



Fig. 2 L3T4 and Lyt-2 antigen expression on cells derived from a single-cell-recolonized thymus lobe demonstrates that a single thymic stem cell can give rise to three marker-defined phenotypes. Lobes recolonized by a single thymic stem cell were produced as described in Fig. 1. Dual immunofluorescence labelling was carried out using the reagents described for rows 5–7 in Table 2. *a*–*c*, Two cells are shown (*a*), the smaller of which labels with both Lyt-2 (*b*) and L3T4 (*c*), whereas the larger cell expresses only Lyt-2 (*b*). *d*, Two adjacent lymphoid cells from the same preparation as that in *a*–*c*. These cells express Lyt-2 (*e*) and L3T4 (*f*) in a mutually exclusive fashion. Thus, all three phenotypes—L3T4⁺ Lyt-2[−]; L3T4⁺ Lyt-2[−]; and L3T4[−] Lyt-2⁺—were present in this lobe.

Table 2 Lyt2 and L3T4 marker expression on the progeny of a single thymic stem cell

Cell count ×10 ⁴	% Lyt 2.2 ⁺ L3T4 ⁺	% Lyt 2.2 ⁺ L3T4 [−]	% Lyt 2.2 [−] L3T4 ⁺	Null
4.2	22.1	9.7	0	68.2
2.0	40.5	17.1	4.5	37.9
6.6	50.5	25.2	0	24.6
1.95	15.1	44.2	1.2	31.5
5.0	37.5	19.2	0	43.3
3.0	20.8	15.8	0	63.6
8.8	52.7	14.5	4.4	28.4

Strain combinations and details for rows 1–4 are as in Table 1. Immunofluorescence reagents for this group were: first step, anti-Lyt 2.2 (mouse IgM) and anti-L3T4 (clone H129.19 rat IgG²²); second step, anti-mouse IgM(Fc)-Rh and anti-rat IgG(Fc)-F1 (Nordic). In rows 5–7 the strain combination consisted of AKR stem cell donor and BALB/c host thymus, and the following reagents were used sequentially: anti-L3T4 (H129.19) anti-rat IgG(Fc)-Rh, 1% normal rat serum wash, anti-Lyt-2-ars (clone 53-6.7), anti-ars-FL. All reagents were tested for specificity and non-cross-reactivity and gave the expected staining patterns on adult T cells. The results show that in both strain combinations tested, in some lobes all three phenotypes were generated, similar results being obtained with the two different sets of reagents used.

Initial studies have shown that the progeny of a single cell can respond to concanavalin A without added growth factors, implying that they can produce their own IL-2 and are capable of helper-type activity. If the same cultures can also generate cells possessing cytotoxic activity, this will indicate that both functional phenotypes can arise from a single cell irrespective of their relationship to the marker-defined populations.

Previous studies on thymocyte populations in bone-marrow-reconstituted, irradiated adults have suggested that entire thymus lobes can be recolonized by the progeny of a small number of donor bone marrow cells^{14,15}. However, the initial pattern of

differentiation of donor-derived cells in this model seems to differ from that seen in ontogeny^{16,17}, and it is not clear whether all the recolonizing cells are equivalent to those populating the thymus during normal development. Our demonstration that a single immature fetal thymus cell can repopulate a thymus lobe, coupled with observations suggesting limited entry of precursors into the thymus during embryonic¹⁸ and adult life¹⁹, is consistent with the need for diversification of the T-cell receptor repertoire within the thymus. Consistent with this, recent studies have shown that thymus development is associated with an increasing proportion of cells showing rearrangement at the β-chain locus¹. However, the possibility of some receptor gene rearrangement at a pre-thymic level^{20,21} and the progressive recruitment of rearranged cells into the developing thymus cannot be excluded. In this context, examination of the pattern of gene rearrangement in the progeny of a single intra-thymic stem cell should show whether there is extensive diversification within the thymus or whether, despite considerable proliferation, only limited rearrangements are generated in the descendants of a single precursor.

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Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype

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Recent advances in molecular genetics have led to the possibility of using large DNA viruses, such as vaccinia virus, as a biological delivery system for immunizing man against unrelated disease-causing agents^{1–7}. When live vaccinia virus recombinants expressing the hepatitis B virus surface antigen (HBsAg)^{8,9}, the influenza A virus haemagglutinin¹⁰, the herpes simplex virus (HSV) type 1 glycoprotein^{11,12}, the rabies virus G glycoprotein^{13,14} and the vesicular stomatitis virus G glycoprotein¹⁵ were used for immunization, animals were protected upon challenge with the appropriate

pathogenic agent. A major concern with using such vaccines, however, stems from the previously documented vaccinia virus-associated post-immunizing complications¹⁶. We present here experimental evidence that thymidine kinase-negative (TK⁻) vaccinia virus recombinants, constructed by inserting a variety of DNA coding sequences into the vaccinia virus *tk* gene, are less pathogenic for mice than wild-type virus.

The vaccinia virus *tk* locus^{4,17} was chosen as a site of insertion for foreign DNA, primarily because it provides an efficient method of selecting infectious recombinants^{6,17}. However, the possibility that this would lead to attenuation of virus virulence was considered because of data obtained with herpesviruses. HSV types 1 and 2 and marmoset herpesvirus encode a TK that is important for virus pathogenicity in certain strains of inbred mice inoculated by the intracerebral or intradermal routes^{18,19}. This effect has been attributed to the role of TK in enhancing herpesvirus replication in non-dividing cells²⁰⁻²². To determine whether inactivation of the vaccinia virus *tk* gene by the insertion of foreign DNA led to attenuation, several independently derived TK⁻ recombinant viruses produced from two closely related TK⁺ strains of vaccinia virus (WR and Wyeth) were inoculated into BALB/cByJ mice by the intracerebral (i.c.), intraperitoneal (i.p.) and intradermal (i.d.) routes. Wyeth is the New York City Board of Health (NYCBH) strain used extensively in the United States and elsewhere for smallpox vaccination. WR, derived from the NYCBH strain by multiple passage in mouse brain²³, has been more extensively studied in the laboratory, and because of its enhanced virulence provides a more sensitive model for studying virus attenuation.

Table 1 lists the amount of virus required to kill half of the mice (50% lethal dose, LD₅₀) by the i.c. route of inoculation. The WR strain TK⁻ vaccinia virus recombinants, which encoded the genes for the haemagglutinin of influenza virus A/Japan/305/57 (vInf1)¹⁰ and herpes simplex virus (strain KOS) type I D glycoprotein (v52)¹² had an LD₅₀ 2.5 × 10⁴–10⁵-fold higher than that of wild-type (WT) virus. Mice that survived i.c. inoculation of WR-WT, WR-vInf1 or WR-6/2 viruses showed no signs of delayed onset of disease by 24 days post-infection, at which time the experiment was terminated. Significantly, insertion of DNA into the *tk* locus of the Wyeth strain of vaccinia virus also had an attenuating effect. The LD₅₀ of Wyeth-v55 (expressing HBsAg) was even higher than that of the WR recombinants because of lower starting virulence of this vaccine strain. Since TK11 (a gift of R. Condit), which lacks a functional *tk* gene but contains no insertion of foreign DNA, showed a higher LD₅₀ than WT, the TK⁻ phenotype and not the foreign gene expression must be the major factor contributing to lowered virus virulence. In summary, insertion of foreign DNA into the *tk* locus attenuates strains of vaccinia virus that vary widely in neurovirulence.

It is of interest that the mutant designated 6/2 (ref. 24), which has a spontaneous 9-kilobase deletion located proximal to the left inverted terminal repetition, yielded the highest LD₅₀ by the i.c. route of any WR strain derivatives examined. A previous study showed that the region of the WT virus genome defined by the 6/2 deletion is also suitable for the insertion and expression of foreign DNA⁶.

Decreased virus pathogenicity also was demonstrated by the i.p. route of inoculation. Lethality by this route is probably a consequence of virus seeding and replication in the internal organs. The LD₅₀ in BALB/cByJ male mice for strain WR-WT by the i.p. route was 1 × 10⁸ ± 1.1 plaque-forming units (PFU); however, doses of viruses vInf1, v52 and TK11 as high as 10⁹ PFU were insufficient to produce a lethal effect (that is, LD₅₀ > 10⁹ PFU; Table 2). A similar result was seen in C3H/HeJ mice with vInf1 virus (data not shown). In a second experiment, i.p. injection of BALB/c mice with 10⁸ PFU of WR-WT or WR-v52 resulted in 20/25 and 0/25 deaths, respectively. Surviving mice recovered rapidly from the virus infection, and as late as 49 days post-inoculation no new signs of disease were observed.

To quantitate further the differences in virulence between WR

Table 1 LD₅₀ of vaccinia and derivative viruses in BALB/cByJ male mice by i.c. inoculation

Virus strain	PFU inoculum at LD ₅₀
WR-wild type	1 × 10 ⁸ ± 1.0
WR-vInf1	4 × 10 ⁶ ± 1.4*
WR-v52	2.5 × 10 ⁵ ± 2.8*
WR-TK11	4.0 × 10 ⁴ ± 2.5*
WR-6/2	6.3 × 10 ⁴ ± 1.2*
Wyeth-wild type	3.2 × 10 ⁶ ± 1.4
Wyeth-v55	9.1 × 10 ⁷ ± 1.1*

Serial 10-fold dilutions of virus (30 µl) purified from cells³⁰ in 1 mM Tris-HCl (pH 9.0) were injected with a tuberculin syringe and 26-gauge needle into the right cerebral hemisphere of six anaesthetized 6-week-old BALB/cByJ male mice. The Spearman-Karber method was used to calculate the average lethal dose for 50% of the population (LD₅₀)³¹. Only mice dying between the 2nd and 14th day after inoculation were used in the calculations.

* Significantly different from animals inoculated with wild-type virus; P < 0.02, Student's *t*-test.

Table 2 LD₅₀ of vaccinia wild-type and derivative viruses in BALB/cByJ male mice by i.p. inoculation

Virus strain	Virus dose*	Mortality	PFU inoculum at LD ₅₀
WR-wild type†	10 ⁹	9/10	1 × 10 ⁸ ± 1.1
	10 ⁸	7/12	
	10 ⁷	0/12	
WR-vInf1	10 ⁹	0/6	>1 × 10 ⁹
	10 ⁸	0/6	
	10 ⁷	0/6	
WR-TK11	10 ⁹	0/3	>1 × 10 ⁹
	10 ⁸	0/6	
	10 ⁷	0/5	

* Serial 10-fold dilutions of virus (100 µl) were injected i.p. into 6-week-old BALB/cByJ male mice. The LD₅₀ was calculated as described in Table 1 legend.

† Results of two experiments.

and Wyeth-WT viruses and TK⁻ recombinants, a direct comparison of virus infectivity in spleen and liver was carried out. The level of TK⁻ recombinant virus infectivity in spleen and liver as compared with that of WT virus at various times following i.p. inoculation is depicted in Table 3. In three independent experiments, TK⁻ recombinant vInf1, v52 and v55 viruses yielded lower levels of infectivity in spleen and liver tissue than the corresponding WT virus. This difference in infectivity levels may be best attributed to a decreased spread of recombinant virus and/or to lower rates of replication in target organs. It is of interest that the levels of infectious WR viruses in the spleen and liver were higher than that of the corresponding Wyeth viruses at all time points examined.

Further evidence supporting either decreased dissemination and/or replication of TK⁻ recombinant viruses comes from measuring the levels of vaccinia virus neutralizing antibody. At 21 days after i.p. inoculation of three groups of mice with 1 × 10⁷ PFU of WR-WT, WR-vInf1 and WR-v52, the latter two groups (which received the recombinant viruses) had significantly lower neutralizing titre than the WT group (data not shown). Taken together, the differences between TK⁻ recombinant and WT viruses in LD₅₀ values, virus infectivity in spleen and liver, and neutralizing antibody titres indicate that the TK⁻ recombinant viruses are less virulent than WT by the i.p. as well as the i.c. route of inoculation.

If the vaccinia virus recombinants are to be used as vaccines, it is important that decreased virulence does not occur at the expense of a marked drop in immunogenicity. Since vaccinia virus vaccines are administered by i.d. scarification, it was necessary to evaluate the immunogenicity and virulence of TK⁻

Table 3 Vaccinia wild-type and TK⁻ recombinant virus infectivity in liver and spleen tissue of BALB/cByJ mice by i.p. inoculation

Virus	Expt	log ₁₀ PFU per g tissue on indicated day post-inoculation*							
		1		2		3		6	
		Liver	Spleen	Liver	Spleen	Liver	Spleen	Liver	Spleen
WR-wild type	1	7.57 ± 0.1	9.52 ± 0.1	7.11 ± 0.1	9.41 ± 0.1	7.15 ± 0.2	8.91 ± 0.2	6.30 ± 0.4	7.18 ± 0.6
WR-vInf1	1	5.00†	5.46†	4.26 ± 0.1	6.08 ± 0.2	3.69 ± 1.5	4.11 ± 0.9	ND‡	ND
WR-wild type	2	6.77 ± 0.2	7.65 ± 0.1	5.52 ± 0.3	5.23 ± 0.8	5.30 ± 1.5	5.50 ± 0.20	ND	ND
WR-v52	2	3.53 ± 0.7	5.04 ± 0.4	3.98 ± 0.2	3.95 ± 0.1	ND	3.45 ± 0.1	ND	ND
Wyeth-wild type	3	4.08 ± 0.0	6.30 ± 0.08	ND	4.20 ± 0.01	ND	ND	ND	ND
Wyeth-v55	3	ND	5.04 ± 0.18	ND	ND	ND	ND	ND	ND

* Eight 4–6-week-old male BALB/cByJ mice were inoculated intraperitoneally with the indicated virus at a multiplicity of infection of 5×10^8 in expts 1, and 5×10^7 in expts 2 and 3. At the indicated times post-inoculation, two animals from each group were killed, and virus infectivity present in spleen and liver was quantitated by standard procedures. ND, No virus detected; detection limit log₁₀ 2.85 and 3.00 in liver and spleen, respectively.

† Based on one mouse only.

Table 4 Vaccinia virus neutralizing antibody titres in BALB/cByJ male mice inoculated i.d. with vaccinia wild-type and TK⁻ recombinant viruses

Virus strain	Reciprocal of neutralizing antibody titre at different times post-inoculation*	
	4 weeks	7 weeks
WR-wild type	4,444 ± 1.2 (1,960–10,780)	12,591 ± 1.4 (4,128–20,740)
WR-v52	5,092 ± 1.1 (2,308–8,780)	—†
WR-vHBs4	3,283 ± 1.4 (1,392–8,052)	8,523 ± 1.3 (2,528–41,792)
Wyeth-wild type	2,729 ± 1.5 (812–21,840)	2,957 ± 1.3 (2,148–10,152)
Wyeth-v55	7,834 ± 1.4 (2,215–20,960)	5,577 ± 1.5 (804–11,932)
None	<160	—†

* A group of 6–8 8-week-old male BALB/cByJ mice was inoculated with 10^7 PFU of each of the indicated strains by scarification using 18 strokes of a 25-gauge needle through 10 µl of virus suspension placed on the dorsal surface of the base of the tail²². At 4 and 7 weeks post-inoculation, each mouse was bled from the orbital sinus. Plasma was heat-inactivated at 56 °C for 30 min, and virus neutralization assays were carried out by standard methods³³. The highest dilution of plasma at which a 50% reduction in the number of indicator virus plaques (intracellular band-purified WT virus) was chosen as the assay end-point. The geometric mean and relative standard error was calculated from six to eight neutralizing titres obtained for each inoculation series. Values in parentheses indicate the titre range.

† Not determined.

recombinants by this route. As can be seen from Table 4, TK⁻ recombinant virus derived either from WR or Wyeth strains of vaccinia virus invoked a level of neutralizing antibody similar to that detected in the WT infection (variations of less than fourfold are not significant). Recombinant WR-vHBs4 and Wyeth-v55 contain an identical DNA insert coding for HBsAg, and induced similar levels of anti-vaccinia neutralizing antibody (Table 4) and anti-HBsAg antibody (data not shown). When the amount of infectious WR-WT and TK⁻ recombinant viruses, vInf1 and v52, present at the site of administration was measured directly by plaque assay, comparable levels of virus were detected between 2 and 10 days post-inoculation. By 42 days, both TK⁻ recombinant and WT virus had been cleared from the primary lesion²⁵. The only difference noted between the TK⁻ recombinant and WT viruses was the delayed appearance of the primary lesion in the TK⁻ recombinant inoculations. By this route, both recombinant virus and WT virus have a low level of virulence for mice.

Reversion of TK⁻ to TK⁺ virus, which readily occurs with point mutations, is unlikely when large insertions are made into the body of the tk gene. In agreement with this expectation, no revertants to TK⁺ virus have been detected (by screening for virus plaques in hypoxanthine/aminopterin/thymidine medium⁴) either after extensive tissue culture passage or in tail tissue isolated from BALB/cByJ mice 8 days after tail scarification with Wyeth-v55. In addition, those recombinants used in this study stably express the foreign genes, as judged by the observation that all screened plaques bind antibody to expressed gene product^{10,12}.

Thus, inactivation of the tk gene of vaccinia virus by the insertion of foreign genes dramatically affects the pathogenesis of vaccinia virus by the i.c. and i.p. routes of inoculation in the mouse. However, by i.d. scarification, which is the preferred route for vaccination, TK⁻ recombinant viruses replicate locally to a level similar to that of WT virus. Our results suggest that TK⁻ recombinant virus has decreased ability to disseminate to, and/or replicate in the internal organs and brain. Thus, by the i.d. scarification route of inoculation, the TK⁻ phenotype may alter the virus pathogenesis in such a manner as to reduce the likelihood of vaccinia-associated complications, such as post-vaccinal encephalitis^{26–28}. One group²⁹ has reported that in 23 out of 40 children diagnosed with postvaccinal encephalitis, vaccinia virus could be detected in the blood, cerebrospinal fluid and pharyngeal secretions. Thus, any genetic modifications to the vaccinia virus that reduce its ability to initiate and maintain a viraemia may potentially lessen the frequency of this most undesirable vaccination-associated complication.

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