A DNA vaccine containing an infectious Marek’s disease virus genome can confer protection against tumorigenic Marek’s disease in chickens

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A DNA vaccine containing the infectious BAC20 clone of serotype 1 Marek’s disease virus (MDV) was tested for its potential to protect against Marek’s disease (MD). Chickens were immunized at 1 day old with BAC20 DNA suspended either in PBS, as calcium phosphate precipitates, incorporated into chitosan nanoparticles, in Escherichia coli DH10B cells, or bound to gold particles for gene-gun delivery. Challenge infection with MDV strain EU1 was performed at 12 days old, and four out of seven birds immunized with BAC20 DNA in saline by the intramuscular route remained free of MD until day 77 after challenge infection. A delay in the development of the disease could be observed in some animals vaccinated with other BAC20 DNA formulations, but clinical MD and tumour formation were evident in all but one bird. Five out of seven animals immunized with the vaccine virus CVI988 were protected against MD, but none out of seven birds survived EU1 challenge infection after injection of negative-control plasmid DNA. In a second animal experiment, five out of 12 chickens immunized with BAC20 DNA and six out of eight birds immunized with virus reconstituted from BAC20 DNA remained free of MD after challenge infection. In contrast, none out of 12 chickens survived challenge infection after immunization with BAC20 DNA lacking the essential gE gene or with gE-negative BAC20 virus. The results suggested that an MDV BAC DNA vaccine has potential to protect chickens against MD, but that in vivo reconstitution of vaccine virus is a prerequisite for protection.

Introduction

Marek’s disease (MD) is a devastating disorder affecting chickens worldwide. It is caused by Marek’s disease virus (MDV), an Alphaherpesvirus, and is characterized by T cell lymphomas, polyneuritis, immunosuppression and, rarely, atherosclerosis (Calnek, 2001). Three MDV serotypes have been identified, out of which only serotype 1 (MDV-1) is pathogenic, whereas MDV serotype 2 (MDV-2) and serotype 3 (herpesvirus of turkeys, HVT) do not cause MD. MDV infection results in the establishment of a latent infection, which is common in Alphaherpesvirinae; however, MDV has unique properties when compared with other members of this virus subfamily because it can cause tumours and integrate its DNA into the host cell genome (Delecluse & Hammerschmidt, 1993; Delecluse et al., 1993).

MD is controlled by immunization, but despite vaccination more and more pathogenic MDV strains have evolved (Witter, 2001). These strains have been classified as so-called virulent (v), very virulent (vv) or very virulent plus (vv+) strains (Schat et al., 1982; Witter, 1983, 1997). Vaccination using HVT protected chickens against infections with vMDV, but failed to protect against newly emerging vvMDV in the late 1970s.
(Witter, 2001). Vaccines containing MDV-2 strains or combinations of HVT and MDV-2 were used successfully in the 1980s (Witter, 1982, 1997; Calnek et al., 1983; Witter & Lee, 1984), but within a few years novel vv + MDV-1 strains such as 648A and 584A were isolated, which could break bivalent vaccination and cause acute transient paralysis as well as a significant increase in mortality within the first 2 weeks after infection (Witter, 1997; Gimeno et al., 1999). The appearance of vv + MDV has led to the introduction of the CVI988/Rispens vaccine in the USA, which has been used in Europe since the 1970s (Rispens et al., 1972a, b; Witter, 1992; Witter et al., 1995).

The isolation of highly virulent strains from vaccinated flocks has been reported not only in the USA but also in Europe. Changes in clinical signs and cellular tropism were reported for strain C12/130 (Barrow & Venugopal, 1999). The mechanisms underlying the steady increase in MDV virulence and the appearance of a more acute neuronal form of MD remain enigmatic, but imperfect vaccines may have an impact on these phenomena (Witter, 2001). MDV exhibits high cell association in vitro, and vaccines, except for some HVT formulations, represent living chicken cells infected with the agent, which are kept in liquid nitrogen until application (Sharma, 1971). Inactivated and recombinant fowlpox virus vaccines expressing various MDV tegument and envelope proteins have also been tested, and some protection against MD was reported. Oil-adjuvant whole cell preparations (Lee & Witter, 1991; Witter, 2001), and especially gB-expressing fowlpox and baculoviruses (Nazerian et al., 1992, 1996; Niikura et al., 1992), were shown to elicit protective immune responses. It was demonstrated that passive immunization after inoculation of serum of birds immunized with inactivated and oil-adjuvant vaccines prevented MD development to a certain degree (Lee & Witter, 1991). Protection against MD appears to be both humoral and cell-mediated, but based on experiments with MDV and taking into account the situation in other herpesviruses, cell-mediated responses appear to play the important role, at least in long-lived anti-tumour immunity (Schat & Markowski-Grimsrud, 2001).

After the introduction of DNA vaccination, a number of preparations were shown to confer protection against infectious diseases (Robinson & Torres, 1997). This is especially true for infectious diseases of chickens, e.g. influenza virus infections (Fynan et al., 1993, 1995). The DNA vaccines contain individual genes of an infectious agent, controlled by relatively strong viral promoters, like the SV40 or human cytomegalovirus (HCMV) immediate-early promoter (Fynan et al., 1993, 1995). Based on a technique that allows cloning and maintenance of large herpesvirus genomes as infectious bacterial artificial chromosomes (BAC) in Escherichia coli (Messerle et al., 1997), an infectious MDV clone (BAC20) was generated by our laboratory (Schumacher et al., 2000). BACs not only allow efficient analysis of individual open reading frames and their effect on virus replication, but may represent a new form of vaccine that combines the advantages of DNA and modified live virus preparations, because vaccine virus can be reconstituted in vivo after administration of infectious DNA (Suter et al., 1999). The results of this study demonstrate that MDV BAC20 DNA applied intramuscularly (i.m.) in saline was able to protect 42–56% of conventional chickens against tumorigenic MD, whereas a replication-deficient gE-negative BAC20 did not induce protection against the disease.

### Methods

- **Animals.** MDV antibody-negative white Lohmann selected leg-horns (LSL) (Lohmann) were used and wing-banded on the day of hatch. Birds were kept on the floor and received food and water ad libitum.

- **Cells and viruses.** The MDV strains used were CVI988 (MarekVac forte, Lohmann), 584Ap80C (Schumacher et al., 2000) and the highly virulent European isolate, EU1, which was isolated from a CVI988-vaccinated flock in Italy and induces MD in approximately 20% of CVI988-vaccinated chickens (Schumacher et al., 2002). Strain 584Ap80C was reconstituted from the infectious BAC20 DNA clone (Schumacher et al., 2000) and is referred to as BAC20 virus (Fig. 1A). BAC20 virus was propagated in chicken embryo cells (CECs) and gE-negative BAC20 virus (20AgE) was propagated in the cell line SoLE, which represents quail muscle QM7 cells constitutively expressing gE (Schumacher et al., 2002).

- **Vaccination experiments.** BAC20 DNA was isolated from E. coli strain DH10B by column chromatography (Qiagen). One preparation used for vaccination was BAC20 DNA diluted in PBS (Sambrook et al., 1989). Calcium phosphate precipitates were generated as described previously (Morgan et al., 1990; Schumacher et al., 2000). DNA-containing nanoparticles were prepared by diluting 100 µg of BAC20 DNA in 1 ml of 50 mM Na₂SO₄ (pH 5.7). The solution was heated at 55 °C and 1 ml of 0.02% chitosan in 25 mM sodium acetate (pH 5.7) was added while vortexing for 1 min (Roy et al., 1999). Chickens were randomly allocated to groups. Immunization at 1 day old was carried out with 10 µg of BAC20 DNA in various formulations and by different routes (Fig. 1B). For i.m. immunizations (200 µl per animal), a 12 mm long 20-gauge sterile needle was used. Intradermal (i.d.) immunizations were carried out using a gene gun (Helios, BioRad) after adsorption of 10 µg BAC20 DNA to 1 mg of gold particles (1.0 μm), according to the supplier’s instructions. Some birds were given i.m. injections of 1 × 10⁴ or 1 × 10⁵ colony-forming units of BAC20-containing DH10B cells. Control groups consisted of animals immunized i.m. with 1 × 10⁶ p.f.u. of CVI988 (positive control group) and chickens immunized with 10 µg of plasmid pDS-pHAl in PBS (Schumacher et al., 2000; Adler et al., 2000) by the i.m. route (negative control group). One group consisted of contact control birds, which were kept together with CVI988-vaccinated birds from day 1. Challenge infection (day 12) was carried out by i.m. injection of 5 × 10⁶ buffy coat cells from the blood and spleens of EU1-infected birds suspended in 200 µl cell culture medium per animal (Fig. 1B). From the day of challenge infection, birds of all groups were kept in one room. The dose of challenge EU1 virus contained 2.5 × 10⁷ infectious units as determined by co-seeding of PBMCs with CECs and the determination of infected cells by indirect immunofluorescence using monoclonal antibody (mAb) 2K11, which is directed against MDV-1 gB (Schumacher et al., 2000).

- **Statistical analyses.** The number of animals per group was calculated using nQuery Advisor (Statistical Solutions), and statistical analyses were carried out using sas, version 9.2 (SAS Institute). As the primary end point, differences of protection against challenge infection...
between vaccination groups were analysed using Fisher’s exact test (Stokes et al., 2000). Protective potentials of individual DNA preparations and survival functions of individual groups were also compared (secondary end points). The statistical model applied for the comparative analyses was a calculation of contrasts of the survival rates (Stokes et al., 2000). Comparisons of survival functions were carried out using the Log–Rank and Wilcoxon tests (SAS Institute Inc., 1984). Lastly, medians of survival times including the 95% confidence intervals were calculated.

■ Analysis of blood samples. Blood (500 µl in 100 µl of 2% sodium citrate) was collected on various days (Fig. 1B). Blood samples of two animals each were pooled and PBMCs and plasma were isolated. One aliquot of PBMCs was used to isolate DNA (Qiagen) and the other aliquot was used to infect CECs. Purified total DNA was eluted with 100 µl H2O and 10 µl each were used in two independent PCR assays. One PCR assay targeted the MDV gB gene (Lee et al., 2000; Tulman et al., 2000), and 100 pmol each of forward (5'-GCAATACGCGTGTCT-ATC 3') and reverse (5'-AACCAATGGGTCTGCTATAAC 3') primer were mixed with DNA and 35 cycles (95 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s) were run. Specificity of the PCR products was confirmed by Southern blotting using digoxigenin-labelled gB sequences as a probe. The second assay targeted the MDV 132 bp repeats. In the case of avirulent MDV strains, amplification of the repeats results in typical laddering of the PCR products on agarose gels and can be used to identify CECs. Free DNA, and 40 cycles (94 °C for 30 s, 68 °C for 30 s, 72 °C for 120 s) were run after a primary denaturation step at 94 °C for 120 s, with a final extension at 72 °C for 300 s. After addition of 1 x 10⁶ PBMCs to CECs, up to three blind passages were carried out, and MDV antigen in CECs was detected by indirect immunofluorescence using pp38-specific mAb H19 (Cui et al., 1990) or convalescent anti-MDV1 chicken serum, and anti-mouse or anti-chicken IgG AlexaFluor 488 (Molecular Probes) (Schumacher et al., 2000).

MDV-specific antibodies in plasma were determined by ELISA exactly as previously described (Schumacher et al., 2002). Total chicken IgG titres in plasma were determined by diluting plasma samples in log₂ steps in 96-well plates, which were then incubated for 16 h at 4 °C. Free binding sites on plates were blocked with 2% skimmed milk in PBS containing 0.05% Tween 20 (PBS-T) for 30 min at 20 °C. After three washes with PBS-T, anti-chicken IgG peroxidase conjugate (Sigma) was added and end-point titres were determined after addition of tetramethylbenzidine substrate and stopping the reaction using 2 M H₂SO₄ (Osterrieder et al., 1995).

Results

Intramuscular immunization with BAC20 DNA in PBS leads to a transient absence of MDV DNA in PBMCs after challenge infection

After two preliminary animal trials, a first comprehensive animal experiment was conducted. Groups of seven to nine animals were formed based on the results of the previous pilot experiments and assuming that: (i) less than 10% of control-vaccinated animals would survive EU1 challenge infection; (ii) greater than 70% would survive EU1 challenge infection after vaccination with CVI988; and (iii) 0–70% of immunized chickens in the various groups would survive EU1 challenge infection. The pilot experiments had suggested that from all routes tested (i.e. the oculonasal, the in ovo, the intracutaneous and the i.m. route), the i.m. route could lead to protection against challenge EU1 infection in vaccinated animals. Therefore, BAC20 DNA was applied by the i.m. route using various delivery systems.

The systemic presence of MDV DNA after vaccination and challenge infection was investigated by isolation of total DNA
### Table 1. Animal groups: routes of immunization and outcome of challenge infection (experiment 1)

<table>
<thead>
<tr>
<th>Group (no. of animals)</th>
<th>Administered vaccine/formulation</th>
<th>Viraemia after immunization (PCR/virus isolation)*</th>
<th>Viraemia after challenge infection (PCR)</th>
<th>Early mortality†</th>
<th>Median time to death (95 % confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (9)</td>
<td>BAC20 DNA/gene gun</td>
<td>Negative/negative</td>
<td>Positive</td>
<td>2</td>
<td>54 days (43;57)</td>
</tr>
<tr>
<td>2 (7)</td>
<td>BAC20 DNA/nanoparticles</td>
<td>Negative/negative</td>
<td>Positive‡</td>
<td>2</td>
<td>49 days (11;55)</td>
</tr>
<tr>
<td>3 (7)</td>
<td>BAC20 DNA/calcium phosphate</td>
<td>Negative/negative</td>
<td>Positive</td>
<td>1</td>
<td>57 days (51;77)</td>
</tr>
<tr>
<td>4 (7)</td>
<td>BAC20 DNA/PBS</td>
<td>Positive(day 8)/negative</td>
<td>Negative days 0–6</td>
<td>0</td>
<td>69 days (55;69)§</td>
</tr>
<tr>
<td>5 (7)</td>
<td>BAC20 DNA/E. coli</td>
<td>Negative/negative</td>
<td>Positive</td>
<td>0</td>
<td>56 days (51;77)</td>
</tr>
<tr>
<td>6 (7)</td>
<td>BAC20 DNA/E. coli</td>
<td>Negative/negative</td>
<td>Positive</td>
<td>0</td>
<td>61 days (57;65)§</td>
</tr>
<tr>
<td>7 (7)</td>
<td>CVI988/diluent</td>
<td>Positive/positive(day 8,10)</td>
<td>Negative days 0–6</td>
<td>0</td>
<td>61 days (57;65)§</td>
</tr>
<tr>
<td>8 (7)</td>
<td>pDS-pHA1 DNA/PBS</td>
<td>Negative/negative</td>
<td>Positive</td>
<td>0</td>
<td>54 days (52;57)§</td>
</tr>
<tr>
<td>9 (7)</td>
<td>None</td>
<td>Negative/negative</td>
<td>Positive</td>
<td>0</td>
<td>44 days (42;57)§</td>
</tr>
</tbody>
</table>

* PCR assays targeted the gB gene and the 132 bp repeat region (see Methods). A negative result in virus isolation means that no MDV was detected by indirect immunofluorescence after three blind passages on CECs.
† Deaths during the first 14 days after EU1 infection.
‡ Virus isolation was negative after challenge infection in all cases except for one positive virus isolation on day 9 in group 2.
§ Censored calculations because individual chickens of groups 4, 6 and 7 survived. Medians were determined for deceased birds only.

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Fig. 2. (A) Scanned digital image of a 1% agarose gel with separated gB-specific PCR products obtained from PBMC DNA isolated on days 3 and 6 after EU1 challenge infection (days 15 and 18 post-vaccination). The 525 bp PCR product is marked by an arrowhead. Lanes correspond to individual immunization groups (Table 1). The negative-control DNA (−) was from an irrelevant blood sample and the positive control (+) was 0.01 ng of BAC20 DNA. (B) Scanned digital image of a Southern blot of the PCR products shown in (A). PCR products were transferred to nylon membranes and probed with a gB-specific probe (Schumacher et al., 2000). Amplification products could be detected in all groups on day 3 and 6 after challenge except for groups 4 (BAC20 in PBS by the i.m. route) and group 7 (CVI988).
Intramuscular immunization with BAC20 DNA in PBS results in sustained production of MDV-specific antibodies after challenge infection

Total IgG and MDV-specific antibodies were determined by ELISA after BAC20 DNA immunization and challenge infection with strain EU1. MDV-specific plasma antibodies were undetectable (< 1:100) in all groups after immunization (Fig. 3). After challenge infection, MDV-specific antibodies were detectable in all groups from day 6 or 9 after infection (Fig. 3). In animals who had received BAC20 DNA in PBS by the i.m. route and the CVI988-vaccinated birds, plasma titres of MDV-specific antibodies gradually increased until day 29 after infection, when titres of 1:25 600 and 1:51 200, respectively, were determined (Fig. 3). In contrast, MDV antibody titres decreased in all other animals from day 16 (Fig. 3). In addition to the reduction of MDV-specific antibodies, an up to 10-fold reduction of total plasma IgG was observed in groups of animals that succumbed to the disease but not in those in which protection against MD was observed (BAC20 DNA in PBS and CVI988) (Fig. 3). From these results we concluded that challenge infection with the highly virulent EU1 strain caused a failure of the B cell immune response from very early times after challenge infection, which led to a generalized reduction of total plasma IgG levels and to an absence of MDV-specific antibodies.

Intramuscular immunization with BAC20 DNA in PBS protects against MD at levels comparable with conventional vaccines

Previous experiments had demonstrated that MDV strain EU1 causes lethal MD in 100% of unvaccinated LSL chickens.

from PBMCs and subsequent PCR analysis. Blood from two birds of each group were collected on the indicated days after immunization and challenge (Fig. 1B) and pooled for further analysis. MDV-specific DNA could be detected after i.m. vaccination of BAC20 DNA in PBS on day 8 after vaccination (Table 1; group 4). Reconstitution of MDV from BAC20 DNA by virus isolation from PBMCs, however, could not be demonstrated (Table 1). We were able, however, to identify amplification of the 132 bp repeats, which is specific for avirulent vaccine MDV strains, in some blood samples after BAC20 DNA application using a PCR assay targeting the repeat region (Table 1). These results suggested that vaccine virus was reconstituted in vivo from cloned DNA. After immunization with CVI988, DNA and infectious virus were detected in some vaccinated birds on days 8 and 10 after immunization (Table 1). After EU1 challenge infection, MDV-specific DNA was detected on days 3 and 6 after challenge in all groups, with the exception of group 4 (BAC20 DNA in PBS by the i.m. route) and group 7 (CVI988) (Fig. 2; Table 1). In PBMCs from chickens of these groups, no viral DNA was detected up to day 6 after EU1 challenge infection by the very sensitive gB-specific PCR and Southern blot analysis (Fig. 2, Table 1). From day 9 after infection, MDV-specific DNA could be amplified from PBMCs of all groups (Table 1). In animals immunized with BAC20 DNA incorporated into nanoparticles by the i.m. route (group 2), infectious MDV was isolated at day 9 after challenge infection. The isolated virus induced large plaques in CEC cultures, which were reactive with the H19 and the MDVI antibody (data not shown). We concluded that this isolate represented reconstituted BAC20 and not EU1 virus because it grew readily in CECs. Contamination with the CVI988 strain was excluded by reactivity with anti-pp38 antibody H19, which does not recognize the CVI988 strain (Cui et al., 1990, 1999).
Pilot vaccination trials had also shown that: (i) BAC20 virus-infected CECs (1 × 10^3 p.f.u.) were able to protect against MD with an efficacy that was comparable with that of CVI988 vaccination; and (ii) BAC20 DNA in PBS has potential to protect against MD after i.m. application.

Animals were vaccinated and monitored for the development of MD for 11 weeks after challenge infection before the experiment was terminated and all surviving birds were killed and scanned by gross pathological examination for MD and/or tumour development. Starting at day 7 after challenge infection, approximately 25% of chickens suffered from transient paralysis. More than 50% of the birds recovered from the paralysis, and early MD mortality (death within 2 weeks after challenge infection), which was associated with atrophy of the thymus and the bursa of Fabricius, was observed in approximately 10% of chickens (Table 1; Fig. 4). None of the CVI988-immunized animals or birds having received BAC20 DNA in PBS by the i.m. route suffered from acute transient paralysis. More than 50% of the birds recovered from the paralysis, and early MD mortality (death within 2 weeks after challenge infection), which was associated with atrophy of the thymus and the bursa of Fabricius, was observed in approximately 10% of chickens (Table 1; Fig. 4). None of the CVI988-immunized animals or birds having received BAC20 DNA in PBS by the i.m. route suffered from acute transient paralysis (Table 1; Fig. 4). Starting at 3 weeks and culminating in weeks 7 and 8 after EU1 challenge infection, chickens were found to be dying from MD as evidenced by severe atrophy of the thymus and bursa of Fabricius, oedematous swelling of peripheral nerves such as the N. ischiadicus, the N. vagus and the Plexus brachialis, and especially tumours of visceral organs and the lymphatic extraorbital tissue (Table 1; Fig. 4). The tumours were confirmed to be caused by MDV by histopathology and in situ hybridization (data not shown). All animals vaccinated with the negative control plasmid pDS-pHA1 and the CVI988 contact birds died within 57 days of challenge infection (Table 1; Fig. 4). Most of the animals vaccinated with BAC20 DNA also suffered and died of MD with similar kinetics as the negative controls, but all birds vaccinated with BAC20 DNA in PBS by the i.m. route and all CVI988-vaccinated chickens survived until day 57 after infection (Table 1; Fig. 4). Four out of seven animals of group 4 (BAC20 DNA in PBS by the i.m. route) and five out of seven birds of group 7 (CVI988) were protected against MD until termination of the experiment, and gross pathology of surviving animals did not identify any other individual in these two groups as suffering from the disease (Table 1; Fig. 4).

Statistical analyses using Fisher’s exact test revealed that the hypothesis of identical survival rates between individual DNA immunization groups had to be rejected (primary end point: \( P < 0.001 \)). Because group 7 (CVI988-vaccinated animals) was excluded from this calculation, the results indicated that at least one of the DNA formulations was able to significantly protect chickens against challenge infection. Analyses of secondary end points showed that group 4 (BAC20 in PBS by the i.m. route) as well as group 7 (CVI988) were highly statistically significantly different \( (P < 0.001) \) from all other groups concerning the number of surviving birds at the end of the experiment using the contrast model. Log–Rank and Wilcoxon tests demonstrated that the survival functions of group 4 \( (P < 0.05) \) and group 7 \( (P < 0.001) \) were significantly different from the negative control groups 8 (pDS-pHA1 in PBS by the i.m. route) and 9 (CVI988 contact birds). No significant differences, however, between survival rates of groups 4 and 7 could be determined. Median times until deaths of animals ranged from 44 to 69 days (Fig. 4; Table 1). It should be noted that in group 6 (BAC20 in E. coli), one out of three animals remained free of MD after EU1 challenge infection (Table 1). In groups 5 and 6 (Table 1; BAC20 in E. coli), two and four animals had died shortly after immunization, respectively, although high resistance against systemic application of E. coli to chickens had been observed in preliminary trials and was also previously reported (Adler & DaMassa, 1979). In addition, in group 3 (BAC20 DNA as calcium phosphate precipitates by the i.m. route) two out of seven animals survived until the end of the experiment (Table 1). These two animals, however, exhibited tumours on post-mortem examination on day 77 after infection, and no significant differences in the survival functions of either group 3 or 6 when compared with the negative control groups 8 and 9 were found. It should be noted that the same findings (i.e. no difference between immunization groups and the negative control groups) was obtained after application of BAC20 DNA in the different formulations by the in ovo and oculonasal routes (data not shown).

**BAC-based vaccination requires inoculation of viral DNA that results in infectious MDV-1**

In a second animal experiment, the question was addressed of whether protection against MDV challenge can also be
Table 2. Comparison of protection after i.m. application of BAC20 and 20ΔgE (experiment 2)

<table>
<thead>
<tr>
<th>Group (no. of animals)</th>
<th>Administered vaccine/formulation</th>
<th>Viraemia after immunization (positive/total)*</th>
<th>Viraemia after challenge (positive/total)†</th>
<th>Early mortality‡</th>
<th>No. (%) of protected birds§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (12)</td>
<td>BAC20 DNA/PBS</td>
<td>3/6</td>
<td>4/6</td>
<td>2</td>
<td>5/12 (42 %)</td>
</tr>
<tr>
<td>2 (12)</td>
<td>20ΔgE DNA/PBS</td>
<td>0/6</td>
<td>0/6</td>
<td>1</td>
<td>0/12 (0 %)</td>
</tr>
<tr>
<td>3 (8)</td>
<td>pDS-pHA1/PBS</td>
<td>0/4</td>
<td>4/4</td>
<td>3</td>
<td>0/8 (0 %)</td>
</tr>
<tr>
<td>4 (8)</td>
<td>BAC20 virus (1×10⁶, CECs)</td>
<td>4/4</td>
<td>1/4</td>
<td>0</td>
<td>0/6 (75 %)</td>
</tr>
<tr>
<td>5 (12)</td>
<td>20ΔgE virus (1×10⁶, SOgE)</td>
<td>0/12</td>
<td>0/12</td>
<td>0</td>
<td>0/12 (0 %)</td>
</tr>
</tbody>
</table>

* As determined by PCR targeting the gB gene and the 132 bp repeat region (see Methods). Viraemia was detected on days 8 and 10 after immunization.
† As determined by PCR targeting the gB gene on day 3 after challenge infection. From day 6 after challenge infection, all blood samples from all groups were positive.
‡ Deaths during the first 14 days after EU1 infection.
§ Numbers of protected birds reflect numbers of birds surviving until day 48 after challenge infection and did not exhibit any sign of MD at post-mortem examination by gross pathology at day 48 after challenge infection.

induced by DNA of a replication-incompetent MDV. Therefore, groups of eight to 12 animals were immunized i.m. with PBS-suspended BAC20 DNA or 20ΔgE DNA, which lacks the essential gE gene. On transfection of 20ΔgE DNA into CECs or QM7 cells, MDV that is not viable on non-complementing cells is reconstituted (Schumacher et al., 2001). Groups of eight and 12 animals, respectively, were also immunized with 1×10⁶ p.f.u. of BAC20 virus or 1×10⁶ 20ΔgE virus propagated on complementing SOgE cells (Schumacher et al., 2002). The results of this second animal experiment are summarized in Table 2 and Fig. 5. Whereas six out of eight animals immunized with reconstituted BAC20 virus survived EU1 challenge infection and did not exhibit signs of MD at post-mortem examination (Table 2, group 4), none of the 12 animals immunized with replication-incompetent 20ΔgE virus remained free of MD (Table 2, group 5). Similarly, all 12 chickens immunized with 10 µg of 20ΔgE DNA as well as all animals immunized with 10 µg of the negative control BAC pDS-pHA1 died as a consequence of EU1 challenge infection or exhibited the tumour form of MD at post-mortem examination (Table 2, groups 2 and 3). In contrast, five out of 12 chickens vaccinated with 10 µg BAC20 DNA survived EU1 challenge infection and did not exhibit signs of MD as verified by gross pathology (Table 2, group 1). It is important to note that no virus could be isolated from PBMCs of animals immunized with either BAC20 virus or BAC20 DNA (Table 2). However, on days 8 and 10 after immunization, MDV DNA was detected by both the gB-specific and the 132 bp repeat-specific PCR in three pooled blood samples of BAC20 DNA-immunized animals and in all pooled blood samples of chickens immunized with BAC20 virus (Table 2, groups 1 and 4). In contrast, no MDV DNA could be PCR-amplified in animals immunized with either 20ΔgE DNA or the gE-negative MDV (Table 2, groups 2 and 5). After challenge infection, MDV DNA was detectable from day 6 post-infection (Table 2) in all blood samples of all groups. It was absent, however, on day 3 p.i. in the groups vaccinated with BAC20 DNA (two out of six samples) or with BAC20 virus (three out of four samples) (Table 2). As described for the first animal experiment, anti-MDV antibody titres gradually increased in chickens that survived challenge infection (Fig. 5). In animals immunized with BAC20 virus, all four pooled blood samples exhibited MDV-specific ELISA titres of > 1:128 000 at the final bleeding at day 46 after challenge infection (Fig. 5). In BAC20 DNA-immunized animals, four out of the six plasma pools reacted in the same manner, whereas the two remaining plasma pools exhibited titres of < 1:4000 on day 46 after challenge. The
four plasma pools with high titres of anti-MDV antibodies all contained plasma obtained from the five surviving birds (Fig. 5).

In summary, from the two animal experiments it was concluded that BAC20 DNA in PBS was able to at least partially protect against MD induced by the highly virulent EU1 virus after i.m. application to 1-day-old chickens. BAC20 DNA in saline led to significantly better protection rates than any other DNA application form, and induced protection in 42–56% of immunized animals in two independent experiments (total: 9/19), whereas none of the control-immunized animals survived challenge infection (total: 0/15). In addition, the results suggested that reconstitution of replication-competent MDV from injected DNA is a prerequisite for protection and leads to sustained anti-MDV antibody production in immunized and challenge-infected birds.

Discussion

The experiments presented in this study have demonstrated that tumorigenic Marek’s disease may be controlled by i.m. vaccination using BAC20 DNA, which contains an infectious genome of the attenuated MDV strain 584Ap80C. Immunization with BAC20 DNA resulted in sustained production of a humoral anti-MDV immune response for at least 7 weeks in animals surviving challenge infection, an absence of MDV-specific DNA in peripheral blood until day 6 after challenge infection, and partial protection of BAC20 immunized birds against development of MD which ranged from 42 to 56% in two independent animal experiments. In addition, in vivo reconstitution of replication-competent vaccine MDV appears critical for protection against MD, because none of the chickens immunized with BAC DNA lacking the essential gE gene (0/12) or with reconstituted gE-negative virus (0/12) survived EU1 challenge infection.

For a primary assessment of the efficacy of immunization of chickens with BAC20 DNA, various routes of immunization and delivery systems were tested. It could be demonstrated that from all tested routes and formulations, only i.m. application of BAC20 DNA in saline led to protection in four out of seven chickens. In contrast, no significant protection against MD was seen in any of the other DNA-vaccinated groups, although moderate effects on the median times to death and survival functions of immunized and challenge-infected birds were observed. The lack of protection after gene-gun vaccination was unexpected because the potency of i.d. delivery of plasmid DNA in protecting laboratory animals and chickens against lethal virus challenge had been demonstrated (Fynan et al., 1993, 1995). Furthermore, immunization of mice twice with 1·5 µg of a replication-defective herpes simplex virus BAC clone by this route led to virus-specific cytotoxic T cell responses, production of neutralizing antibodies and protection against lethal challenge infection (Suter et al., 1999). The exact mechanisms by which i.m. vaccination with BAC20 DNA in saline caused a significant reduction in tumour formation and incidence of MD after EU1 challenge infection is not known. It is possible that BAC20 DNA in PBS may have been taken up more efficiently by macrophages in muscular tissue and may have replicated in these cells, which are primary targets of MDV (Calnek, 2001). In the muscle, high numbers of macrophages have been demonstrated after injection when compared with other tissues (Davis et al., 1994). Macrophages also attract and activate B and/or T lymphocytes, which themselves are targets of lytic MDV replication (Calnek, 2001). In this respect, it is important to note that only application of BAC DNA of replication-competent MDV led to protection against subsequent challenge, indicating that in vivo replication of the vaccine is important for protection. There was evidence for MDV replication after BAC20 administration, because a PCR assay that was able to discriminate between cloned BAC20 DNA and virus reconstituted from BAC20 gave positive results in some blood samples of chickens immunized with BAC20 DNA. This assay was also able to distinguish between vaccine and virulent virus, because in the latter only a single band and no laddering is observed (Schumacher et al., 2000). In addition, infectious MDV was isolated at day 9 after challenge from PBMCs of animals that had been immunized by the i.m. route with BAC20 incorporated into nanoparticles (group 2). Since we have not been able to isolate EU1 in CECS or chicken kidney cells so far, despite numerous trials, we speculate that latent BAC20 virus was reactivated by EU1 challenge infection in these animals. MDV DNA was also detected by PCR in PBMCs of chickens after i.m. immunization with BAC20 DNA in saline in both animal experiments, but not in birds immunized with 20AgE DNA or reconstituted 20AgE virus (Schumacher et al., 2001). MDV-specific DNA in PBMCs was detected on only two days after CVI988 or BAC20 virus vaccination. Low amounts of infectious virus could be isolated in the case of CVI988 but not after application of BAC20 virus, indicating that levels of viraemia after application of vaccine viruses may be determined by the virus strain used rather than the dose of virus or DNA applied. However, the positive PCR results after application of BAC20 DNA or virus and the sustained antibody response indicate that low levels of lytically replicating and/or latent or reactivated virus are present in the chicken after administration of BAC20 DNA. Future experiments will address virus replication and reactivation in various compartments of vaccinated birds, especially in lymphatic organs (thymus, spleen and bursa of Fabricius) after injection of infectious BAC20 DNA.

The protection of immunized chickens against MDV challenge was shown to be irregularly associated with an absence of MDV DNA in PBMCs on days 3 and 6 after challenge infection. A sustained MDV-specific antibody response with continuously rising titres up to day 46 after challenge infection, however, was observed in all immunized birds that survived EU1 challenge and did not develop MD, as
assessed by gross pathology examination. An up to 10-fold reduction of total IgG was also observed in chickens that were not protected against EU1 challenge infection. In contrast, in chickens immunized with BAC20 in PBS by the i.m. route or avirulent BAC20 or CVI988 virus, total IgG exhibited a moderate increase (up to fourfold) after challenge infection. These results can be interpreted in several ways, the most likely being that uncontrolled infection with MDV strain EU1 caused a depletion of B cells, which represent the major target of lytic MDV replication (Calnek, 2001). It is conceivable that total IgG levels were reduced by lytic replication in and destruction of B cells, especially because plasma IgG levels were decreasing in unprotected birds from day 6 after infection, which corresponds well to the reported half-life of chicken IgG in serum (Yokoyama et al., 1993). It is important to stress that a clear correlation between antibody production and the absence of signs of MD was observed. The fact that BAC20 DNA-immunized birds only survived when high antibody titres were determined after challenge infection, and that none of the animals having received replication-incompetent 20AgE virus or 20AgE DNA was protected strongly suggests that in vivo reconstitution of MDV and replication are imperative for protection. These findings are in contrast to those seen with a replication-incompetent HSV BAC that was able to induce protection in a murine model (Suter et al., 1999).

Important advantages of a DNA-based vaccine over conventional vaccines against MD are the stability and ease of handling of DNA vaccines (Davis & McCluskie, 1999; Krishnan, 2000). MD vaccines consist of infected CECs and have included serotype 1, 2 and 3 strains alone or in various combinations (Witter, 2001). For these reasons it is necessary to maintain the liquid nitrogen cold chain from the vaccine manufacturer to the chicken, and vaccine production cannot easily be standardized because primary CECs have to be used. In future experiments, alternative DNA delivery systems and application routes will be tested in an attempt to combat this important infectious disease of the domestic chicken.

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