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A crucial role for B cells in neuroinvasive scrapie

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Although prion proteins are most efficiently propagated through intracerebral inoculation, peripheral administration has caused the diseases kuru, iatrogenic Creutzfeldt–Jakob disease (CJD), bovine spongiform encephalopathy (BSE) and new-variant CJD[1,2]. The development of neurological disease after peripheral inoculation depends on prion expansion within cells of the lymphoreticular system[3,4]. Here we investigate the identity of these cells by using a panel of immune-deficient mice inoculated with prions intraperitoneally: we found that defects affecting only T lymphocytes had no apparent effect, but that all mutations that disrupted the differentiation and response of B lymphocytes prevented the development of clinical scrapie. As an absence of B cells and of antibodies correlates with severe defects in follicular dendritic cells, a lack of any of these three components may prevent the development of clinical scrapie. However, we found that scrapie developed after peripheral inoculation in mice expressing immunoglobulins that were exclusively of the M subclass and without detectable specificity for the normal form of the prion PrP^{c}, and in mice which had differentiated B cells but no functional follicular dendritic cells. We conclude that differentiated B cells are crucial for neuroinvasion by scrapie, regardless of the specificity of their receptors.

The effect of combined immune defects on the pathogenesis of scrapie was studied in mice deficient in Rag-2 (*rag-2*$^{0/0}$) (ref. 5) and Rag-1 (*rag-1*$^{0/0}$) (ref. 6), which lack B and T cells, in SCID (severe combined immune deficient) mice, and in Agr-deficient (*agr*$^{0/0}$) mice (M.S., unpublished results), which lack Rag-2 as well as the receptors for interferon-α/β[7] and interferon-γ[8]. For controls, we inoculated inbred mice of strains C57BL/6 and 129Sv, which are the genetic backgrounds of all other mouse strains used. To investigate the role of T cells, we used mice with targeted disruption of the genes encoding CD4 (ref. 9), CD8 (ref. 10), β_2-microglobulin[11] or perforin[12]. Selective ablation of B lymphocytes was studied in μMT mice[13], which have a targeted disruption of the transmembrane exon of the immunoglobulin μ-chain gene, do not produce any immunoglobulins and suffer from a B-cell differentiation block at the large-to-small pre-B-cell transition, yet bear complete and functional T-cell subsets.

After intracerebral (i.c.) challenge with scrapie prions, all immune-deficient mice developed clinical symptoms of scrapie. This was confirmed by histopathological analysis (not shown) and by transmission of disease to indicator *tga20* mice, which overexpress the normal prion protein (PrP^{C}) and are hypersensitive to scrapie[14] (Table 1). Transmission to *Prnp*$^{0/0}$ mice[15], which do not express PrP^{C} and are resistant to scrapie[16] ($n = 4$), did not induce disease after >240 days, as expected for bona fide scrapie. In all groups, latency times from inoculation to first appearance of clinical symptoms and to terminal disease (Table 2), as well as brain prion infectivity titres (Table 1), were similar to those of control mice. Thus, if prions were delivered to the central nervous system, scrapie pathogenesis and prion expansion in the brain proceeded without any detectable influence of the immune status of the host.

When mice were exposed to prions through the intraperitoneal (i.p.) route, mice with homozygous ablations of CD4, CD8, β_2-microglobulin or perforin developed the initial symptoms of disease and terminal scrapie with latency periods similar to those of 129Sv and C57BL/6 mice (Table 2), and reached analogous prion titres in both spleen and brain (Table 1). We conclude that CD8^{+} cytotoxic and CD4^{+} helper T lymphocytes are not rate-limiting for scrapie after peripheral inoculation of prions, in agreement with the observation that nude mice develop scrapie normally after i.p. inoculation[3].

In contrast, no disease appeared after i.p. inoculation in μMT and in Rag-deficient (*rag-1*$^{0/0}$, *rag-2*$^{0/0}$ and *agr*$^{0/0}$) mice, and no prion infectivity was detectable in their spleens (Table 1). In SCID C57BL/6 mice, disease was marginally prolonged, which disagrees with earlier results[4,17] and may be due to incomplete immune deficiency of SCID mice in specific genetic backgrounds[18,19], because SCID C.B-17 mice (whose immune defect is less leaky) did not develop disease (Table 2).

Histopathological examination of brain sections revealed generalized spongiform encephalopathy in all wild-type and immune-

deficient mice clinically diagnosed as scrapie-sick (Fig. 1). In addition, and despite lack of clinical symptoms, spongiform encephalopathy was seen in 1/7 Rag-deficient and 1/6 μMT mice (at random sampling) 342 and 436 days after i.p. inoculation (Fig. 1), and significant prion titres were found in brains of 3/7 Rag-deficient mice and 1/3 μMT mice (Table 1). Western blot analysis revealed accumulation of the pathological form of the prion protein, PrP^{Sc}, in the brains of 2/6 Rag-deficient and 2/6 μMT mice inoculated i.p. (Fig. 2). The remaining Rag-deficient and μMT mice did not accumulate PrP^{Sc} as late as 504 days after inoculation.

The latter findings are compatible with incipient scrapie in a fraction of B-cell-deficient mice. Therefore, although it prevents 'neuroinvasion' of the scrapie agent in most cases, absence of B cells uncovers a slower, <50% efficient mechanism of pathogenesis which may cause scrapie in situations of immune deficiency. Even then, B-cell deficiency prolongs the delay between PrP^{Sc} accumulation,

Table 1 Scrapie symptoms, scrapie histopathology and infectivity titres in individual immunodeficient mice

	Primary infection (route of inoculation: i.c.)			Infectivity bioassay	
Genotype	Incubation (d)	Symptoms	Pathology	Brain	Spleen
$CD4^{0/0}$	176	+	+	7.2* (65 d ± 2†)	6.6 (71 d ± 2)
$CD4^{0/0}$	154	+	+	7.4 (63 d ± 1)	7 (67 d ± 2)
SCID	167	+	+	7.3 (64 d ± 1)	5.5 (81 d ± 2)
SCID	167	+	+	7.6 (62 d ± 2)	5.2 (83 d ± 1)
rag-2$^{0/0}$	171	+	+	7.3 (64.5 d ± 2)	<0 (>200 d)
agr$^{0/0}$	182	+	+	7.3 (64.5 d ± 1)	<0 (>145 d)
μMT	175	+	+	7.9 (59 d ± 5)	<0 (>200 d)
	Primary infection (route of inoculation: i.p.)			Infectivity bioassay	
$CD4^{0/0}$	191	+	+	7.3(64 d ± 1†)	ND
$CD4^{0/0}$	195	+	+	7.5 (62.5 d ± 2)	ND
SCID	214	+	+	7.3 (64 d ± 1)	5.2 (83 d ± 1)
SCID	249	+	+	7.7 (61 d ± 2)	5 (85 d ± 1)
rag-2$^{0/0}$	286	–	+	6.5 (72 d ± 2)	<0 (>200 d)
rag-2$^{0/0}$	286	–	–	<0 (>200 d)	<0 (>200 d)
rag-2$^{0/0}$	339	–	–	<1 (>122 d)	<2 (>115 d)
rag-2$^{0/0}$	342	–	+	7.5 (65 d ± 1)	<2 (>115 d)
rag-1$^{0/0}$	222	–	–	<1 (>122 d)	<2 (>115 d)
agr$^{0/0}$	284	–	+	7.2 (65 d ± 0)	<0 (>139 d)
agr$^{0/0}$	349	–	–	<0 (>139 d)	<0 (>139 d)
μMT	286	–	–	<0 (>200 d)	<0 (>200 d)
μMT	286	–	–	<0 (>200 d)	<0 (>200 d)
μMT	375	–	–	7.8 (60 d ± 1)	<0 (>200 d)
μMT	436	–	+	ND	ND
$TNFR1^{0/0}$	211	+	+	7.7 (61 d ± 1)	ND
$TNFR1^{0/0}$	212	+	+	7.7 (60 d ± 1)	ND

For infectivity bioassays, brain or spleen homogenates were injected i.c. into groups of four *tga20* mice. ND, not determined.
* Prion titres expressed as log LD_{50} per g of spleen or brain tissue.
† Incubation time, in days, of indicator *tga20* mice (average ± standard deviation).

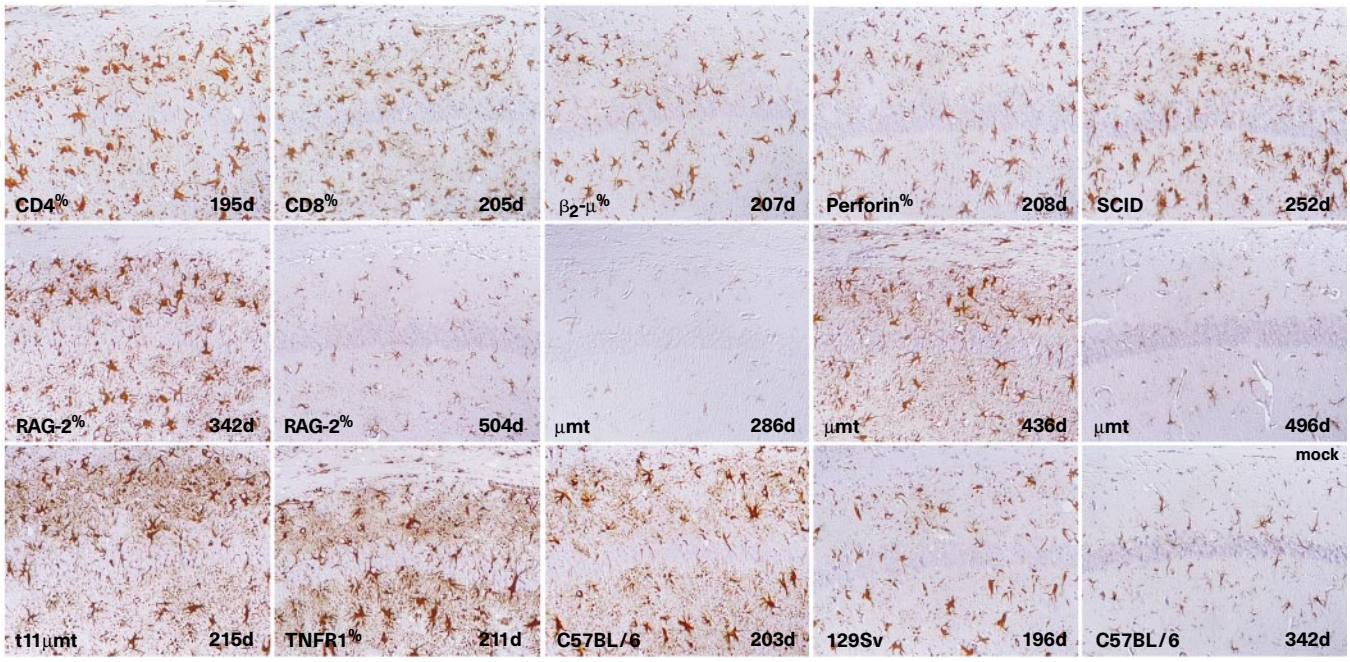

Figure 1 Brain histopathology of immune deficient and control mice after i.p. inoculation of scrapie prions. The hippocampal formation was immunostained for glial fibrillary acidic protein, and identical segments of the pyramidal cell ribbon were microphotographed (200×). Intense, diffuse gliosis was visible in brains of T-cell-deficient, SCID, $TNFR1^{0/0}$, *t11μMT*, and infected control mice. Some *rag-2*$^{0/0}$ and *μMT* mice showed spongiform encephalopathy, but others of the same genotype did not display any pathology after similar time periods following i.p. inoculation, and were indistinguishable from mock-infected C57BL/6 mice.

onset of scrapie histopathology and clinical symptoms, arguably beyond the typical life expectancy of mice.

These results suggest that B cells may 'transport' prions from lymphoid organs to nervous tissue. Alternatively, the apparent protection of B-cell-deficient mice from prions administered i.p. may result from the absence of immunoglobulins. Complexing of PrP^{Sc} with antibodies may favour nucleation (a process proposed to underlie the formation of prion infectivity[20]) or may opsonize PrP^{Sc} and enhance access to lymphoid sites of prion expansion. To clarify this question, we inoculated *t11μMT* mice (μMT mice expressing a rearranged IgM transgene directed against the glycoprotein of vesicular stomatitis virus) and found that they could support normal B-cell differentiation (U.K., unpublished) but exclusively expressed the transgenic IgM heavy chain, had a heavily skewed and very limited antibody repertoire, and lacked immunoglobulins of the D, G, E and A subclasses.

After i.p. inoculation with prions, *t11μMT* mice developed disease with a latency comparable to that of wild-type mice (Table 2)

Table 2 Latency of scrapie in different immunodeficient mice

		Intracerebral route		Intraperitoneal route	
Defect	Genotype	Scrapie	Time to terminal disease (d)	Scrapie	Time to terminal disease (d)
T	$CD4^{0/0}$ *	7/7	159 ± 11	8/8	191 ± 1
T	$CD8^{0/0}$ *	6/6	157 ± 15	6/6	202 ± 5
T	$\beta_2\text{-}\mu^{0/0}$ *	8/8	162 ± 11	7/7	211 ± 6
T	*Perforin*$^{0/0}$ *	3/4†	171 ± 2	4/4	204 ± 3
T and B	SCID *	7/8†	160 ± 11	6/8‡	226 ± 15
T and B	SCID§	4/4	152 ± 1	1/4‖	289
T and B	*rag-2*$^{0/0}$ *	7/7	167 ± 2	0/7	Healthy (>504)
T and B	*rag-1*$^{0/0}$ *	3/3	175 ± 2	0/5	Healthy (>258)
T and B	*agr*$^{0/0}$¶	6/6	184 ± 10	0/7	Healthy (>450)
B	μMT*	8/8	181 ± 6	0/8	Healthy (>534)
IgG	*t11μMT**	5/5	170 ± 3	4/4	223 ± 2
FDC	$TNFR1^{0/0}$#	7/7	165 ± 3	9/9	216 ± 4
Controls	129Sv	4/4	167 ± 9	4/4	193 ± 3
	C57BL/6	4/4	166 ± 2	4/4	206 ± 2

All mice developed spongiform encephalopathy after i.c. inoculation. In contrast, B-cell-deficient mice stayed healthy after i.p. inoculation of RML scrapie prions.
* Genetic background was inbred C57BL/6.
† One *Perforin*$^{0/0}$ and one SCID mouse suffered from intercurrent death 135 and 141 days after inoculation, respectively.
‡ Two SCID C57BL/6 mice remained healthy and were killed 303 and 323 days after inoculation.
§ Genetic background was inbred C.B-17.
‖ 3/4 SCID C.B-17 mice remained healthy (>340 d). Four further SCID C.B-17 mice were challenged with 100 μl of a 10^{-4} dilution of RML prions, and all remained healthy (>340 d).
¶ Genetic background was C57BL/6 × 129Sv.
# Genetic background was inbred 129Sv.

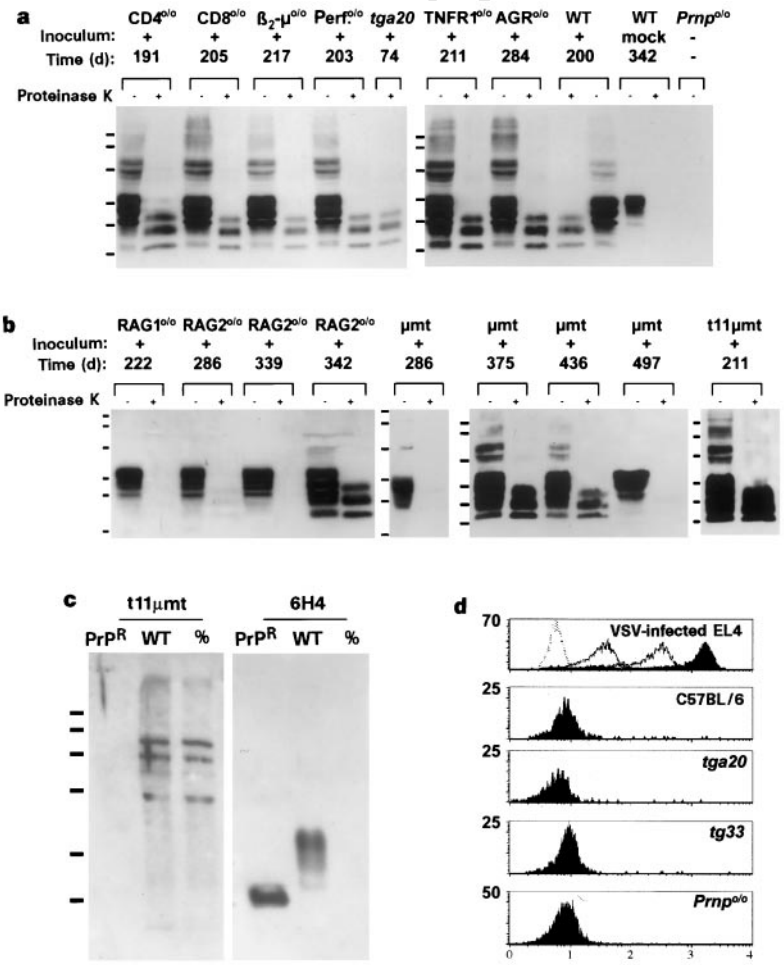

Figure 2 Western blot analysis of brains of immune-deficient mice after i.p. inoculation with scrapie prions and lack of specific antibodies against PrP in *t11μMT* mice. **a**, **b**, Western blots of brain material electrophoresed native (–) or after digestion with proteinase K (PK) (+). Large amounts of PK-resistant prion protein (PrP^{Sc}) were detected in all mice that had developed scrapie, as well as in one *agr*$^{0/0}$ (**a**) two *rag-2*$^{0/0}$ and two μMT mice (**b**). One further B-cell-deficient mouse proved negative for PrP^{Sc} (not shown), and no clinical symptoms of scrapie were detected in any B-cell-deficient mice irrespective of accumulation of PrP^{Sc}. **c**, Western blot prepared with recombinant murine PrP from *E. coli* (PrP^{R}), total brain protein extract from a wild-type mouse (WT), and total brain protein extract from a *Prnp*$^{0/0}$ mouse (*0/0*)[15]. Blots were incubated with serum from a *t11μMT* mouse inoculated with prions i.p. (left), stripped and reprobed with monoclonal antibody 6H4 to recombinant PrP (right). The presence of PrP-specific antibodies, as indicated by a 20K band in lane PrP^{R} and by a cluster of bands present in lane WT but absent from lane *0/0*, is evidence with 6H4 antibody but undetectable in *t11μMT* serum. Relative molecular mass markers (top to bottom): 105K, 82K, 45K, 37.3K, 28.6K, 19.4K. **d**, FACS analysis of immunoreactivity of *t11μMT* serum. Ordinate: cell counts; abscissa: logarithm of fluorescence intensity. Serum from a *t11μMT* mouse 210 days after i.p. inoculation with prions was diluted 1:10 and 1:100, stained VSV-infected EL4 cells (top panel, unfilled area) almost as strongly as VSV-specific monoclonal antibody VI24 (filed area). In contrast, immunoreaction of *t11μMT* serum (1:10) with $CD3^{+}$ T cells from C57BL/6, *tga20*, *tg33* (ref. 29) and *Prnp*$^{0/0}$ mice (lower panels) did not exceed background, like normal C57BL/6 serum on EL4 cells (top panel, dotted line). The same profiles were obtained when probes were stained with serum of untreated *t11μMT* mice (data not shown).

and accumulated PrP^{Sc} in their brains (Fig. 2b). Serum from both uninfected and terminally scrapie-sick *t11μMT* mice inoculated i.p. was shown by western blotting and by fluorescence-activated cell sorting (FACS) analysis not to crossreact with PrP^{C} (Fig. 2c, d), suggesting that IgGs are not the effectors of prion 'neuroinvasion', and that a specific humoral immune response (at least as assessed by FACS and western-blot analysis) cannot be correlated with peripheral pathogenesis of scrapie. However, we cannot exclude the possibility that IgMs below the threshold of detectability, or indirect effects of antibodies, may be involved in scrapie pathogenesis.

B cells are required for maturation of follicular dendritic cells (FDCs) and formation of germinal centres. Protection of B-cell-deficient mice may therefore result from the absence of FDCs, especially as FDCs accumulate PrP^{Sc} extensively in i.p.-inoculated mice[3] and in the tonsils of patients suffering from new-variant CJD[21]. We inoculated mice lacking tumour-necrosis factor receptor-1 ($TNFR1^{0/0}$)[22], which have virtually no germinal centres in lymphatic organs and very few, if any, FDCs[23], despite differentiation of functional B and T lymphocytes. These mice developed scrapie after both i.c. and i.p. inoculation, as did control mice (Table 2), thus disproving a prime role for FDCs in peripheral pathogenesis and supporting our previous finding that adoptive transfer of fetal liver cells (which does not efficiently replace FDCs[24]) can restore high spleen prion titres after i.p. inoculation[25].

We have identified B cells (or B-cell-dependent processes) as a limiting factor in the development of scrapie after peripheral inoculation. The suspicion that B cells may be the physical carriers of prions may eventually call for a re-evaluation of the safety of blood products. The greatly increased time between peripheral inoculation and detection of prions in the central nervous system, as well as the complete suppression of clinical symptoms, suggest that suppression of B cells could mitigate the course of spongiform encephalopathies. □

Methods

Scrapie inoculation. Mice were inoculated with a 1% homogenate of heat- and sarcosyl-treated brain prepared from mice infected with the Rocky Mountain laboratory (RML) scrapie strain. 30 μl were used for i.c. injection, whereas 100 μl were administered i.p. Mice were monitored every second day, and scrapie was diagnosed according to standard clinical criteria.

Western blot analysis. 10% brain homogenates were prepared as described[16] and, where indicated, digested with 20 μg ml^{-1} proteinase K for 30 min at 37 °C. 80 μg of total protein were then electrophoresed through 12% SDS–polyacrylamide gels, transferred to nitrocellulose membranes, probed with monoclonal antibody 6H4 (a gift from B. Oesch, Prionics AG, Zurich) or polyclonal antiserum IB3 (ref. 26) against mouse PrP, and developed by enhanced chemiluminescence (Amersham).

FACS analysis. Peripheral blood cells were incubated with serum from *t11μMT* mice, washed, incubated with anti-mouse IgM-FITC conjugate followed by anti-CD3-PE (Pharmingen), and analysed with a Becton-Dickinson FACScan instrument after erythrocyte lysis and fixation. For analysis, cells were gated on CD3-positive T cells. EL4 cells infected with vesicular stomatitis virus (VSV) were stained with 5 μg VSV-specific monoclonal antibody VI24 (ref. 27) and with FITC-labelled antibody to mouse IgG2a (Southern Biotechnology), or with serum of *t11μMT* mice, and with FITC-labelled $F(ab')_2$ antibody to mouse IgM (anti-IgM-FITC, Tago), or with serum of C57BL/6 mice and anti-IgM-FITC. All data acquisition and analysis were performed with CellQuest software (Becton Dickinson).

Generation of *t11μMT* mice. The *V*-gene segment of the immunoglobulin heavy chain of the immunoglobulin heavy chain of the B-cell hybridoma VI41 (ref. 27) secreting a VSV-neutralizing antibody was cloned into an expression vector encoding the mouse μ-chain of allotype a. Transgenic mice were generated and backcrossed to μMT mice. *t11μMT* mice exclusively expressed the transgenic μ-chain of the allotype a; endogenous IgM of the allotype b and immunoglobulins of other subclasses were not detected in their serum (not shown).

Detection of anti-PrP antibodies. Brain lysates from wild-type and $Prnp^{0/0}$ mice[14], as well as recombinant *E. coli* PrP, were electrophoresed through a 12% SDS–polyacrylamide gel and transferred to nitrocellulose. Membranes were incubated with serum from infected, terminally scrapie-sick mice (diluted 1:100). Visualization was achieved as described above.

Immunohistochemical studies. Brain tissue from each mouse was fixed, inactivated for 1 h with 98% formic acid, embedded in paraffin, and subjected to conventional staining and to immunostaining for glial fibrillary acidic protein according to standard procedures. Gliosis (a nonspecific but early indicator of brain damage) was detected by the presence of large immunostained reactive astrocytes. In terminally scrapie-sick mice, widespread vacuolation was consistently seen throughout the CNS.

Infectivity bioassays. Brain and spleen homogenates (10% in 0.32 M sucrose) were prepared from infected animals as described, and 30 μl (diluted 1:10 in PBS and 1% BSA) were administered i.c. to groups of at least four *tga20* mice for each sample. The incubation time until development of terminal scrapie sickness was determined and infectivity titres were calculated[28] using the relationship $y = 14.37 - 0.11x$, where y is the ID_{50} and x is the incubation time (in days) to terminal disease[14].

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