA)-tagged ubiquitin eukaryotic expression vector, HeLa thymidine kinase-negative (TK<sup>-</sup>) cell transfections, purification of Jun-ubiquitin conjugates, and immunoblot analysis were as described (6)
- 20 The cytomegalovirus-based expression vectors for HA-tagged c-Jun and JNK1 have been described (6, 14). The c-Jun substitution mutants were generated as described (11). NIH 3T3 cells were transfected by calcium phosphate precipitation (17) with 0.25 µg of the respective c-Jun expression vector and 3 µg of JNK expression vector as indicated. After 24 hours, cells were radioactively labeled for 30 min with 75 mCi of <sup>35</sup>S-methionine and <sup>35</sup>S-cysteine per milliliter of medium, followed by incubation in a medium that contained 2 mM each of unlabeled methionine and cysteine for 0, 90, 180, or 270 min. The cells that had been transfected with the JNK1 expression vector were treated with 10 mM anisomycin during the labeling and chase periods to induce kinase activity. Cells were lysed in RIPA buffer [10 mM Tris (pH 7.5), 45 mM β-glycerophosphate, 50 mM NaF, 5 mM sodium molybdate, 0.1% SDS, 1 mM EDTA, 1% NP-40, 0.5% deoxycholate] supplemented with 1 mM phenylmethylsulfonyl fluoride and 10 mg each of leupeptin, aprotinin, and pepstatin per milliliter of buffer. Samples containing equal amounts of acid-

insoluble radioactivity were incubated with protein G- and protein A-agarose (Oncogene Science) and then incubated with the monoclonal antibody 12CA5 to HA. Precipitates were collected on protein G- and protein A-agarose, washed once with buffer A [10 mM Tris (pH 7.5), 45 mM β-glycerophosphate, 50 mM NaF, 5 mM sodium molybdate, 0.1% SDS, 1 mM EDTA, 0.5% NP-40], once with buffer B [10 mM Tris (pH 7.5), 410 mM NaCl, 45 mM β-glycerophosphate, 0.1% SDS, 1 mM EDTA, 0.5% NP-40], once

with 10 mM Tris (pH 7.5), and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE, 10% gel) and autoradiography  
21 We thank S. Gutkind for plasmid vectors, L. Staszewski for technical assistance, and I. Mattaj, S. Cohen, T. Graf, A. Isaksson, L. Kockel, A. Papavassiliou, and C. Ovitt for comments on the manuscript. Supported by a grant from the CNR to A.M.M.

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## Transmission of the BSE Agent to Mice in the Absence of Detectable Abnormal Prion Protein

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The agent responsible for transmissible spongiform encephalopathies (TSEs) is thought to be a malformed, protease-resistant version (PrPres) of the normal cellular prion protein (PrP). The interspecies transmission of bovine spongiform encephalopathy (BSE) to mice was studied. Although all of the mice injected with homogenate from BSE-infected cattle brain exhibited neurological symptoms and neuronal death, more than 55 percent had no detectable PrPres. During serial passage, PrPres appeared after the agent became adapted to the new host. Thus, PrPres may be involved in species adaptation, but a further unidentified agent may actually transmit BSE.

One of the distinct features of the BSE agent is its high ability to infect other species (1-3), whereas other TSE agents are easily transmitted only within a species. This species barrier leads to considerable prolongation of the incubation period during interspecies transmission (4). During subsequent experimental passages, TSE agents adapt to the new host: the incubation period shortens and stable pathological properties are acquired (5). According to the prion hypothesis, PrPres (the pathological, protease-resistant isoform of the prion protein) constitutes the infectious agent in TSEs, and replication involves the homotypic interaction between a pathological PrP molecule and the endogenous native protein to produce a conformational conversion to the abnormal isoform. The mag-

nitude of the species barrier would thus be a condition of the extent of congruency between the PrP of the donor species and that of the new host (6). However, this mechanism cannot account for the exceptional ability of the BSE agent to cross the species barrier. This agent has original properties and is suspected to have contaminated humans (2, 7). Thus, we examined BSE transmission and PrPres during primary transmission to mice and in subsequent passages to other mice.

Thirty C57BL/6 mice were inoculated by intracerebral injection of a 25% BSE-infected cattle brain homogenate. After 368 to 719 days, all of the inoculated animals exhibited symptoms of a neurological disease encompassing mainly hindlimb paralysis, tremors, hypersensitivity to stimulation, apathy, and a hunched posture. Biochemical analysis of their brains showed no detectable PrPres accumulation in more than 55% of the mice; these mice were termed PrPres<sup>-</sup> (Figs. 1 and 2) (8). Histological examination revealed neuronal death in all mice, but other classical changes associated with TSEs—that is, neuronal vacuolation and astrocytosis—were limited to the PrPres<sup>+</sup> mice (Fig. 3). Neuronal loss was most obvious in the Purkinje cells of the cerebellum, but degenerated neurons were also observed, to a smaller extent, in the CA1 region of the hippocampus. No sign of local inflammation was present. Electron microscopic examination of degenerated cells

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showed marginalization and clumping of the chromatin, a characteristic of type I apoptosis (Fig. 3E) (9).

The PrPres<sup>-</sup> mice were infected with a TSE agent because they could transmit a disease exhibiting the classical features of TSE, that is, PrPres accumulation and spongiform lesions (Fig. 2). The brains of PrPres<sup>+</sup> mice (for example, B1) and PrPres<sup>-</sup> mice (for example, B4) were used to inoculate a second series of mice. Most of the mice inoculated with PrPres<sup>-</sup> brains developed a classical TSE, but a few presented the PrPres<sup>-</sup> pattern again and the incubation periods remained spread. However, as was observed at primary passage, PrPres<sup>+</sup> and PrPres<sup>-</sup> mice had the same range of incubation periods (Fig. 2) (10). Transmission from PrPres<sup>+</sup> mice led to an important reduction of incubation time that was very homogeneous ( $167 \pm 2$  days, mean  $\pm$  SEM) with detectable PrPres in all mice (Fig. 2).

A third passage was performed with one mouse from the B1 lineage and two mice from the B4 lineage, only one of which had detectable PrPres (Fig. 2). After inoculation with the PrPres<sup>-</sup> brain, incubation periods were shortened and less variable and all but one of the mice had detectable PrPres at the terminal stage of disease. Transmission from PrPres<sup>+</sup> mice gave very similar incubation periods, whether originally inoculated with brain homogenate from the PrPres<sup>-</sup> or PrPres<sup>+</sup> lineages. Finally, as a result of this third passage, the PrPres<sup>-</sup> pattern had almost disappeared (Fig. 2). Thus, the PrPres<sup>+</sup> pattern had a selective advantage and was associated with the short and homogeneous incubation periods. Therefore, PrPres could be associated with the adaptation of the agent to its new host.

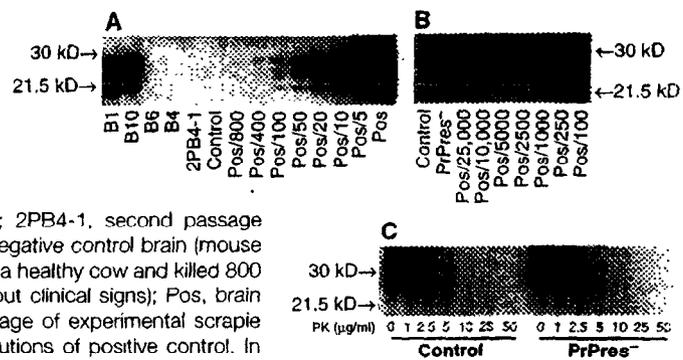
Because we were able to transmit a TSE agent without detectable PrPres upon three passages, infectivity and PrPres can be dissociated [see also (11)]. The similarity of the clinical signs in PrPres<sup>-</sup> and PrPres<sup>+</sup> mice suggests that neuronal death was the major determinant of central nervous system function impairment. However, the presence of spongiform lesions and overt gliosis was directly linked to that of PrPres (12). The role of PrPres in the pathogenesis of cerebral damage has been shown in vitro (13), as has the requirement for normal PrP in the development of disease and pathological lesions (14, 15). Thus, PrPres is clearly involved in the pathogenic process of TSEs. However, it may not be the transmissible component of the infectious agent.

This concept is supported by the multiplicity of TSE strains. For example, more than eight different strains can replicate in syngeneic C57Bl/6 mice but exhibit specific properties (incubation periods, distribution of the lesions, and biochemical features), even though the PrP of the host is

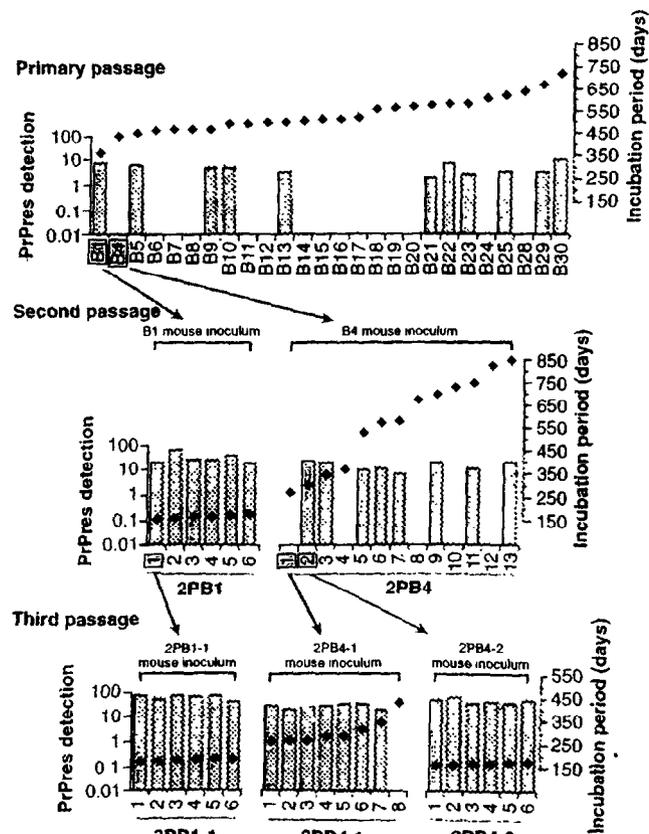
the same (16, 17). Some strains are even able to retain their specific properties upon transmission to different hosts with different PrP molecules (16), whereas others undergo phenotypic changes when passaged in a single host (18). Finally, when mice lacking PrP were inoculated with either the Chandler scrapie strain or the mouse-adapted Fukuoka-1 strain of Creutzfeldt-Jakob disease, they did not develop clinical disease, but several brains contained a transmissible agent 20 weeks after inoculation (14, 19).

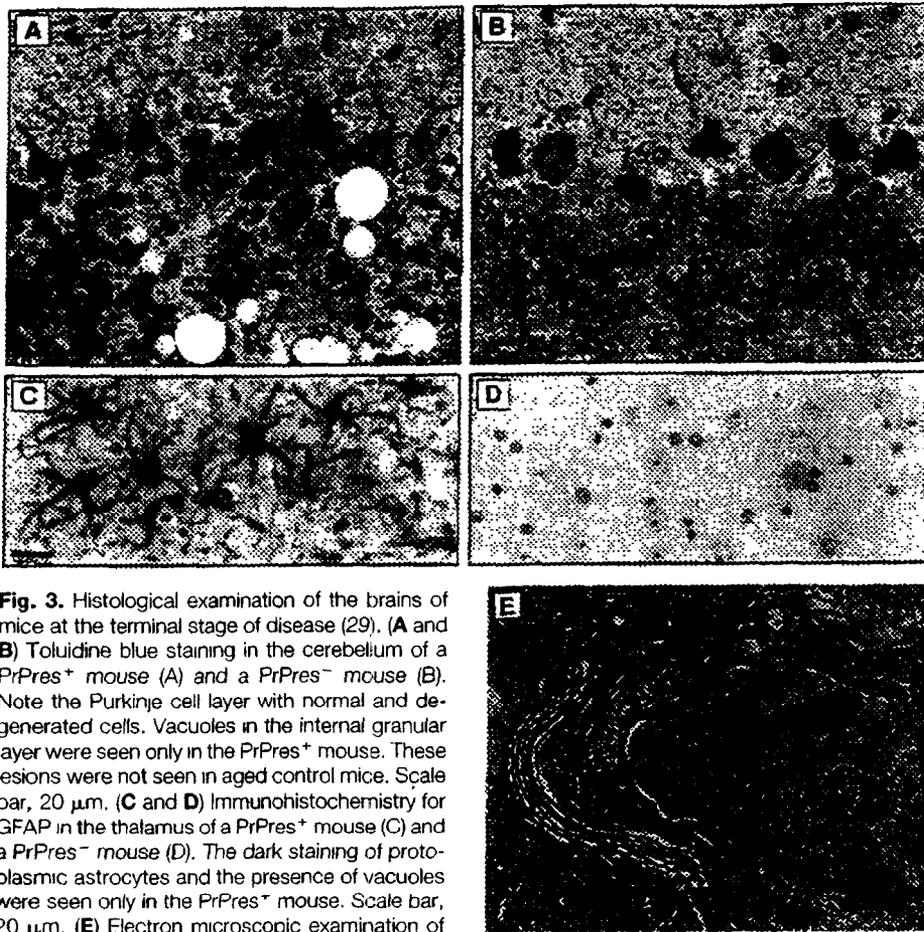
Because we could transmit a TSE without detectable cerebral PrPres accumulation in the case of interspecies transmission of the BSE agent, the hypothesized existence of an infectious agent in addition to PrPres becomes more likely in view of the complexity of TSE strain properties, this agent may be a nucleic acid. Moreover, our results suggest a pathogenic mechanism that may account for the peculiar efficacy of the BSE agent in crossing the species barrier. The BSE agent is virulent enough to replicate in the new host

**Fig. 1.** (A and B) PrPres detection by protein immunoblot (26). In (A), brains of mice at the terminal stage of the disease (4 mg brain equivalent) were analyzed. B1, B10, B6, and B4, first passage from cattle brain; 2PB4-1, second passage from B4 mouse; Control, negative control brain (mouse inoculated with the brain of a healthy cow and killed 800 days after inoculation without clinical signs); Pos, brain pool of mice at terminal stage of experimental scrapie (strain C506M3); Pos/x, dilutions of positive control. In (B), under conditions of maximal sensitivity, the PrPres signal can be detected at a 1:10,000 dilution of the positive control (2.5  $\mu$ g brain equivalent). Pos, control, and PrPres<sup>-</sup> samples correspond to 25 mg brain equivalent. (C) Similar degradation pattern of PrP with a range of doses of PK in a normal mouse brain and a PrPres<sup>-</sup> brain, showing the absence of PrPres with less resistance to protease than usual in PrPres<sup>-</sup> brains (27).



**Fig. 2.** Transmission features of BSE into mice at first, second, and third passage (28). Histograms represent the amount of PrPres (expressed as a percentage of the positive control) in the brains of mice at the terminal stage of neurological disease. Diamonds represent the incubation period for each individual mouse tested for PrPres. The positive control corresponds to a brain pool of mice at the terminal stage of experimental scrapie (strain C506M3). At primary passage, individual mice were scored from B1 to B30 according to their incubation periods. The brains of B2, B3, B26, and B27 could not be analyzed and are not represented. The brains of B1 and B4 were inoculated to a second series of mice called, respectively, 2PB1 and 2PB4. At third passage, the recipient mice were called, respectively, 3PB1 and 3PB4. Second passages were also performed with B6, B10, and B15 and are not shown for the sake of clarity, they were consistent with the passages from B1 and B4





**Fig. 3.** Histological examination of the brains of mice at the terminal stage of disease (29). (A and B) Toluidine blue staining in the cerebellum of a PrPres<sup>+</sup> mouse (A) and a PrPres<sup>-</sup> mouse (B). Note the Purkinje cell layer with normal and degenerated cells. Vacuoles in the internal granular layer were seen only in the PrPres<sup>+</sup> mouse. These lesions were not seen in aged control mice. Scale bar, 20  $\mu$ m. (C and D) Immunohistochemistry for GFAP in the thalamus of a PrPres<sup>+</sup> mouse (C) and a PrPres<sup>-</sup> mouse (D). The dark staining of protoplasmic astrocytes and the presence of vacuoles were seen only in the PrPres<sup>+</sup> mouse. Scale bar, 20  $\mu$ m. (E) Electron microscopic examination of an apoptotic Purkinje cell in the cerebellum of a PrPres<sup>-</sup> mouse (30). Note the clumping and marginalization of the chromatin, as well as the normal aspect of the nuclear membrane (arrows) and cytoplasmic organelles (arrowheads show the Golgi apparatus and mitochondria). Scale bar, 0.5  $\mu$ m.

without PrPres accumulation. Hence, it is not eliminated, and during replication the agent may acquire the capacity to convert the new host PrP into PrPres. As a result of this adaptation, the transmissible agent would be tightly associated with PrPres, which would confer enhanced virulence and induce the development of classical spongiform lesions.

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8. It could be argued that we killed our mice too early, when infectivity was not maximal in the brain. However, mice were killed at the pre-mortem stage (that is, just before they would have died of disease). Moreover, it is known from experimental models that PrPres accumulation precedes the appearance of pathology and is detectable several months before clinical signs (17).
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10. It could be argued that the mice that died of a neurological disease without detectable PrPres had been contaminated with a conventional agent during the inoculation process. This is unlikely because (i) control mice injected with normal cow brain remained healthy, and (ii) histological and electron microscopy examination of brains did not show classical encephalitis (complete lack of inflammatory cells or edema, absence of viral particles) but rather neuronal death, which is a hallmark of TSE and is particularly prominent in cattle BSE (20). It might also be argued that these findings are the result of laboratory contamination with prions during serial passage, but (i) the PrPres<sup>-</sup> trait was maintained and exhibited specific pathological features, and (ii) the mouse-adapted BSE strain obtained from the series of passages described here has been characterized and is clearly different from the scrapie strain C506M3 handled in our laboratory (17).
11. A dissociation of PrPres and infectivity has been reported in fractionation and time course experiments as well as with amphotericin B treatment (27). Also, the absence of detectable PrPres has been described in several models of transgenic mice overexpressing a modified PrP and after some Creutzfeldt-Jakob disease transmissions in hamsters (22).
12. These results are complementary to the observa-

tions made in PrP<sup>+/0</sup> mice that PrPres accumulation and spongiform lesions reach their maximum extents more than 6 months before the animals die, hence they are dissociated from clinical condition and death (23).

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26. Mice were killed at the pre-mortem stage by cervical fracture, and brains were immediately removed. One hemisphere (including the cerebellum) was frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for PrP analysis. (The other hemisphere was fixed for pathological examination.) For PrPres purification, the whole brain hemisphere was homogenized to 10% (w/v) in a 5% glucose solution. Briefly, proteinase K (PK) was used at 10  $\mu\text{g/ml}$  (1 hour at  $37^{\circ}\text{C}$ ) and digestion was blocked with phenylmethylsulfonyl fluoride (5 mM). After addition of sarkosyl to 10% and tris (pH 7.4) to 10 mM, samples were incubated for 15 min at room temperature. They were then centrifuged at 245,000g for 4 hours at  $20^{\circ}\text{C}$  on a 10% sucrose cushion (Beckmann Ti 100 ultracentrifuge). Pellets were resuspended in Laemmli buffer (24) and run on a 12% polyacrylamide gel. Protein immunoblotting procedures using chemiluminescence were as described (17). The standard conditions correspond to the load of samples equivalent to 4 mg of brain and a 1-min exposure time. Sensitivity of the detection can be increased by a higher loading of the gel (up to 25 mg) and a longer exposure time (up to 30 min).
27. PK doses are expressed in micrograms of 10% brain homogenate per milliliter. Digestion was performed as described above with increasing doses of PK. After denaturation in Laemmli buffer, homogenates equivalent to 1 mg of brain were electrophoresed.
28. Thirty adult male C57BL/6 mice were injected intracerebrally with 20  $\mu\text{l}$  of 25% BSE-infected brain homogenate. Ten control mice were injected similarly with control cow brain. Subsequent mouse-to-mouse passages used 20  $\mu\text{l}$  of 10% mouse brain homogenates (corresponding to about 1/200 of a mouse brain), except for the 2FBI-1 mouse inoculum (1% homogenate). Twenty mice were injected with a 1% brain homogenate of a mouse infected with experimental mouse scrapie, strain C506M3, constituting the positive control group. Negative control mice were kept in the same room and did not develop any neurological disease. The incubation periods correspond to survival times assessed according to the criteria in (25).
29. Whole brain hemispheres were fixed in buffered 10% formalin. Pieces of brain were then embedded either in paraffin for immunohistochemistry (7- $\mu\text{m}$  sections) or in Araldite (4- $\mu\text{m}$  sections) for fine morphological examination. Antibodies were a polyclonal antibody to mouse glial fibrillary acidic protein (GFAP) and a horseradish peroxidase-conjugated secondary anti-

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5. A. detailed description of this study is in press in *Endocrinology*. Please contact S. Safe for more information

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*Response:* It is difficult to compare the results of the study by Ramamoorthy *et al.* to ours because the assays they used, while appearing to be similar to ours, were in each case different. The differences, however, have been instructive in helping us frame some of the parameters that may be important in determining the synergistic action of weakly estrogenic chemicals.

In our mammalian and yeast cell assays (1), as well as in the ligand-binding experiments, the concentration of receptor molecules was low, while in the study by Ramamoorthy *et al.* the concentrations were high. For example, our mammalian cell culture experiments used Ishikawa uterine cancer cells that lack detectable ER and were transfected with only 20 ng of hER cDNA. In contrast, Ramamoorthy *et al.* used MCF-7 breast cancer cells that contained high levels of endogenous ERs [MCF-7 cells typically contain endogenous ER levels in the range of 30,000 ERs per cell (2) to 200,000 ERs per cell (3)] and that were transfected with an additional 4 to 5 µg of hER cDNA. Likewise, in the yeast-based assay used in our report, the number of expressed hERs was estimated to be 500 to 1000 receptors per cell, but the study by Ramamoorthy *et al.* appears to contain well in excess of 1000 ERs per cell. Finally, our in vitro competitive binding conditions used 0.4 nM concentrations of ERs (monomer concentrations), whereas the concentration of ER used by Ramamoorthy *et al.* was considerably higher and the assays were not performed according to our report (1). Therefore, because our results showed synergy and theirs did not, ER concentration may play an important role in the ability of mixtures of chemicals to synergize.

With regard to the animal studies, our earlier work showed synergistic responses to weakly estrogenic chemicals in turtles that were treated early in development (4). The study by Ramamoorthy *et al.* was performed in the uterus of female mice that had already undergone sexual differentiation. Our contention has been that developmentally exposed animals are more likely to demonstrate synergistic responses to estrogenic chemicals. Nonetheless, inspection of the data provided by Ramamoorthy *et al.* sug-

body (Dako). Seven PrPres<sup>+</sup> and six PrPres<sup>-</sup> brains were examined. Spongiform lesions and gliosis could not be seen in any brain region of PrPres<sup>-</sup> mice. The absence of localized PrPres deposits was confirmed by PrP immunohistochemistry.

30. Whole brain hemispheres were fixed overnight with a solution of 1% glutaraldehyde and 1% paraformaldehyde in 0.12 M phosphate buffer (pH 7.4). After 1 hour postfixation with 2% osmic acid, they were stained en bloc with uranyl acetate and embedded in Araldite. Ultrathin sections were stained with uranyl

acetate and lead citrate before examination with a Philips CM10 electron microscope

31. We thank R. Bradley for BSE-infected cattle brain homogenate, C. Weissmann and F. H. Kimberlin for helpful discussions, and R. Roux and J. C. Mascaro for expert animal care. We also thank P. Fritch and M. Wasowicz, as well as L. Court, who encouraged our research on TSE. Supported by a grant from D.R.E.T. (Paris).

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TECHNICAL COMMENTS

Potency of Combined Estrogenic Pesticides

Steven F. Arnold *et al.* found 150- to 1600-fold synergistic interactions between binary mixtures of the weakly estrogenic pesticides endosulfan, dieldrin, toxaphene, and chlordane in competitive estrogen receptor (ER) binding assays and in an estrogen-responsive assay in yeast (1). Less dramatic synergistic interactions between two weakly estrogenic hydroxy polychlorinated biphenyl congeners were also observed in the yeast assay and in human endometrial cancer cells. On the basis of these data, it was suggested "that the estrogenic potency of some environmental chemicals, when tested singly, may be underestimated" (1, p. 1491). The purported synergistic interactions of these compounds have important mechanistic and public health consequences (2). We reassessed the potential synergistic interactions of two weakly estrogenic pesticides, dieldrin and toxaphene, using the following estrogen-responsive assays: induction of uterine wet weight, progesterone receptor (PR) levels and uterine peroxidase activity in the immature female mouse; induction of cell growth and two estrogen-responsive reporter gene assays in MCF-7 human breast cancer cells; induction of reporter gene activities in two yeast-based assays that expressed either the human or mouse ER, and competitive binding to human and mouse ER. For these 10 different estrogen-responsive assays, the combined activities of dieldrin plus toxaphene were essentially additive. Moreover, interactions of all the binary mixtures of organochlorine pesticides reported by Arnold *et al.* (1) were reinvestigated in the two yeast-based assays.

The results we obtained in yeast transfected with an expression plasmid that contained the wild-type mouse ER and a reporter plasmid containing a single ERE linked to the LacZ gene (3) indicate that the estrogenic activities of all the binary mixtures of organochlorine pesticides were

additive. These same binary mixtures were also investigated in a yeast-based human ER assay (4), which used the same yeast strain and reporter gene construct used by Arnold *et al.* (1). In contrast to that study, synergistic activity was not observed for any pesticide combination. The differences between our results and those reported by Arnold *et al.* (1) cannot be accounted for by differences in total ER expression, because varying this expression did not have any effect on synergy. These results demonstrate that synergism between weakly estrogenic chemicals is not universal, even within the same strain of yeast. The recent scientific, regulatory, and public concern regarding the potential adverse environmental and human health impacts from synergistic estrogen responses induced by organochlorine pesticide mixtures should be tempered by our results, which demonstrate that these compounds are weakly estrogenic and, in combination, their activities are additive (5).

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