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Induction of anti-viral immune responses by immunization with recombinant-DNA encoded avian coronavirus nucleocapsid protein

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Immune responses to the infectious bronchitis virus (IBV) nucleocapsid protein were studied using a recombinant-DNA expression product. In mice, a lymphocyte proliferative response and a delayed-type hypersensitivity reaction to IBV were induced upon immunization with this nucleocapsid protein. Next, we studied the role of the expressed nucleocapsid protein in induction of a protective immune response to IBV in chickens. Chickens were primed with nucleocapsid protein and subsequently boosted with inactivated IBV, strain M41. Proliferative responses of blood mononuclear cells corresponded with increased mean haemagglutination inhibition and virus neutralization titres. Finally, an increased tracheal protection against challenge with live IBV was observed. These results indicate that infectious bronchitis virus nucleocapsid protein is a relevant target for immune recognition in both the mouse and the chicken.

Keywords: Infectious bronchitis virus; mice; chickens; nucleocapsid; recombinant DNA

INTRODUCTION

Infectious bronchitis virus (IBV) is the prototype of the Coronaviridae. The virus consists of a lipid-containing membrane, a single-stranded RNA genome and three structural proteins. Apart from the internally localized nucleocapsid protein (N), the virus consists of two glycoproteins anchored in the lipid membrane[1,2]. The integral matrix protein (M) protrudes only slightly from the membrane in contrast to the spike protein (S) exposed at the surface which gives the virus its coronaviral image. The virus can cause an acute respiratory disease in young chickens and a reduction in egg production in laying hens. Attention has been focused primarily on responses to the S protein. This was based on the observation that neutralizing antibody showed specificity for the S protein[3]. By generating antigenic variants of the S protein the virus is capable of avoiding elimination by virus neutralizing antibody[4]. These distinct antigenic variations pose a problem in IBV vaccine design[5]. To circumvent the problem of the observed antigenic variability of the S protein we directed our attention to the IBV N protein which is more conserved among IBV strains[1,6]. Observations in other pathogenic virus systems indicate that internal viral antigens can contribute significantly to the induction of protective immunity[7–9]. Protection is not only induced by generating cytotoxic T cells (36), but also by generating T helper cell responses that augment the activity of B cells in production of virus-neutralizing antibody[11–13]. Recently, we have shown that two murine CD4-positive T cell hybridomas generated from an IBV-specific T cell line were responsive to N proteins of several IBV strains[14]. Now the immunogenicity of recombinant-DNA encoded N protein in relation to the cellular immune response to IBV was studied. First it was shown that delayed-type hypersensitivity (DTH) and lymphocyte proliferative responses to IBV were induced upon immunization of mice with the expressed N protein. The purpose of the work described in this paper was secondly, to assess the role of the N protein in induction of cellular immune responses to IBV in the chicken, thirdly to test whether the N protein can accelerate the induction of virus-neutralizing (VN) and haemagglutination-inhibition (HI) antibodies, and finally to ascertain whether priming of chickens with the N protein results in increased tracheal protection against challenge with IBV.

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MATERIALS AND METHODS

Antigens

IBV strain M41 was obtained from egg-grown virus and gradient purified as described[15]. The M42 strain, an IBV laboratory strain, was grown in Vero cells

0264-410X/92/020119-06

(multiplicity of infection 0.1)[16]. Supernatant of infected cells was harvested after 36 h and stored at −80°C.

Expression of the nucleocapsid fusion protein

The IBV nucleocapsid pEX clone was constructed as described by Kusters[17]. Briefly, the DNA encoding the N protein was isolated from the IBV M41 cDNA library and thereafter cut with restriction enzymes and cloned in the expression vector pEX[18]. The recombinant plasmid was expressed in *Escherichia coli*. In this system heterologous expression leads to the synthesis of a C-terminal extension of the cro-beta-galactosidase protein (CGZ). The sequence of the insert was checked by sequencing using the dideoxy termination method. The expressed fusion protein included amino acids 2–405 of the IBV M41 N protein. The protein expressed from pEX11, the vector without insert, contained only the CGZ protein and was used as control.

Induction of IBV-specific T-cell responses in mice

To analyse cellular immune responses to the IBV N protein we used a lymphocyte proliferative assay and a classical DTH assay.

Lymph node cell proliferative assay. Groups of five mice were immunized subcutaneously in the footpad with either 5 μg gradient-purified, inactivated IBV M41, N fusion protein (pXM41-EP) or CGZ (pEX11 control) mixed with 100 μg dimethyl dioctadecyl ammonium bromide (DDA, Kodak). Antigens were injected in 50 μl volumes. At day 7 after immunization mice were killed to obtain the popliteal lymph nodes. Lymph node cell suspensions were prepared in Iscove's modification of Dulbecco's medium containing 1% fetal calf serum (FCS; Gibco, Breda, The Netherlands). Cells were washed and distributed into round-bottomed 96-well microtitration plates in 0.2 ml volumes of Iscove's modification of Dulbecco's medium (Gibco) supplemented with 10% FCS, antibiotics and 2-mercaptoethanol (2×10^{-5} M) containing 10^5 cells. Antigens were added in 0.01 ml volumes to triplicate wells and the plates were incubated at 37°C in a humidified 5% CO_2 atmosphere for 3 and 4 days. [^{3}H]-thymidine (1 μCi/well, 1 Ci mmol^{-1}, Amersham) was added and the incorporated radioactivity was measured 18 h later.

Immunization and assay for DTH. The immunization and DTH assay were performed as described[19]. Briefly, groups of five Balb/c mice were immunized intracutaneously, either with purified inactivated IBV strain M41 (0.3 μg), or with pEX fusion protein (pXM41-EP or pEX11, 0.2 or 5.0 μg). Antigens were diluted in PBS, subsequently mixed with DDA (100 μg/animal) and injected in the vicinity of axillae and groins. Control mice received PBS and DDA. The footpad immunization was followed 6 days later by an injection of antigen in PBS in a 50 μl volume in the left hind footpad. To determine IBV-specific DTH responses, antigen preparations of different sources were used. DTH reactions were measured as the increase in footpad thickness of the left hind footpad between 0, 24 and 48 h. The footpad swelling was measured using an electronic footpad meter.

Induction of IBV-specific immunity in chickens

Immunization. Groups of 20, four-week-old, female White Leghorn chickens, derived from a specified pathogen-free (SPF) flock from Intervet (Boxmeer, The Netherlands) were immunized intramuscularly in the leg with the IBV M41 preparation (formalin inactivated according to standard procedures), the N fusion protein, the CGZ protein or with PBS. All antigens were mixed 1:1 with Freund's complete adjuvant (FCA; Difco). Each chicken received ≈100 μg of protein. All birds were boosted with the formalin-inactivated IBV M41 preparation 6 weeks after the first immunization.

Blood mononuclear cell proliferative assay. Two weeks after primary and secondary immunization, chickens were bled by cardiac puncture and blood mononuclear cells were isolated to perform proliferative assays. A modified procedure as described by Timms[20] was used. Heparinized blood samples were centrifuged three times at low speed (7.5 min at 50*g* at 18–20°C) in glass capillaries containing 0.8 ml heparinized blood. The subsequent plasma layer and white cells were then collected. Lymphoid cells (10^6) were cultured in 150 μl HEPES buffered RPMI 1640 (Dutch modification) supplemented with 1 g l^{-1} $NaHCO_3$ and 200 IU ml^{-1} penicillin and 200 μg ml^{-1} streptomycin in round-bottomed wells of a microtitration plate. Antigens were added in 50 μl volumes to triplicate wells and plates were incubated for 3 days at 41°C in a humidified 5% CO_2 atmosphere. Six hours prior to harvesting, 0.5 μCi [^{3}H]-thymidine (5 Ci mmol^{-1}) was added to the wells.

Humoral immune responses. Serum was collected at 2, 4 and 6 weeks after the first immunization and at 1, 2 and 4 weeks after the booster immunization. Two techniques were applied to analyse anti-S protein antibodies: the haemagglutination inhibition assay (HI)[22,22] and the virus neutralization (VN) assay[23]. For the latter assay the constant virus/diluted serum microneutralization technique on primary chicken embryo kidney cells was applied as described[23] except that the presence or absence of virus in the cultures was assayed by antigen capture ELISA on the culture supernatants[24]. The HI and VN data were analysed using a two-sample *t* test (STATISTIX).

Induction of tracheal protection. From each vaccination group chickens were transferred to separate isolators and challenged by eye-drop administration of 10^5 egg infectious doses (EID_{50}) of the IBV M41 challenge strain (originally supplied by CVL, Weybridge, UK) at 4 weeks after primary immunization and at 4 weeks after secondary immunization. Four days post-challenge the chickens were killed and the tracheae removed. To assess protection of the trachea following challenge with live M41 virus, two methods were used, the ciliostasis assay[25,26] and a more sensitive method to detect viral antigen in tracheal sections by an indirect immunofluorescence test[27] using an IBV M protein-specific monoclonal antibody (obtained from G. Koch, Central Veterinary Institute, Lelystad, The Netherlands). The data were analysed using a linear model of logistic regression (STATISTIX).

RESULTS

Induction of IBV-specific T-cell responses in mice

The immunogenicity of the recombinant-derived N protein in the mouse system was evaluated in the following assays.

Lymph node cell proliferative assay. The induction of proliferative cellular immune responses to IBV by immunization with the N fusion protein was studied. Immunization of mice in the footpad resulted in popliteal lymph node cell proliferative responses to IBV (*Table 1*). IBV-specific proliferation was not observed following immunization with the control protein CGZ.

Assay for DTH. IBV-specific DTH responses peaked at 24–30 h after the second injection (*Table 2*, only 24 h responses are shown). Control mice injected with PBS showed no DTH to IBV M42. Sensitization with M41 induced a DTH response specific for the N protein. No response to CGZ was induced. The reverse experiment showed that mice immunized with the N protein showed DTH responses to IBV, indicating that the N fusion protein induced responses which could be recalled by intact virus.

Induction of IBV-specific immunity in chickens

Subsequently, the immunogenicity of the expressed N protein was analysed in the chicken.

Table 1 Proliferative responses of murine popliteal lymphocytes[a]

Antigen used for immunization	Con A[b] (2.5 μg ml^{-1})	NA	M41[b] (5 μg ml^{-1})	M42[b] (1:50)
M41	8.0[a]	0.1	1.1	4.8
pXM41-EP	10.3	0.1	1.7	3.7
pEX11	8.9	0.3	0.5	0.4

[a]Results are expressed as counts min^{-1} × 10^{-3}. Values represent the mean of triplicate measurements with a standard deviation <30%
[b]Antigens used in proliferative assay: Con A = concanavalin A; NA = background proliferation

Table 2 Detection of delayed-type hypersensitivity to IBV or the IBV N fusion protein

Antigen used for primary immunization	Antigen used for footpad injection	Mean footpad swelling[b] 24 hr after footpad immunization
IBV M41	M42 (1:5)	**6.9 ± 0.6**
PBS	M42 (1:5)	0.7 ± 0.2
IBV M41	Vero sup. (1:5)[a]	0.7 ± 0.2
PBS	Vero sup. (1:5)	0.8 ± 0.3
IBV M41	pXM41-EP (2.5 μg)	**5.3 ± 0.4**
IBV M41	pXM41-EP (10.0 μg)	**2.9 ± 0.4**
IBV M41	pEX11 (2.5 μg)	0.6 ± 0.3
IBV M41	pEX11 (10.0 μg)	0.1 ± 0.3
pXM41-EP (0.2 μg)	M42 (1:5)	**4.0 ± 0.4**
pXM41-EP (5.0 μg)	M42 (1:5)	**4.0 ± 0.9**
pEX11 (0.2 μg)	M42 (1:5)	1.9 ± 0.3
pEX11 (5.0 μg)	M42 (1:5)	1.3 ± 0.3

[a]Vero sup. = uninfected supernatant of Vero cells
[b]The footpad swelling reaction is presented in 0.1 mm ± standard error of the mean (s.e.m.). Values regarded positive are printed in bold type

Blood mononuclear cell proliferative assay. After single immunization with IBV only modest proliferative responses to IBV were observed. Out of the group of eight chickens only one, 5083, showed a distinct proliferative response to IBV with a stimulation index (SI) value (antigen-specific counts min^{-1}/control counts min^{-1}) of 5 (*Table 3*). One out of eight chickens immunized with the N fusion protein showed a moderate response to IBV. In contrast, none of the CGZ immunized control chickens responded to IBV.

Following the booster immunization with IBV, two out of eight chickens of the IBV primed group showed an IBV-specific proliferative response with SI values of 6 and 17. The data indicate that priming with inactivated IBV followed by an IBV booster induces cellular responses in only 25% of chickens tested.

Priming with recombinant-derived N protein followed by a booster immunization with IBV resulted in a proliferative response to IBV in four out of eight chickens with SI values ranging from 5 to 17, suggesting that the N protein can efficiently prime cellular immune responses to IBV. These results should be compared to priming with CGZ, which did not provoke proliferative responses to IBV.

Humoral immune responses. To assess an effect of the priming antigen on the kinetics of antibody induction to the IBV S protein, which is the main target of both VN antibody and HI antibody[28], we monitored the humoral immune response to the S protein in chickens before and after the booster injection with IBV. We hypothesized that activation of N protein-specific T-helper cells would accelerate antibody synthesis to the IBV S protein. As expected, the effect of priming with whole IB virus was most prominent. Mean HI and VN titres (VN titres not shown) rose within 2 weeks after primary immunization and rose to maximum levels upon secondary immunization. Chickens primed with N fusion protein, CGZ or PBS (data not shown) showed, as anticipated, no anti-S responses before the booster with IBV (*Figure 1*). A significant priming effect of immunization with the N protein was detected within 2 weeks after administration of the IBV booster using the HI assay ($p = 0.0009$). An anti-S protein antibody response was mounted more rapidly than in control groups, suggesting a role for activated T-helper cells in the anti-S antibody response. These results were confirmed using the VN assay. VN titres rose in weeks 8–10 only in N primed chickens ($p = 0.0025$).

Induction of tracheal protection. Four weeks after primary IBV immunization one out of eight chickens showed a protection to challenge with live IBV based on ciliary activity (*Table 4*). In the other groups none of the chickens resisted challenge.

After revaccination a minimal percentage (20–40%) of protection was expected in all groups due to single vaccination with inactivated IBV[29,30]. In the IBV primed group all chickens challenged showed protection on the basis of ciliary activity. However, using a more sensitive immunofluorescence assay, virus was detected in one out of eight chickens primed with IBV. In the N protein primed group of chickens eight out of ten showed protection on the basis of ciliary activity. Virus was detected by immunofluorescence in only three out of ten chickens, suggesting that 70% of N-primed chickens

Table 3 Proliferative responses of chicken blood mononuclear cells

		Proliferative response[a]					
		PHA (250 $\mu g\ ml^{-1}$) (weeks post primary immunization)		NA[b] (weeks post primary immunization)		IBV M41 (100 $\mu g\ ml^{-1}$) (weeks post primary immunization)	
Immunization	Animal	2	8	2	8	2	8
IBV M41	5081	97.6	ND*[c]	0.1	ND	0.1	ND
	5082	81.9	10.6	0.1	0.2	0.1	**3.4**
	5083	77.9	19.4	0.2	0.2	**1.1**	**1.3**
	5084	111.4	59.4	0.3	0.1	0.9	0.2
	5085	125.2	21.3	0.2	0.03	0.6	0.05
	5086	117.6	120.9	0.2	0.1	0.3	0.1
	5087	46.9	95.0	1.0	0.1	2.1	0.2
	5088	132.6	94.4	0.1	0.1	0.4	0.1
	5089	ND	64.1	ND	0.1	ND	0.2
pXM41-EP	5040	104.7	14.7	0.1	0.2	**0.5**	**3.3**
	4042	78.4	99.9	0.1	0.2	0.1	**1.0**
	5043	74.1	ND*	0.1	ND	0.0	ND
	5044	113.9	78.9	0.1	0.2	0.1	0.2
	5045	36.4	ND*	0.1	ND	0.1	ND
	5046	102.4	40.7	0.1	0.2	0.2	**3.4**
	5047	61.6	107.1	0.1	0.1	0.2	0.3
	5048	60.5	69.0	0.1	0.1	0.1	0.1
	5049	ND	76.1	ND	0.1	ND	0.3
	5051	ND	171.9	ND	0.3	ND	**1.6**
pEX11	5061	101.9	17.7	0.1	0.02	0.1	0.03
	5062	94.5	46.1	0.2	0.1	0.2	0.1
	5063	82.7	120.9	0.3	0.1	0.2	0.1
	5064	97.6	151.4	0.1	0.1	0.3	0.1
	5065	100.1	88.9	0.1	0.1	0.1	0.2
	5066	134.2	7.6	0.2	0.05	0.2	0.06
	5067	138.4	105.6	0.1	0.04	0.2	0.05
	5068	87.7	58.6	0.2	0.1	0.2	0.1

[a]Responses are expressed as counts $min^{-1} \times 10^{-3}$. The values represent the mean of triplicate measurements with standard deviations <30%. Values printed in bold are regarded as positive

[b]NA, no antigen

[c]ND, not done. *, Animals did not survive cardiac puncture and were substituted by other chickens which had received the same pretreatment

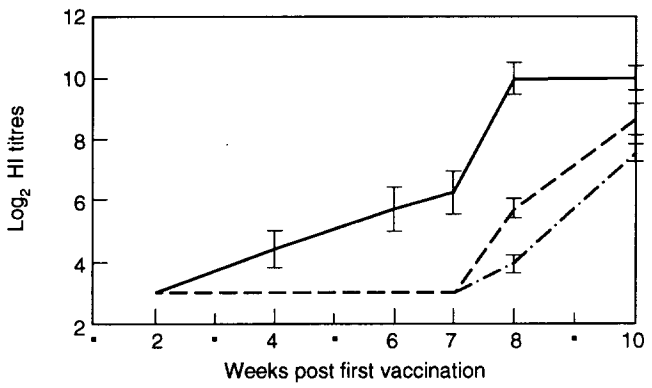

Figure 1 Mean $\log_2$ HI titres ± s.e.m. of chickens vaccinated at day 0 with the indicated antigens. At week 6 all chickens were boosted with inactivated IBV. Antigens: ——, M41; – – – –; pXM41-EP; –·–·, pEX11

showed protection in this group. In the control group several animals showed ciliostasis after challenge and in eight out of 12 chickens virus was detected in tracheal tissue. This indicated that, at the most, 33% demonstrated signs of resistance to live IBV in this group, which equals the expected percentage of protection due to one vaccination with inactivated IBV[29]. To test the hypothesis that priming of chickens with IBV or IBV N protein contributes to protection we analysed the data obtained by immunofluorescence microscopy using a linear model

Table 4 Induction of tracheal protection

	Protected chickens, ciliostasis assay challenge at day		Protected chickens, immunofluorescence (%) challenge at day	
Primary immunization	28	70	28	70
IBV M41	1/8	8/8	ND	7/8 (88) ($p = 0.013$)
pXM41-EP	0/8	8/10	ND	7/10 (70) ($p = 0.083$)
pEX11	0/8	8/12	ND	4/12 (33)

ND, not done

of logistic regression. We compared the induction of protection following priming with IBV or IBV N protein to priming with the control CGZ protein. A significant effect of IBV priming on tracheal protection was shown ($p = 0.013$). Analysis of the data in these small groups suggested also an increased protection to tracheal challenge in chickens primed with recombinant-derived N protein ($p = 0.083$).

DISCUSSION AND CONCLUSIONS

We have found that the N protein of IBV produced in a bacterial expression system is capable of priming an immune response to intact virus, both in mice and in chickens. Upon immunization of mice with the N protein,

lymph node cell proliferative responses and DTH reactions specific for IBV were demonstrated. Thus the data indicated a role for the N protein in activation of T-cells in the response to intact IBV.

Subsequently, the role of the N protein in induction of protective immunity to IBV in chickens was explored. Three main findings emerged from this study. First, a priming effect of the N protein on proliferative responses of blood mononuclear cells was seen 2 weeks after secondary immunization. Primary N protein vaccination did not result in a detectable proliferative response to IBV. Primary immunization with inactivated virus showed responses to IBV in 25% of chickens, similar to previous reports[31]. After secondary immunization of chickens with IBV, the priming effect of the N protein became manifest and exceeded the outcome of priming with virus twofold. In 50% of chickens a proliferative response to IBV was shown. No responses were observed in chickens vaccinated with CGZ which demonstrates the specificity of the IBV sensitization by N protein priming.

Secondly, we have shown that immunization with N protein resulted in accelerated antibody induction to the IBV S surface protein as measured in the HI and VN assays. The observed variations of individual $\log_2$ HI and VN titres agree with those reported by Darbyshire[32]. Both assays showed increased mean titres specific for the S protein within 2 weeks after secondary immunization. Since it is expected that in the chicken T and B cells follow the rules of cognate interaction as described for mammalian species[33] we explain the present observation by the action of an expanded population of N protein-specific T cells which could accelerate the expansion and differentiation of primary virus-specific B cells. In individual chickens, however, proliferative responses did not always coincide with increased HI and VN titres, an observation made earlier by Timms and Bracewell[31]. This finding can be explained by the notion that not all proliferative antigen-specific cells are T-helper cells[34].

Finally, a role for the N protein in protection to tracheal challenge was implicated. Single vaccination with inactivated IBV results in little or no protection in the trachea against challenge infection, whereas two vaccinations may result in up to 80–100% protection[29,30]. We hypothesized that a priming effect of immunization with the N protein should be visible within these ranges. Indeed, the data obtained by immunofluorescence microscopy indicated that 70% of N-primed and IBV-boosted chickens had resisted tracheal challenge. Of the chickens primed with CGZ, 33% had resisted challenge. The ciliostasis assay indicated a higher percentage of protected chickens compared to the data obtained by immunofluorescence microscopy. This discrepancy is explained by the observation that in older chickens tracheal symptoms due to virus replication are less severe than in young chickens[30].

It has been suggested that local immunity of the respiratory tract, the primary target organ of IBV infection, is of fundamental importance in IBV resistance[35]. Our data confirm earlier data[36] that systemically induced responses to IBV can support protection at a local level.

Recombinant-derived proteins have been successful in the induction of cellular immune responses to viral antigens[19,37,38]. In our study we demonstrated the immunogenicity of the IBV N protein fused to CGZ. The CGZ control protein, although known for its capacity to induce T helper and T suppressor responses in the mouse[34] did not influence the response to IBV in chickens. It should be considered, however, that this complex protein could influence the efficacy of a given recombinant vaccine.

Little is known about the role of coronaviral N proteins in the response to coronaviral infection. Until now, cell-mediated immune responses to coronaviral N proteins in the target species have not been reported. Our data have shown a role for the IBV N protein in the activation of T-helper cell responses in the chicken. Our findings add to the pivotal role of internal viral antigens in the induction of protective immunity by activation of cytotoxic or helper T-cell responses[10,12,13]. These internal antigens, usually less subject to antigenic variation than surface proteins, have been shown to generate cross-reactive protective immunity[8,9,38]. The N proteins of IBV strains also show highly conserved regions[2,6]. In a previous paper we reported on T-cell hybridoma responses to N proteins of several IBV strains, supporting the data that indicate a stable antigenicity of the N protein[14].

For future vaccine design it would be useful to gain insight into the determinants recognized by T-helper cells. We hypothesize that the N protein on the basis of its immunogenicity and relatively constant antigenicity is relevant for further study.

ACKNOWLEDGEMENTS

The authors are grateful to B.A.M. van der Zeijst, J.G. Kusters (Department of Bacteriology, Institute of Infectious Diseases and Immunology, Utrecht), A. Snijders and C.A. Kraaijveld (Section of Experimental Virology, Eijkman–Winkler Laboratory, Utrecht) for helpful discussion and for their contribution to the work described above, and to K.A. Zwaagstra (Department of Bacteriology, Institute of Infectious Diseases and Immunology, Utrecht) and T. Loeffen (Intervet, Boxmeer) for technical assistance. The authors are indebted to I. Joosten for critical reading of the manuscript.

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