JOURNAL OF VIROLOGY, Sept. 1993, p. 5664–5667
0022-538X/93/095664-04$02.00/0
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Vol. 67, No. 9

Bovine Herpesvirus 1: Immune Responses in Mice and Cattle Injected with Plasmid DNA†

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Received 3 May 1993/Accepted 4 June 1993

Mice and cattle injected with plasmids encoding bovine herpesvirus 1 (BHV-1) glycoproteins developed gene-specific antibody responses capable of neutralizing BHV-1. The ability of animals to respond serologically to DNA injections was in part dependent on the quantity of DNA injected and was also negatively affected by carrier DNA. Calves injected with a plasmid encoding BHV-1 gIV developed significant antibody titers to gIV and shed less virus than did the control calf after challenge. This report indicates the potential of DNA injection as a method of vaccination.

Protection from infectious disease has traditionally relied on the use of either attenuated or killed vaccines. However, many such vaccines are inadequate for reasons of efficacy, safety, and cost-effectiveness. Live-attenuated vaccine viruses may be immunosuppressive (4, 10) or cause clinical disease if not attenuated sufficiently (15) or, alternatively, if attenuated to the extent that their ability to generate immunity is limited (13). A major concern regarding the use of live vaccines is the possibility, however remote, of reversion to a more virulent, potentially disease-causing phenotype (17, 26). Killed vaccines are often unable to generate protective levels of immunity for reasons of antigen load and loss of important epitopes during inactivation (22). In addition, they are frequently inconvenient, as repeated immunizations are often necessary to achieve effective levels of immunity. Further, because killed viral vaccines do not provide endogenously synthesized proteins, they are, in general, unable to induce cytotoxic T cells (16), possibly a required component of a truly effective vaccine (12, 19).

While modern molecular methodologies have provided solutions to certain vaccine-related problems, in general they have fallen short of expectations. Subunit vaccines, although safe, are costly and poorly immunogenic. Live recombinant vaccine vectors are effective, but their repeated use in the same host may be limited by vector immunity (7); further, they are subject to reversion events and can cause disease or death in immunocompromised hosts (18). Clearly, the need for better vaccines exists. A new methodology with the potential to address many of the shortcomings of present-day vaccines involves the injection of genes encoding protective antigens directly into the host. First described in 1990 (14, 28), direct gene injection and expression has since been demonstrated with many genes in several tissues and in species as diverse as mice and fish (1, 2, 9, 11, 21, 27, 29). Recently, the potential of DNA injection as a vaccine for influenza in a mouse model was demonstrated (21).

To explore the utility of DNA injection as a means of immunizing against a large animal pathogen, we chose to use glycoprotein genes from the Cooper strain of bovine herpesvirus 1 (BHV-1). BHV-1 is an economically important pathogen of cattle which causes a variety of diseased states, most notably as a contributor to a respiratory disease complex and abortions (31). We have previously demonstrated the protective capacity of BHV-1 glycoprotein I (gI), gIII, and gIV in a bovine challenge model (3, 23, 24); we have also demonstrated that of the three, gIV is the prime subunit vaccine candidate for its ability to induce the greatest levels of neutralizing antibody and abrogate virus shedding (3, 23, 24). Each of these three genes has been expressed in vitro under the control of the Rous sarcoma virus long terminal repeat in plasmids derived from pRSVCAT (8, 20).

Our first objective was to investigate the ability of these plasmids (pRSVgI, pRSVgIII, and pRSVgIV) to induce gene-specific antibody responses in mice. Plasmids were grown in *Escherichia coli* JM105, purified by equilibrium banding in CsCl and ethanol precipitation, and resuspended in 0.85% saline at a concentration of 0.5 mg/ml. Groups of BALB/c (H-2^d) mice were injected with 50 μg of a single plasmid type in the quadriceps muscle and again 4 weeks later. Control groups included mice injected with vector (pRSV0) or with saline. Serum samples were collected from mice every 2 weeks for a 10-week period and tested for protein-specific immunoglobulin G (IgG) by enzyme-linked immunosorbent assay (ELISA), using purified BHV-1 glycoprotein-coated plates as described previously (3) except that the secondary antibody used was horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:2,000.

Each gene was demonstrated to function in vivo by the development of antibody against the corresponding gene product (Table 1). The specificity of the antibody response was further evaluated by immunoblotting (6, 25) using pooled serum samples of each group from weeks 6, 8, and 10 at a 1:25 dilution. Control immunoblots using pooled monoclonal antibodies at a dilution of 1:500 specific for each glycoprotein are shown in Fig. 1A. Immunoblot results supported the ELISA data which showed that the antibody generated in the DNA-injected mice was protein specific. Mice injected with pRSV0 or saline did not at any time contain BHV-1 glycoprotein-specific antibody detectable by ELISA or immunoblotting, and at no time throughout the

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† Published with the permission of the director of the Veterinary Infectious Disease Organization as publication no. 163.
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TABLE 1. Gene-specific antibody responses in mice after plasmid injection[a]

Plasmid	Protein-specific ELISA titer[b] (mean ± SD)				SN titer[c] (mean ± SD)		
	4 wk	6 wk	8 wk	10 wk	6 wk	8 wk	10 wk
pRSV0	0	0	0	0	<4	<4	<4
pRSVgI	200	400	1,600	1,600	<4	16	16
pRSVgIII	200	2,400 ± 1,131	2,400 ± 1,131	1,600	20 ± 17	32	24 ± 11
pRSVgIV	300 ± 141	4,400 ± 3,464	9,067 ± 6,466	6,933 ± 5,619	117 ± 121	181 ± 129	192 ± 111

[a] Mice were injected with 50 μg of plasmid on weeks 0 and 4.
[b] Expressed as the reciprocal of the highest dilution resulting in a reading of 2 standard deviations above the control value. In all cases, values at 0 and 2 weeks were 0.
[c] Expressed as a 50% endpoint, using 100 PFU of BHV-1.

experiment did mice seroconvert to BHV-1 proteins other than to that which was intended by the injections. This result confirmed that the glycoprotein-specific antibody responses resulted from the plasmid injections and not from inadvertent infection with BHV-1 or other cross-reactive herpesviruses. The effectiveness of the gene-specific antibodies to neutralize BHV-1 was determined by a plaque reduction assay as described previously (5). All mice which seroconverted had serum-neutralizing (SN) antibody within 4 weeks of the booster injection (Table 1). pRSVgIV-injected mice developed the highest SN titers, followed by pRSVgIII- and pRSVgI-injected mice. While the level of protein expression for each plasmid type in vivo was not known, the pattern of antigenicity of gIV protein relative to gI and gIII has been

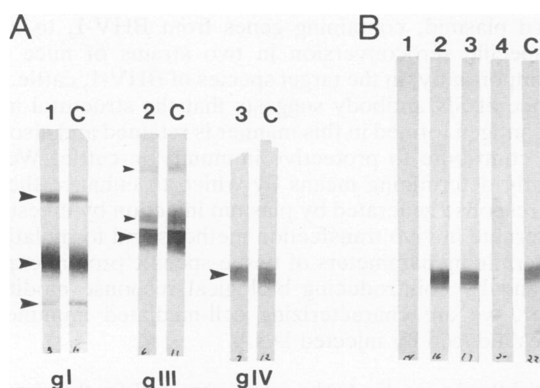

FIG. 1. (A) Immunoblots of sera from mice injected with plasmid DNA encoding BHV-1 gI (lane 1), gIII (lane 2), or gIV (lane 3). Sera from mice seropositive by ELISA were pooled and used at a 1:25 dilution. Pooled monoclonal antibodies against the specific proteins were used at a dilution of 1:500 (lanes C). Immunoblots were developed with horseradish peroxidase-conjugated goat anti-mouse IgG (1:2,000) and 4-chloro-1-naphthol. Arrowheads in lane 1 indicate the forms of gI under reduced conditions. The apparent molecular weights, from top to bottom, are 130,000, 74,000, and 55,000. In lane 2, the large arrowhead indicates gIII protein with an apparent molecular weight of 91,000 and small arrowheads indicate gIII immunoreactive species observed in some immunoblots. The arrowhead in lane 3 indicates gIV protein with an apparent molecular weight of 71,000. (B) Immunoblots of sera from calves injected with pRSVgIV DNA encoding BHV-1 gIV. Calves were injected with either 125 μg of pRSVgIV (lane 1), 500 μg of pRSVgIV (lanes 2 and 3), or vector DNA (lane 4). Sera from DNA-injected calves were used at a dilution of 1:10. Control serum (lane C) was obtained from a calf immunized with affinity purified BHV-1 gIV protein. Immunoblots were developed by using horseradish peroxidase-conjugated rabbit anti-bovine IgG (1:1,000) and 4-chloro-1-naphthol.

observed in animals injected with purified BHV-1 glycoproteins (3, 23). Some pRSVgIV-injected mice, kept for 24 weeks, retained mean ELISA and SN titers of 6,400 and 128, respectively (data not shown). This and other reports suggest that DNA injection can generate long-lived immunity (2, 14, 27).

In a second experiment, our first objective was to determine whether a DNA-mediated immune response could be elicited in a mouse strain other than the strain used in the first experiment. Further, we wished to determine whether the responses were dose dependent and affected by carrier DNA. It was anticipated that the number of transfected cells would be determined in part by the quantity of DNA injected and therefore ultimately be reflected in the level of antibody generated against the protein encoded by the plasmid. In addition, it was anticipated that carrier DNA would increase the effectiveness of transfection and thus lower the amount of plasmid necessary to achieve an immune response. In this experiment, groups of C3H (*H-2*^{k}) mice were injected with various quantities of either pRSVgIV or pRSVgIV DNA and sonicated salmon sperm DNA as carrier to bring the final quantity of DNA injected in each mouse to 100 μg. All injection volumes were normalized with saline to 0.1 ml and injected into the quadriceps muscle at weeks 0 and 6. Control groups were injected with either vector, saline, or carrier DNA. Serum was collected from each mouse every 2 weeks and tested for the presence of gIV-specific IgG by ELISA as indicated above. For clarity, only those groups containing mice which seroconverted are included in the results (Table 2). Mice injected with as little as 25 μg of pRSVgIV seroconverted; however, the number of mice responding and the antibody titers achieved were higher in groups injected with more pRSVgIV DNA. Groups which showed no evidence of seroconversion were those injected with 5 μg of pRSVgIV, 5 μg of pRSVgIV plus 95 μg of carrier, or 25 μg of pRSVgIV plus 75 μg of carrier and all

TABLE 2. Effects of DNA dose and carrier DNA on the antibody responses of mice injected with pRSVgIV[a]

Dose of pRSVgIV (μg)	No. of mice that responded	gIV-specific ELISA titer[b] (mean ± SD)			
		4 wk	6 wk	8 wk	10 wk
100	5	175 ± 50	280 ± 110	800 ± 490	1,360 ± 1,117
50	4	133 ± 58	250 ± 100	450 ± 252	375 ± 310
25	2	0	150 ± 71	250 ± 212	250 ± 212
50 + 50 of carrier DNA	2	0	150 ± 71	300 ± 141	300 ± 141

[a] Five mice were injected on weeks 0 and 6.
[b] Expressed as in Table 1. In all cases, values at 0 and 2 weeks were 0.

TABLE 3. BHV-1 gIV-specific antibody in calves after pRSVgIV injection[a]

Dose	gIV-specific ELISA titer (SN titer)[b]			
	8 wk	12 wk	16 wk	20 wk
500 μg of pRSV0	0 (<4)	0 (<4)	0 (<4)	0 (<4)
125 μg of pRSVgIV	0 (<4)	0 (<4)	100 (<4)	400 (<4)
500 μg of pRSVgIV	200 (8)	200 (4)	200 (8)	800 (16)
500 μg of pRSVgIV	400 (16)	200 (16)	800 (32)	1,600 (32)

[a] Calves were injected on weeks 0, 4, 12, 14, and 16.
[b] Expressed as in Table 1. In all cases, values at 0 and 4 weeks were 0 (<4).

control mice. Only mice injected with 100 and 50 μg of pRSVgIV had detectable levels of antibody which was neutralizing (data not shown). The only mixed-DNA group to seroconvert was the group injected with pRSVgIV and carrier DNA at 50 μg of each (Table 2). The kinetics of the immune response of this group was protracted; this group contained fewer responding mice, all with lower antibody titers, relative to the group injected with 50 μg of pRSVgIV alone. The response of the mixed-DNA groups is clearly not what was expected and, in fact, suggests that sonicated salmon sperm DNA negatively affects in vivo transfection. It is interesting to note the considerable difference in antibody titers between the groups injected with 50 μg of pRSVgIV from each experiment. While many possibilities for this variability exist (29), it is quite likely due in part to the varied injection schedules.

To complete our preliminary investigation, we sought to determine the ability of DNA injection to induce an immune response in cattle. Four calves, seronegative for BHV-1, were used in this experiment; two received 500 μg of pRSVgIV (high dose) at each immunization, one received 125 μg (low dose) at each immunization, and the last received 500 μg of pRSV0 at each immunization as a control. The calves were injected five times over a period of 5 months and monitored for the presence of gIV-specific IgG by ELISA as previously described (3). Table 3 indicates that calves which received the high dose of pRSVgIV produced relatively high ELISA titers of gIV-specific IgG. The specificity of the antibody response was also confirmed by immunoblotting using a 1:10 dilution of serum obtained 4 weeks after the last immunization (Fig. 1B). Serum from a calf immunized with affinity-purified gIV was used at a dilution of 1:100 as a control. Figure 1B shows that the two calves which received a high dose of pRSVgIV seroconverted in a gene-specific fashion, indicating that the gIV-specific antibody was not the result of infection with BHV-1. The calf injected with a low dose of pRSVgIV attained levels of IgG detectable by ELISA but not by immunoblotting. The calf sera were then tested for the ability to neutralize BHV-1 in plaque reduction assays (Table 3). The two high-dose calves exhibited SN titers in the protective range reported for BHV-1 (3, 23, 24), while the low-dose and control calves were without SN antibody. At the end of the experimental period, all four calves were challenged with 10^7 PFU of BHV-1 strain 108, which is antigenically similar to the Cooper strain (30), intranasally by aerosol as previously described (3). The calves were observed for 10 days postchallenge for clinical symptoms, and both serum and nasal secretions were collected for analysis. Clinical symptoms including fever, nasal mucosal lesions, and inappetence were reduced in all pRSVgIV-injected calves compared with the pRSV0-injected calf. Increases in gIV-specific antibody titers were observed at day 6 postchallenge and continued to the end of the experiment (Table 4). It is interesting to note that the low-dose calf, which had only very low ELISA titers prior to challenge, achieved titers equal to those of one of the high-dose calves. By comparison with the titer achieved by the control calf after challenge, it is clear that all three pRSVgIV-injected calves were primed to respond to BHV-1. This conclusion is supported by the similar development of SN antibody over the challenge period (Table 4). Following challenge, there was considerable difference in the amount of virus shed into nasal secretions of the four calves. Plaque assay showed that either no virus or much lower levels of virus were shed for fewer days by pRSVgIV-injected calves than by the control calf (Table 4).

In conclusion, we have demonstrated the ability of an injected plasmid, containing genes from BHV-1, to cause gene-specific seroconversion in two strains of mice and, more importantly, in the target species of BHV-1, cattle. The presence of SN antibody suggests that the structural integrity of antigen formed in this manner is retained and also that it can contribute to protective immunity in cattle. We are presently determining means by which to enhance the immune response generated by plasmid injection by investigating alternate in vivo transfection methods and formulations, by determining parameters of tissue-specific protein expression, and by cointroducing biological response modifiers. Further, we are characterizing cell-mediated immune responses induced by injected DNA.

Special thanks are due to the animal care staff for their involvement throughout this work. The technical assistance of B. Chow, G. Harry, M. Snider, and J. van den Hurk is gratefully acknowledged. Affinity-purified glycoproteins were kindly provided by S. van den

TABLE 4. BHV-1 gIV-specific antibody and virus shedding of pRSVgIV-injected calves after challenge[a]

Dose	gIV-specific ELISA titer (SN titer)[b]						Titer of virus in nasal secretions (log PFU/ml)[c]			
	0[d]	2	4	6	8	10	2	4	6	8
500 μg of pRSV0	0 (<4)	0 (<4)	0 (<4)	400 (4)	400 (16)	800 (32)	3.7	6.5	7.1	4.3
125 μg of pRSVgIV	100 (<4)	100 (<4)	100 (<4)	800 (8)	1,600 (64)	3,200 (128)	2.7	4.8	<1	<1
500 μg of pRSVgIV	400 (8)	200 (8)	400 (8)	1,600 (64)	3,200 (128)	3,200 (256)	2.5	3.9	<1	<1
500 μg of pRSVgIV	800 (16)	400 (16)	400 (16)	1,600 (32)	6,400 (128)	6,400 (512)	<1	<1	<1	<1

[a] Calves were challenged by aerosol exposure to 10^7 PFU of BHV-1.
[b] Expressed as in Table 1.
[c] Determined by plaque assay. In all cases, values at 0 and 10 days were <1.
[d] Day postchallenge.

Hurk. We thank M. Hagen for preparation of the manuscript and S. Tikoo and M. Morsey for helpful advice.

Financial support was provided by the Medical Research Council of Canada.

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