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Localization of a T-cell epitope within the nucleocapsid protein of avian coronavirus

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SUMMARY

In a previous study, two murine T-cell hybridomas generated after immunization with infectious bronchitis virus (IBV) were shown to be responsive to the internally localized viral nucleocapsid protein. In the present study, the antigenic determinants were mapped using recombinant expression products and synthetic peptides. Both hybridomas recognized the region spanning amino acid residues 71 to 78 of the nucleocapsid protein. The experimentally determined epitope corresponded with predicted motifs. Both an I-E^d binding motif and a predicted cleavage site for the aspartyl protease cathepsin D were contained within the sequence. The epitope was shown to prime cellular immune responses to IBV in the chicken.

INTRODUCTION

Infectious bronchitis virus (IBV) is the causative agent of an acute respiratory disease in chickens. The viral RNA genome encodes three structural proteins: the spike glycoprotein (S), protruding from the viral envelope, the integral membrane glycoprotein (M) and the nucleocapsid protein (N).[1-3] Many serotypes of IBV have been defined and there is a constant emergence of new serotypes generated by various mechanisms, causing a problem in vaccination programmes.[4-6] In general, new serotypes show variations in the spike glycoprotein which is the main target for the IBV-neutralizing antibodies.[7,8]

To challenge this problem a new generation of IBV vaccines is required. These new vaccines should be designed with an emphasis on induction of both cellular and humoral immune responses. So far, only B-cell determinants have been identified on proteins of IBV.[7,8] To obtain the information necessary to develop synthetic IB vaccines we started to identify T-cell epitopes in the IBV field strains. In a previous paper, we described two MHC class II (E^d)-restricted, CD4 positive T-cell hybridomas with a specificity for IBV.[9] The hybridomas were responsive to several IBV strains of both serotype A and D.[10] It was found that both T-cell hybridomas (MJB100 and MJB101) responded to the internal N protein of IBV.[9] Subsequently, the gene encoding the N protein was cloned into the expression vector pEX.[7,11] Both hybridomas were responsive to the expressed N protein.[12]

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To delineate the antigenic determinants recognized by MJB100 and MJB101, we used expression products of cDNA fragments of the N gene. The expressed fragments were tested and this indicated recognition of a site close to amino acid residue 75 of the N protein. Several approaches were used to delineate the fine specificity of both T-cell hybridomas.

The first coronaviral T-cell epitope defined was found in the amino acid sequence 71 to 78 of the N protein of IBV.

MATERIALS AND METHODS

Virus

IBV strain M41 was originally obtained from the Poultry Health Institute in Doorn, The Netherlands. Viral RNA was isolated and used to prepare the cDNA library as described elsewhere.[13] The M42 Salk strain,[14] an IBV laboratory strain, was grown on Vero cells (multiplicity of infection 0·1). The supernatant of the infected cells was harvested after 36 hr and used as a soluble source of antigen.

T-cell hybridomas and antigen-presenting cells (APC)

T-cell hybridomas MJB100 and MJB101 were raised from a mouse T-cell line specific for IBV after fusion to C58.[15] Both clones recognize the N protein of avian coronavirus IBV and produce IL-2 upon stimulation with specific antigen.[9]

L-cell transfectants expressing the I-E^d molecule (RT 10.3B-C1) were used as a reproducible source of APC.[16]

In some experiments the L-cells were fixed by a 20-min incubation of 6×10^6 cells/10 ml paraformaldehyde [0·5% in phosphate-buffered saline (PBS)] at room temperature.[17] Cells

Table 1. Expressed fragments of the IBV N protein

Subclone†	Original clone	Fragment	Enzymes used to prepare digest	Vector preparation	Amino acid‡ residues
pXM41-EP	cDNA in M13		*Pst*I*S1	pEX2**Sma*I**Pst*I	2–405
pUC-EP	pXM41-EP		*Eco*RI**Pst*I	pUC **Eco*RI**Pst*I	
pXM41-EB	pUC-EP	*Eco*RI-*Pst*I	*Bgl*II	pEX11**Eco*RI**Bam*HI	2–127
pXM41-BP	pUC-EP	*Eco*RI-*Pst*I	*Bgl*II	pEX11**Bam*HI**Pst*I	127–405
pXM41-AA	pXM41-EB	*Eco*RI-*Pst*I	*Alu*I	pEX11**Sma*I	15–74
pXM41-AB	pXM41-EB	*Eco*RI-*Pst*I	*Alu*I	pEX1**Sma*I**Pst*I	76–127
pXM41-MB	pXM41-EB	*Eco*RI-*Pst*I	*Mnl*I	pEX12**Sma*I**Pst*I	51–127

† The fragments were ligated into linearized pEX as indicated.
‡ Amino acid residues of the N protein expressed as fusion protein.

were washed three times in cold PBS supplemented with 10% foetal calf serum (FCS; Gibco, Breda, The Netherlands). Subsequently, 5×10^5 fixed cells were plated in flat-bottomed 96-well plates and incubated with 2×10^4 hybridoma cells and antigen. IL-2 production was measured as described previously by growth of an IL-2-dependent cell line.[9,18]

Construction of the IBV nucleocapsid pEX clones

The DNA encoding the N protein was isolated from the IBV M41 cDNA library.[13] Restriction enzyme fragments were cloned into the pEX expression vector[7] (Fig. 1). *E. coli* pop 2136 was used as host strain for the pEX plasmids. Recombinant plasmids were introduced into the cells via the $CaCl_2$ transformation procedure. Recombinants were screened by polyacrylamide gel electrophoresis of their expression products. Clones synthesizing hybrid proteins of the expected molecular weight were selected for further characterization. Also, the nucleotide sequence of the insertion was determined by the dideoxynucleotide termination method. The lengths of the cloned restriction fragments are shown in Table 1. The obtained fragments were tested for their capacity to induce IL-2 production by the IBV specific T-cell hybridomas.

Synthetic peptides

The peptides comprising the IBV N protein amino acid residues 67 to 83 and 71 to 83 and a 16-mer control peptide SP111 (EPKSCDKTHICPPCPA) were synthesized via the solid phase Fmoc peptide synthesis devised by Merrifield.[19] The peptides were purified by high-performance liquid chromatography (HPLC) and checked via amino acid analysis. The sequence of the IBV peptides is shown in Table 2. A set of twelve IBV peptides each of ten residues in length was synthesized via the modified PEPSCAN method.[20,21] These peptides overlapped the IBV N protein amino acid residues 61 to 81 and were synthesized with a C-terminal extension, the tripeptide Asp–Pro–Gly (DPG) (Table 2). The presence of the tripeptide allows removal of the peptide from the resin via selective cleavage with formic acid between aspartic acid and proline. This method has been shown to be applicable in T-cell assays.[21–23] All peptides were tested for induction of IL-2 by the IBV specific T-cell hybridomas.

Chicken blood mononuclear cell proliferative assay

Four-week-old female White Leghorn chickens, derived from a specified pathogen-free (SPF) flock from Intervet (Boxmeer, The Netherlands) were used for immunization and housed under isolated conditions. The animals received food and water *ad libitum*. Groups of 20 chickens were immunized intramuscularly in the leg, with either 100 μg of free peptide 67–83, control peptide SP111 or a formalin-inactivated IBV M41 standard preparation. Antigens were mixed 1:1 in Freund's complete adjuvant (FCA). At six weeks after primary immunization all birds were boosted with the formalin-inactivated IBV M41 preparation (Intervet).

Two weeks after primary and secondary immunization, five chickens of each group were bled by cardiac puncture and blood mononuclear cells were isolated to perform proliferative assays. A modified procedure as described by Timms and co-workers was used.[24] Briefly, the white blood cells were separated from the red cells by centrifugation at low speed in glass capillaries. After extensive washing 10^6 lymphoid cells were dispensed in 0·15-ml volumes of HEPES-buffered RPMI-1640 (Dutch modification) supplemented with 1 g $NaHCO_3$/l and 20·000 IU/ml penicillin and 200 μg/ml streptomycin in round-bottomed wells of a microtitration plate. Dilutions of antigen were added in 0·05-ml volumes to triplicate wells and the plates were incubated at 41° in a humidified 5% CO_2 atmosphere for 3 days. Prior to the last 6 hr of culture, 0·5 μCi [^{3}H]thymidine (5 Ci/mM, Amersham, s'Hertogenbosch, The Netherlands) was added to the wells. DNA of cells was harvested and the incorporated radioactivity was measured by liquid scintillation counting. Results are expressed as counts per minute (c.p.m.).

RESULTS

Construction of IBV N pEX recombinants

To correlate the specificity of the T-cell hybridomas with a physical map of the IBV N protein, a cDNA clone encoding the N gene of IBV strain M41 was isolated and sequenced. The derived amino acid sequences of the M41[13] and the M41H[1] N proteins were compared and were shown to be 96·6% identical. cDNA fragments from the N protein of strain M41 were expressed in pEX plasmids as described by Kusters.[7] In this

Table 2. Sequence of the IBV N peptides tested for reactivity with hybridoma MJB100. IL-2 responses are presented as SI values. Background: 580 c.p.m. # # # = I-E^d binding motif.[32] Between residues indicated with ** a cleavage site for cathepsin D was predicted.[25] Selective cleavage of the Tyr_{70} and Trp_{71} bond was confirmed upon analysis of the *in vitro* degradation of IBV N 67–83 by purified cathepsin D (J. M. van Noort, personal communication)

		μg/ml	MJB100*
Synthetic peptides			
IBV N (67–83)	QHGYWRRQARFKPGKGG	5·0	129·0
	** # # #	1·0	175·0
IBV N (71–83)	WRRQARFKPGKGG	5·0	141·0
	# # #	1·0	9·4
PEPSCAN peptides			
IBV N (61–70)	NIKPSQQHGY(D)	1·0	1·0
IBV N (62–71)	IKPSQQHGYW(D)	1·0	1·5
IBV N (63–72)	KPSQQHGYWR(D)	1·0	1·5
IBV N (64–73)	PSQQHGYWRR(D)	1·0	1·0
	#		
IBV N (65–74)	SQQHGYWRRQ(D)	1·0	1·1
	#		
IBV N (66–75)	QQHGYWRRQA(D)	1·0	1·3
	#		
IBV N (67–76)	QHGYWRRQAR(D)	1·0	1·0
	# #		
IBV N (68–77)	HGYWRRQARF(D)	1·0	1·0
	# #		
IBV N (69–78)	GYWRRQARFK(D)	1·0	36·6
	# # #		
IBV N (70–79)	YWRRQARFKP(D)	1·0	1·3
	# # #		
IBV N (71–80)	WRRQARFKPG(D)	1·0	1·0
	# # #		
IBV N (72–81)	RRQARFKPGK(D)	1·0	1·0
	# # #		

* IL-2 response in SI values.

system heterologous expression leads to the synthesis of a C-terminal extension of the cro-beta-galactosidase protein. The relative position of the M41 cDNA clone spanning the N gene and the positions of the restriction enzyme sites are shown in Fig. 1a. From this clone, six fragments were derived (Fig. 1c) and inserted in the plasmid pEX (Table 1). The proper insertion of all inserts was checked by the dideoxynucleotide termination method.

Localization of a T-cell epitope using recombinant expression products

The hybridoma cell lines MJB100 and MJB101 responded to the first 127 amino acids of the N protein (pXM41-EB) in the presence of the I-E^d expressing L-cells as APC (Table 1, Fig. 1). The pXM41-BP clone (residues 127–405) was not recognized. Subfragments of the pXM41-EB fragment were tested, which resulted in the recognition of the pXM41-MB (residues 51–127) fragment. Both the pXM41-AA (residues 15–74) and the pXM41-AB (residues 76–127) clones were not recognized (Fig. 1).

These data suggest that the site recognized by both T-cell hybridomas is encoded by the DNA region containing the *AluI* restriction site at a position corresponding to amino acid 75 of the N protein.

T-cell reactivity to synthetic peptides

This led us to synthesize peptides 67–83 and 71–83. Both MJB100 and MJB101 (not shown) showed a response to these peptides (Table 2). The hybridomas produced IL-2 in a dose-dependent manner. Concentrations of 0·5 μg/ml or less of the smaller peptide no longer resulted in significant IL-2 production (not shown). To define the localization of the epitope more precisely, 12 PEPSCAN peptides, 10 residues in length, were tested. Only one peptide (amino acid residues 69–78 + a C-terminal D) was able to induce an antigen-specific IL-2 response by MJB100 (Table 2) and MJB101 (not shown). Thus, all positive peptides shared residues 71–78 of the N protein.

Peptide 67–83, in contrast to peptide 71–83, contains a cleavage site for the proteolytic enzyme cathepsin D[25] (J. M. van Noort, personal communication, 1990). To test the requirement for processing of the two peptides, we used APC which were mildly fixed in order to prevent intracellular processing. It was

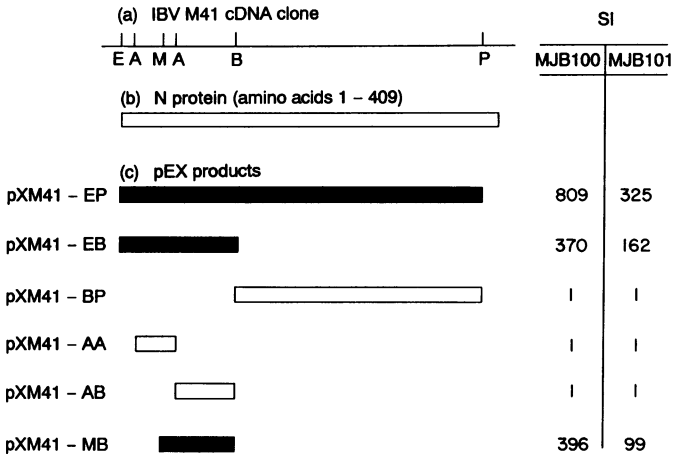

Figure 1. Map of pEX constructs containing fragments of the IBV M41 N gene. (a) The IBV N clone. The capital letters indicate the restriction site of the *Eco*RI (E), *Alu*I (A), *Mnl*I (M), *Bgl*II (B) and *Pst*I (P) restriction enzymes. (b) The N protein. (c) pEX inserts spanning the N gene. pEX products significantly recognized by T-cell hybridomas MJB100 and MJB101 are indicated by solid bars. Twenty μg expression product per well was used in the IL-2 assay. Responses of T-cell hybridomas are presented as SI values (antigen specific counts per minute (c.p.m.)/control c.p.m.).

Table 3. IL-2 responses of MJB100 after stimulation with antigen in the presence of untreated or paraformaldehyde-fixed L-cells as APC. Results are presented as SI values. Background 122 c.p.m.

	IL-2 response (SI) of MJB100	
Antigens	Untreated	APC fixed
IBV N 71–83 50 μg/ml	949	723
IBV N 71–83 5 μg/ml	862	267
IBV N 67–83 50 μg/ml	1023	849
IBV N 67–83 5 μg/ml	1000	849
pXM41-MB	608	1
M42	923	1

shown that fixed cells could present both peptides to either of the hybridoma cell lines. In contrast the M42 whole virus or the pXM41-MB expression product could not induce an IL-2 response under these conditions (Table 3). This suggested that both peptides can bind to E^d sufficiently to be presented by APC to T cells without further intracellular processing.

Table 4. Proliferative responses of chicken blood mononuclear cells*

		Antigen in μg/ml							
		NA		M41 (100)		71–83 (100)		67–83 (100)	
Immunization	Animal	2†	8	2	8	2	8	2	8
IBV N 67–83	5003	0·23	0·24	0·39	0·38	0·70	0·61	0·30	0·31
	5004	0·36	0·27	0·98	1·01	2·98	3·46	0·82	0·97
	5005	0·22	0·18	0·43	1·03	3·01	4·37	0·90	1·45
	5006	0·49	0·17	0·56	0·18	0·40	1·21	0·30	1·00
	5007	0·17	0·20	0·15	0·58	0·18	2·80	0·13	0·57
SP111	5021	0·20	0·16	0·32	0·22	0·30	0·34	0·19	0·27
(control)	5023	0·19	0·12	0·54	0·17	0·48	0·24	0·29	0·13
	5024	0·35	0·13	0·66	0·19	0·38	0·05	0·33	0·35
	5025	0·12	0·22	0·27	0·41	0·24	0·56	0·21	0·23
	5028	0·25	0·38	0·33	0·54	0·36	0·74	0·25	0·45
IBV M41	5082	0·07	0·17	0·14	3·45	0·11	0·96	0·10	0·46
	5083	0·21	0·17	1·08	1·28	0·23	0·70	0·26	0·26
	5085	0·17	0·03	0·60	0·05	0·28	0·06	0·22	0·03
	5086	0·19	0·09	0·29	0·11	0·19	0·13	0·19	0·10
	5088	0·10	0·06	0·36	0·10	0·17	0·08	0·11	0·06

* Chickens were immunized with either inactivated IBV M41, peptide 67–83, or the control peptide SP111 mixed 1:1 with FCA. Chickens were boosted with inactivated IBV M41 at 6 weeks after primary immunization. Proliferative responses of blood mononuclear cells were determined at 2 weeks after primary (Week 2) and secondary immunization (Week 8). Results are expressed as c.p.m. $\times 10^{-3}$. The values represent the mean of triplicate measurements. Standard deviations did not exceed 35%. Underlined values were regarded as positive. NA = no antigen. Responses to phytohaemagglutinin demonstrated the vitality of the blood mononuclear cells of each chicken tested (data not shown).

† Weeks post-primary immunization.

Proliferative responses of chicken blood mononuclear cells

The T-cell antigenic determinant of IBV, defined in a mouse model, was verified for its relevance in the chicken. We expected that the large 17-mer sequence (67–83) would be most successful in the induction of a cellular immune response to IBV.[26,27] Following priming of chickens with the 17-mer peptide, two out of five chickens (5004 and 5005) showed proliferative responses to both the 17-mer and the 13-mer (Table 4). Chicken 5004 also showed a moderate response to IBV. Three out of five chickens did not respond to any of the tested antigens. Primary immunization of chickens with inactivated IBV M41 induced proliferative responses to IBV in only one out of five chickens (5083). No proliferative response to either peptide antigen was seen, suggesting a low frequency of peptide responsive cells upon primary immunization. None of the chickens immunized with the control peptide responded to the IBV antigens (Table 4).

All immunization groups were boosted with inactivated IBV. The proliferative response to peptide and viral antigen were again determined. Now, four out of five chickens of the peptide-primed group responded to the 13-mer sequence. In three out of these four chickens, responses to the 17-mer were also noticed. In chickens 5004 and 5005 only, a proliferative response to intact IBV was observed. In the IBV-primed group two out of five chickens (5082 and 5083) responded to IBV. Both chickens also showed a moderate response to the 13-mer peptide, while no response to the 17-mer was observed.

DISCUSSION

This paper describes the localization of a T-cell site within the N protein of avian coronavirus IBV using two murine T-cell hybridomas. In a few cases it has been convincingly shown that T-cell epitopes can associate with different MHC haplotypes[28–30] including MHC types of different animal species.[27,31] Therefore we hypothesized that a T-cell antigenic determinant defined in the BALB/c mouse is a first step in the delineation of T-cell epitopes which may also be recognized by chicken T cells in the context of one or several chicken MHC types. In support of this assumption, our data showed recognition of this T-cell epitope after immunization of chickens with IBV. More importantly, the epitope was shown to prime cellular immune responses to IBV in the chicken.

The antigenic determinant recognized by the two T-cell hybridomas specific for the N protein of IBV was determined using recombinant expression fragments and synthetic peptides. Both hybridomas recognized a site close to amino acid residue 75 of the N protein. We have compared our experimental data with prediction methods for T-cell epitopes, taking into account the fact that the antigenic determinant of the IBV N protein was recognized in the context of I-E^d. The sequence 72–78 fits precisely the motif described by Sette.[32] Also, we speculated that processing could be another factor determining the sequence recognized by the T-cell hybridomas. Since cathepsin D, an endosomal protease, seems to play a role in antigen processing, we searched for a cleavage site for this enzyme.[25] Such a site was predicted between Tyr_{70} and Trp_{71}, suggesting that the naturally selected fragment during antigen processing would start at position Trp_{71}. On the basis of these predictions we tested whether presentation of peptides 67–83 and 71–83 to T cells was influenced using fixed APC. However, peptides 67–83 and 71–83 were both efficiently presented. It appeared that the N-terminal extension of the four residues Gln_{67}, His_{68}, Gly_{69} and Try_{70} did not influence binding to E^d sufficiently to warrant antigen processing. Further studies are needed to clarify whether N-terminal extensions beyond residue Gln_{67} would influence binding to E^d and would introduce a requirement for processing.

In contrast to these results, the PEPSCAN data indicate that flanking regions, N- as well as C-terminal extensions, influence recognition of the 71–78 sequence, since only the 69–78 sequence is recognized. Several explanations may be considered for this observation. Obviously, there is some variability accepted concerning the fit of an antigenic peptide within the trimolecular complex of MHC, peptide and T-cell receptor. It appears that not a single minimal length is critical.[33] In the case of the PEPSCAN peptides the presence of proline (Pro_{79}) at the penultimate position may have affected the conformation of the peptide sufficiently to prevent either efficient binding to I-E^d or recognition by both T-cell hybridomas. Further studies are required to clarify this issue. Nevertheless our data support our idea that the context of the peptides used is critical.

The epitope found in this study is shared by several IBV strains as was shown in a previous study.[9] This indicates the presence of this sequence or related sequences among the N proteins of different IBV strains and implies that the N protein may be relatively conserved in contrast to the external spike proteins. Unfortunately, little sequence data are available to answer this question.

Milich[34] and co-workers have shown in the study on hepatitis B virus that priming with a single T-cell determinant from the core protein and subsequent boosting with the intact virus resulted in antibodies generated to a protein in the viral envelope. In the case of IBV, our data show that an epitope of the IBV N protein defined in a mouse model can induce cellular immune responses to IBV in both mice and chickens. Influences on the kinetics of induction of humoral immunity are currently under investigation. Our data, together with those of others,[27,31,35] have shown the possibility of using a mouse model to detect T-cell epitopes that are of relevance in other species.

Finally, our data add to the evidence for the pivotal role[22,35,36] of viral nucleocapsid proteins in induction of cellular immune responses.

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