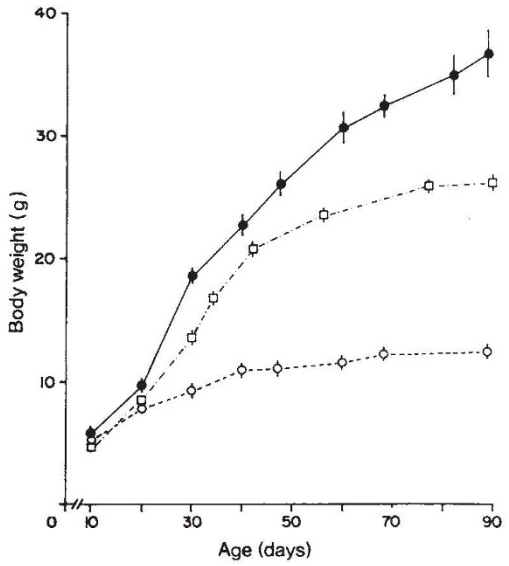

Fig. 2 Growth curves of MT–rGH *lit/lit* mice, nontransgenic littermates and control C57Bl/6J mice. One litter (eight pups) from the F_2 generation of 54-1 (see Fig. 1) was marked 1 week after birth and their weights were recorded periodically. Hybridization of tail DNA with a 0.38-kb *Xho*I–*Pvu*II *rGH* probe revealed five offspring inherited the *MT–rGH* gene (●) and three did not (○). □, Weights of C57Bl/6J mice. Mean body weights ± s.e.m. are indicated at each point.

normal with 11 out of 12 siring multiple litters. In contrast, the fecundity of F_0 transgenic females was dramatically reduced: only 1 of 10 of the *MT–GH* females bred was fertile. Thus whereas excess GH in transgenic mutant males improves fertility and has a negligible effect on the fertility of other *MT–GH* males, it dramatically reduces the fertility of both transgenic mutant and +/+ females. The mechanisms by which GH influences fertility are unexplored.

Several different *Drosophila* null mutants have been corrected recently by introducing wild-type gene counterparts into the germ line by their injection as part of P-element vectors into mutant eggs[14–16]. In the case described here, the primary defect is unknown. The *lit* mutation probably affects the regulation of expression of *GH* gene rather than gene itself[2,3]. Therefore, we attempted to correct the phenotypic defect in GH production by supplying a *GH* gene attached to an alternate promoter so that it would be independent of normal *GH* regulation. Although this approach has remedied the diminutive size of *lit/lit* mice as well as improved the fertility of males, we have uncovered an unsuspected deleterious effect of chronic elevated GH production on female fertility.

Although it seems feasible to correct certain genetic defects in animals, the application of the present technology to humans is inappropriate for several practical reasons. First, the techniques are inefficient; only about 1% of the injected eggs develop into mice that express the gene. Second, because the integration site of the foreign DNA is unpredictable, it is not possible to replace mutant DNA sequences with normal sequences. Thus the mutant gene will not be eliminated from the gene pool and will probably segregate independently of the foreign gene in the next generation. Furthermore, the unpredictability of integration means that it is possible that the foreign DNA could disrupt a normal gene, thereby creating a new mutation[10,17,18]. Current estimates are that about 20% of transgenic mice harbour recessive mutations[10,17]. These considerations, coupled with the fact that for most genetic diseases only one of four offspring are at risk, argue against application to humans.

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Live recombinant vaccinia virus protects chimpanzees against hepatitis B

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Hepatitis B virus (HBV) is an important human pathogen responsible for over 200 million cases of chronic infection, many of which progress to hepatocellular carcinoma. Although HBV cannot be propagated in tissue culture, highly effective subunit vaccines obtained from the plasma of chronically infected patients have been developed and licensed[1–3]. Such vaccines are safe but their expense and limited quantities make them unavailable to most Third World countries. Other approaches to vaccine construction, including purification of the HBV surface antigen (HBsAg) from genetically engineered eukaryotic cells[4–13] and the synthesis of peptides predicted from the nucleotide sequence of the HBsAg gene[14–19], are still under evaluation. Another potential application of recombinant DNA technology to vaccine development is the use of live virus vectors to express foreign genes[20–25]. An infectious vaccinia virus recombinant that expressed the HBsAg in animal cells and which stimulated the production of antibody to HBsAg (anti-HBs) in rabbits represented a novel candidate vaccine of this class[22,23]. As a continuation of our earlier study, we now present evidence that chimpanzees vaccinated with a live recombinant vaccinia virus were protected against hepatitis following challenge with HBV.

The construction of vHBs4, the recombinant vaccinia virus used for these experiments, has been described elsewhere[22]. Briefly, a 1,350-base pair DNA fragment of HBV strain *adw*, containing the entire gene coding for HBsAg, was fused to an early vaccinia virus promoter and inserted into the coding sequence of the vaccinia virus thymidine kinase (*tk*) gene. The chimaeric gene was inserted into vaccinia virus by homologous recombination *in vivo* and infectious TK^- recombinant virus was isolated.

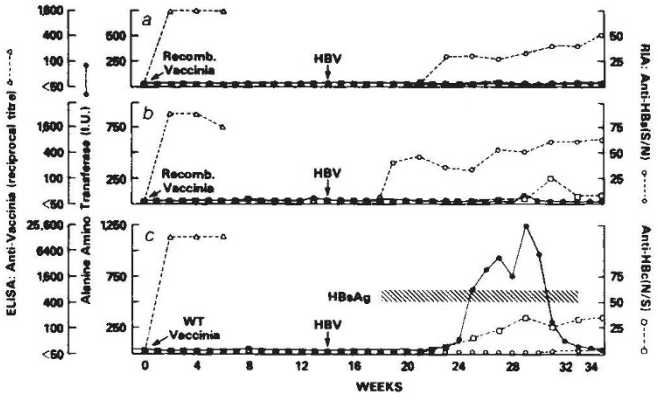

Fig. 1 Response of chimpanzees to intradermal vaccination with live recombianant virus vHBs4 or wild-type vaccinia virus, followed by challenge with live HBV. Chimpanzee 66 (*a*) and chimpanzee 67 (*b*) received recombinant vaccinia virus; chimpanzee A-98 (*c*) received wild-type vaccinia virus. Alanine aminotransferase activity is expressed in $IU\,l^{-1}$. Antibodies to vaccinia are expressed as reciprocal dilutions; anti-HBs and anti-HBc are expressed as the ratio of sample c.p.m. to negative control c.p.m. (S/N) and negative control c.p.m. to sample c.p.m. (N/S) respectively. Positive HBsAg values (>2.1 P/N) are shown as cross-hatching; between weeks 19 and 32, chimpanzee A-98 had P/N values ranging from 56 to 155.

Three chimpanzees (*Pan troglodytes*) weighing 14–25 kg were housed and maintained as described previously[26]. None showed serological evidence of prior exposure to HBV, as determined with commercial radioimmunoassays for HBV markers (HBsAg, AUSRIA; anti-HBs, AUSAB; anti-HB core antigen, anti-HBc, CORAB; anti-HBc IgM, CORZYME-M, Abbott Laboratories). Also, the animals had no other evidence of hepatitis, as judged by normal levels of alanine aminotransferase, aspartate aminotransferase and isocitric dehydrogenase enzymes.

To test the ability of vaccinia virus recombinant vHBs4 to protect chimpanzees against hepatitis, 10^8 plaque forming units (PFU) in 0.1 ml were administered intradermally in the upper back of two animals (66 and 67). A third chimpanzee (A-98) was vaccinated with 10^8 PFU of wild-type vaccinia virus. All three chimpanzees developed primary vaccinia lesions at the sites of vaccination. The two chimpanzees vaccinated with the recombinant virus had dermal lesions of diameters 24 and 22 mm, respectively. The chimpanzee vaccinated with the wild-type vaccinia developed a larger primary lesion of 37 mm diameter. A secondary vaccinia lesion developed in the right axilla of this animal. The greater severity of infection in the chimpanzee receiving wild-type virus, compared with that of chimpanzees receiving recombinant virus, was also reflected in a four- to eight-fold higher antibody titre to vaccinia virus when tested by enzyme-linked immunosorbent assay (ELISA)[27] (Fig. 1). Correlation of the TK^- phenotype of recombinant vaccinia virus with attenuated pathogenecity will be presented elsewhere.

Following vaccination, weekly serum samples were obtained from the three chimpanzees and tested for biochemical evidence of liver disease and serological evidence of exposure to hepatitis B viral antigens as described above. All sera were negative for elevations of liver enzyme activity and for evidence of exposure to HBV antigen except for one weak positive but repeatable 2.5 P/N value (ratio of positive c.p.m. to negative control c.p.m.) for anti-HBs 8 weeks after vaccination of chimpanzee 67 (Fig. 1).

Fourteen weeks after vaccination, all three animals were challenged intravenously with $10^{3.5}$ chimpanzee ID_{50} units of live HBV (subtype *ayw*, strain MS-2), a dose and strain of HBV which has consistently produced hepatitis B in chimpanzees[26,28]. Figure 1 shows that chimpanzee A-98 (vaccinated with wild-type vaccinia) developed typical hepatitis B. Circulating HBsAg appeared 4 weeks after HBV challenge and reached a peak P/N value of 155. Anti-HBc and biochemical evidence of hepatitis occurred after 8 and 9 weeks, respectively. HBsAg levels declined and disappeared by 19 weeks after challenge, coincident with the appearance of anti-HBs.

In contrast, chimpanzees 66 and 67 (vaccinated with recombinant virus) had no detectable HBsAg or biochemical evidence of hepatitis. Instead, anti-HBs appeared at 4–7 weeks after challenge and rapidly rose to high levels that persisted throughout the experiment. Both chimpanzees developed low levels (3.8–8.8 N/P) of anti-HBc, 21 or 27 weeks post-challenge, indicating that they had experienced inapparent infections with HBV. As a follow-up, the livers of the two chimpanzees inoculated with recombinant vaccinia virus were biopsied at 11 months after HBV challenge. There was no evidence of acute or chronic hepatitis.

Sera from chimpanzees 66 and 67 were analysed for the immunoglobulin class of anti-HBs by sucrose gradient centrifugation followed by radioimmunoassay of the fractions containing IgM or IgG immunoglobulins for anti-HBs activity. Two weeks after detection of anti-HBs in chimpanzees 66 and 67, IgM and IgG anti-HBs activities were present in approximately equal proportions (data not shown). Late convalescent sera (14–17 weeks after seroconversion) contained only IgG anti-HBs. Thus, the immune reaction had features characteristic of both primary and anamnestic responses. Sera obtained from chimpanzees 66 and 67 during the early phase of the immune response (2–6 weeks after seroconversion) were tested by radioimmunoassay[29] for the subtype specificity of the anti-HBs: only anti-*a* was detected.

This study demonstrated that chimpanzees receiving a single intradermal vaccination with a live recombinant vaccinia virus that expresses HBsAg were protected against hepatitis B. Following a challenge with HBV of a heterologous subtype, the vaccinated animals experienced a mild inapparent infection characterized only by seroconversion to anti-HBs and anti-HBc. The marginal primary antibody response of chimpanzees 66 and 67, when compared with the anti-HBs response of rabbits following vaccination with this same recombinant vaccinia virus, is in keeping with the relatively poor response of primates to a single dose of HBsAg in the absence of adjuvants[30]. Although the chimpanzees had little or no circulating anti-HBs after vaccination, they were immunologically 'primed'. Consequently, they showed a brisk and sustained antibody response, presumably due to newly synthesized HBsAg, following challenge with live HBV. The early detection of IgG anti-HBs and the anti-*a* subtype specificity are consistent with the anamnestic nature of the response. Priming of neutralizing antibody production by vaccination of rabbits with synthetic peptides of poliovirus structural protein VP1 has been reported[31], but has not been demonstrated previously for HBV.

In conclusion, these studies indicate the feasibility of using a recombinant vaccinia virus as a live hepatitis B vaccine. Current research is directed towards increasing HBsAg expression by using stronger vaccinia virus promoters.

Since submission of this manuscript, additional vaccinia virus recombinants that express prokaryotic chloramphenicol acetyltransferase[32], herpesvirus glycoprotein D (ref. 33), HBsAg[33] and malaria sporozoite antigen[34] have been described.

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Defect in the generation of light-chain diversity in bursectomized chickens

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The avian bursa of Fabricius has been regarded as a central organ for B-cell development[1,2], but there is controversy about the existence of other sites for differentiation of B cells[3–6]. We have recently shown that chickens surgically bursectomized as early embryos, before the bursal primordium appears, can produce cytoplasmic, surface and serum immunoglobulins of IgM, IgG and IgA classes but are unable to generate specific antibodies in response to antigen[7–9]. We have therefore examined the structure and diversity of immunoglobulins of bursectomized chickens. Analysis of serum IgG revealed normal γ-heavy chains but altered light chains with more basic and less diverse isoelectric points than normal. These light chains may represent germ-line specificities not diversified by somatic mutations. Thus the bursa of Fabricius appears not to be necessary for the production of immunoglobulin molecules as such but to function in the creation and expansion of the antibody repertoire, possibly by providing a microenvironment for somatic mutations.

Chickens were surgically bursectomized after 60 h incubation by cutting off the tail caudally to the leg buds. Bursectomy was ascertained by careful microscopic examination at the autopsy at 10 weeks of age[7,8]. All bursectomized chickens had detectable amounts of serum immunoglobulins; mean concentrations of serum IgM and IgA were normal, whereas that of serum IgG was 1/10 of the control value[9]. In spite of immunoglobulin production, the bursectomized chickens did not respond to repeated immunizations with levan, *Escherichia coli* lipopolysaccharide, tetanus toxoid, dinitrophenyl-bovine serum albumin, brucella, diphtheria, pertussis, salmonella, sheep red blood cells and human γ-globulin[7,9]. Also all the tests made to detect natural antibodies to major histocompatibility complex (MHC) products, rabbit red blood cells, faecal bacteria and phosphorylcholine, and autoantibodies to the kidney, thyroids and liver, were totally negative. Antibodies to bursal structures were not observed[9]. The total lack of inducible or spontaneous antigen-specific antibody responses, given near normal immunoglobulin levels, suggested that the immunoglobulins produced by bursectomized chickens might be abnormal in structure. To examine this possibility, we compared serum IgG of the bursectomized and control chickens at the age of 10 weeks. Serum proteins were electrophoresed in SDS-polyacrylamide gel electrophoresis (PAGE)[10] or electrofocused in thin layer agarose gel[11], and then

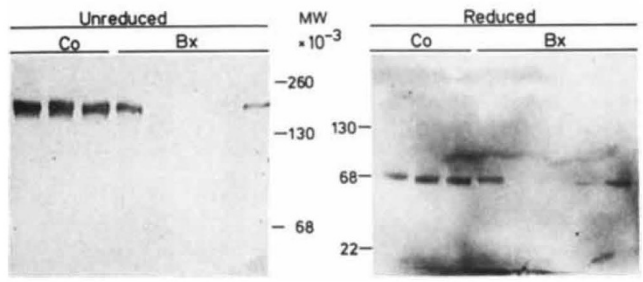

Fig. 1 Molecular weight of serum IgG and γ-chain of the control (Co) and bursectomized (Bx) chickens. Serum samples (1 μl) were examined in non-reducing (left) or reducing (right) conditions by SDS–PAGE[10] by using a 5% homogeneous or 5–22.5% gradient gel, respectively. After electrophoresis, proteins were transferred to a nitrocellulose sheet[12] (HAWP 000 10; Millipore). The sheet was saturated for 1 h with 20% fetal calf serum (FCS) diluted in Tris-buffered 0.9% NaCl (TBS) and then incubated for 2 h with rabbit antiserum against chicken γ-chain[8] diluted in 20% FCS–TBS. After extensive washings with 0.1% Triton-X-100-TBS for 1 h, alkaline phosphatase conjugated pig anti-rabbit IgG (Orion Diagnostica) was added and incubation was continued overnight. After washings with 0.1% Triton-X-100–TBS phosphatase activity on the sheet was visualized with α-naphthyl phosphate as a substrate and 4-amino-diphenylamine diazonium sulphate as a staining dye[11]. Pepsin-digested collagen β- and α-chains of type 1 of molecular weight (MW) 260,000 (260K) and 135K, bovine serum albumin (68K) and trypsin inhibitor (22K) were used as molecular weight standards.

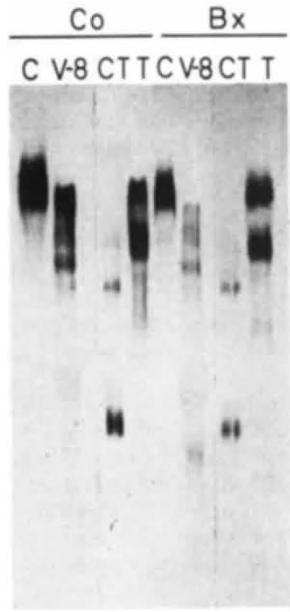

Fig. 2 γ-Chain fragments of one control (Co) and one bursectomized (Bx) bird after enzymatic digestion with *Staphylococcus aureus* V-8 protease (V-8), chymotrypsin (CH) or trypsin (T); control incubation without any enzyme (C). Sodium sulphate precipitated serum immunoglobulins in SDS–buffer were subjected to enzymatic digestion for 2 h at 37 °C according to Cleveland *et al.*[21] All enzymes were used in concentrations of 25 μg ml^{-1}. After digestion proteins were fractionated by using a 15% homogeneous SDS–PAGE. Transfer to a nitrocellulose sheet and identification of γ-chain fragments were performed as described in Fig. 1 legend.

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