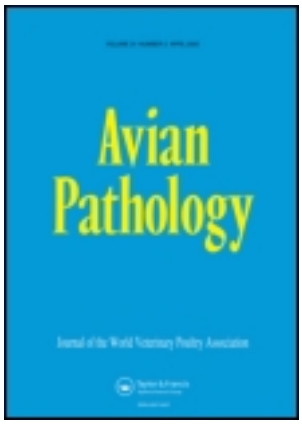

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Tissue tropism in the chicken embryo of non-virulent and virulent Newcastle diseases strains that express green fluorescence protein

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Tissue tropism in the chicken embryo of non-virulent and virulent Newcastle diseases strains that express green fluorescence protein

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The tissue tropism of non-virulent and virulent Newcastle disease virus (NDV) was investigated using 8-day-old and 14-day-old embryonating chicken eggs (ECE), inoculated with an infectious clone of the non-virulent La Sota strain (NDFL-GFP) or its virulent derivative (NDFLtag-GFP). Both strains expressed the gene encoding jellyfish green fluorescence protein (GFP) as a marker. The GFP was readily expressed in chicken embryo cells infected with the NDV strains indicating virus replication. Whereas both strains replicated in the chorioallantoic membrane (CAM) and infected the skin of 8-day-old ECE, only the virulent strain (NDFLtag-GFP) spread to internal organs (pleura/peritoneum). In 14-day-old ECE, the initial target organs appeared to be the CAM and the lungs for both strains. At 48 h after inoculation, the virulent strain (NDFLtag-GFP) had also spread to the spleen and heart and was detected in a wide-range of embryonic cell types. The kinetics of virus replication and spread in the CAM closely resembled each other in both the 8-day-old and 14-day-old ECE. Infection of 8-day-old and 14-day-old ECE forms a convenient model to investigate tissue tropism of NDV, as well as the kinetics of viral infection. The advantage of using GFP is that samples can be easily screened by direct fluorescence microscopy without any pre-treatment.

Introduction

Newcastle disease virus (NDV) is the causative agent of a devastating disease of poultry known as pseudo-fowl pest. It causes high mortality, severe respiratory distress, haemorrhagic intestinal lesions, nervous disorders and a decrease of egg production (Alexander, 2000). Strains of NDV are divided into virulent (viscerotropic velogenic, neurotropic velogenic and mesogenic viruses) and non-virulent pathotypes (lentogenic and asymptomatic enteric viruses) (Office International des Epizooties, 2000). Its pathogenicity largely depends on cleavage of the precursor fusion glycoprotein (F_0), into F_1 and F_2 subunits, which renders the virus particles infectious for target cells (Collins *et al.*, 1993; Peeters *et al.*, 1999). This cleavage is mediated by host cell proteases (Choppin & Scheid, 1980; Collins *et al.*, 1993). The significance of F_0 cleavage is demonstrated by the inability of non-virulent strains to produce plaques in cell culture systems in the absence of trypsin (Nagai *et al.*, 1976). Recently, Peeters *et al.* (1999) confirmed that changing the amino acid sequence around the F_0 cleavage site using genetic engineering of an infectious clone could increase the pathogenicity of NDV.

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The virus grows in cells lining the allantoic cavity when injected in embryonated eggs. Rapid destruction of the allantoic cells is accompanied by release of the virus in the allantoic fluid reaching high titres in approximately 24 h. Most embryos die from 42 h after inoculation of virulent NDV strains. However, no remarkable histopathological changes can be seen in embryos at any stage of infection. Apoptosis of the heart and brain cells is probably involved in their acute death (Lam *et al*., 1995). Death caused by less virulent strains may result from severe pneumonia (Liu & Bang, 1953).

Varying mean death time values for NDV strains probably reflect different abilities to spread in embryonic tissues and their diverse pathogenicity and infectivity. Variations in amino acid sequences of the NDV fusion protein cleavage site most probably represent the virulence factor that is involved. Absence of proteases in most of the visceral organs at early developmental stages might limit the cleavability of the fusion protein and thus might influence viral infectivity.

NDV has been successfully used as an expression vector to express chloramphenicol acetyltransferase (CAT) (Krishnamurthy *et al*., 2000; Huang *et al*., 2001), jellyfish green fluorescence protein (GFP) (Engel-Herbert *et al*., 2003) and influenza haemagglutinin. Different insertion sites were used. Krishnamurthy *et al*. (2000) inserted the CAT and Engel-Herbert *et al.* (2003) inserted the GFP gene between the fusion (F) and haemagglutinin-neuraminidase (HN) genes, whereas Huang *et al.* (2001) inserted the CAT gene upstream to the nucleoprotein (NP) gene and between the F and HN genes and Nakaya *et al.* (2001) inserted the CAT and the influenza haemagglutinin gene between the phosphoprotein and matrix gene of NDV.

We inserted the jellyfish GFP upstream to the NP gene of recombinant avirulent La Sota strain and a virulent derivative thereof using reverse genetics. The viruses only differed in the sequence at the cleavage site. The effect of the difference on the tissue tropism and kinetics of infection was studied in 8-day-old and 14-day-old embryonating chicken eggs (ECE). Autofluorescence and immunohistochemistry were used to identify the target organs and cells for viral replication

Materials and Methods

NDV strain characteristics

An infectious clone, designated NDFL, of the La Sota vaccine strain (ATCC VR-699; Goldhaft, 1980) was used. A mildly virulent variant of NDFL that contains the multiple basic amino acid sequence motif of virulent NDV at the cleavage site of the fusion protein (NDFLtag) was constructed as described previously (Peeters *et al*., 1999). Strain NDFL has an intracerebral pathogenicity index of 0.0, and is classified as a lentogenic strain, whereas strain NDFLtag has an intracerebral pathogenicity index value of 1.3, and is classified as a mesogenic strain. The DNA sequence encoding the GFP gene was inserted into the NDV genome between nucleotides 109 and 110 (*Xmn* I site)—that is, between the transcription-start box of the NP gene and the NP open reading frame—by using plasmids pNDFL$^+$ and pNDFLtag (Peeters *et al*., 1999). In order to allow transcription of downstream genes, a synthetic nucleotide sequence corresponding to the consensus transcription-end and transcription-start box (ATTAAGAAAAAA t ACGGGTAGAAG) of NDV was inserted behind the GFP gene. The resulting plasmids were used to rescue recombinant NDV as described previously (Peeters *et al*., 1999). Rescued viruses were propagated in the allantoic cavity of 10-day-old to 11-day-old specific pathogen free ECE that were incubated at 37 to 38°C. Allantoic fluid was harvested 48 h after inoculation. The virus titres expressed as median tissue culture infectious doses ($TCID_{50}$/ml) were $10^{7.9}$ for strain NDFL-GFP and $10^{8.4}$ for strain NDFLtag-GFP. Single-step growth curves indicated that replication of the viruses that carried the GFP gene did not differ significantly from the parent strains NDFL or NDFLtag (data not shown).

Embryo inoculation and tissue preparation

ECE of specific pathogen free White Leghorn chickens obtained from SPAFAS (Charles Rivers, The Netherlands) were used. The eggs had been incubated at 37 to 38°C for 8 or 14 days before use and then were inoculated with a suspension containing $10^{4.2}$ median embryo infective dose (EID_{50})/ml NDFL-GFP strain or $10^{4.7}$ EID_{50}/ml NDFLtag-GFP strain. From each age group, 20 ECE per strain were inoculated in the allantoic cavity (Nagai *et al*., 1979) with 0.2 ml each suspension diluted in phosphate-buffered saline (PBS). As a control, another 20 ECE of each age group were inoculated via the same route with sterile allantoic fluid diluted 1:10 in PBS. Samples were collected from five embryos at 24, 48, 72, and 96 h after inoculation. The 8-day-old ECE were harvested completely intact, whereas the lung, spleen and heart were individually collected from the 14-day-old ECE. The adherent chorioallantoic membrane (CAM) was Swiss-rolled, and all tissues were individually snap-frozen with OCT compound (Tissue-Tex, Sakura, Japan) in liquid nitrogen and stored at −70°C.

To determine the kinetics of the NDV infections in CAM, four ECE per time point were inoculated with each of the virus strains as previously described. Allantoic fluid and the CAM were harvested 4, 6, 10, 14, and 24 h after inoculation. The allantoic fluid was used to determine the viral content by the haemagglutination test according to EU Council directive 92/66 (1992) "Introducing community measures for the control of Newcastle disease".

Cryostat sections of 8 μm thick were cut at −21°C. From the 8-day-old ECE, coronal sections were cut from the anterior part of the coelom or body cavity, at distances of 100–200 μm to the end of the body. Six to eight cross-sections per embryo were adhered to slides. For the 14-day-old ECE, the internal sampled organs were separately sectioned with three to four sections placed together on one slide. Subsequently, all the slides were allowed to dry at room temperature, fixed for 5 min in acetone and air-dried for 30 min.

Antigen detection

All sections were screened by direct fluorescence microscopy for autofluorescence of GFP. Moreover, NDV antigen was examined in sections by immunohistochemical staining. In short, first endogenous peroxidase activity in the tissue sections was inhibited by 0.01% H_2O_2 for 5 min followed by rinsing with PBS containing 1% bovine serum albumin (B-PBS). Viral antigen was demonstrated using a monoclonal antibody, ID-NDV-134.1 (IgG_1 isotype), against the HN protein of the NDV (Maas *et al*., 2000). Bound monoclonal antibody was detected by incubation with peroxidase-conjugated rabbit-anti-mouse Ig (DAKO, Denmark) after washing with B-PBS. After another rinsing with B-PBS, the slides were incubated with 0.5 mg 3,3-diaminobenzidine-tetrahydrochloride (Sigma, St Louis, Missouri, USA) per ml Tris–HCl buffer (0.05 M, pH 7.6) containing 0.01% H_2O_2 to visualize peroxidase activity. All sections were lightly counterstained with eosin methyl blue for 2 sec, dehydrated and mounted in DePex (BDH, Poole, UK). Viral staining was categorized semiquantitatively: negative, −; dispersed positive cells or spots, +; medium positive areas, ++; and various groups of positive-stained cells, +++. Photographs were taken with the E990, Nikon digital card colour camera (Tokyo, Japan).

Results

Expression of the GFP

GFP expression could easily be seen by fluorescence microscopy of cryostat sections for both virus strains but only after incubation times of 24 h or more. This lag time was expected and was most probably due to the time required for onset of virus replication and expression of GFP in quantities large enough to be visible. A major advantage of GFP was that it enabled the visualization of virus replication without any pre-treatment of the tissues or cells. Figure 1a shows the fluorescence of the GFP in lung.

Tissue tropism of NDV

In situ immunohistochemistry was used to check the observations made by fluorescence microscopy. In the 8-day-old ECE, both virus strains were easily detected in the CAM and the superficial layer of the skin after 24 h (Figure 1b). In the embryos injected with the NDFL-GFP strain, the infection remained limited to these tissues at 48, 72 and 96 h after inoculation. However, after inoculation with the NDFLtag-GFP strain, viral antigen was demonstrated in the capsular layer (the pleura/peritoneum) of the lungs, kidney, liver, and proventriculus and in the pericardium (Table 1). Thus, the NDFLtag-GFP strain appeared to have spread from the CAM to the coelomic epithelium and the capsules of the visceral organs in 8-day-old ECE.

Concerning the 14-day-old ECE, between 24 and 96 h after inoculation of the NDFL-GFP strain, viral antigen was only detected in the CAM and lung (Table 2). The spleen and heart remained negative throughout the experimental period. However, viral antigen was found in the CAM, lung, spleen, and heart of the embryos inoculated with the NDFLtag-GFP strain (Figure 1c,d). The spleen and heart were positive from 48 h after inoculation.

Target cells of NDV

NDV appeared to multiply in a wide range of embryonic cells. After infection with both strains, epithelial cells of the inner site of the CAM were positive in both age groups. In the 8-day-old ECE inoculated with the NDFLtag-GFP strain, positive staining was also observed in the epithelium of the

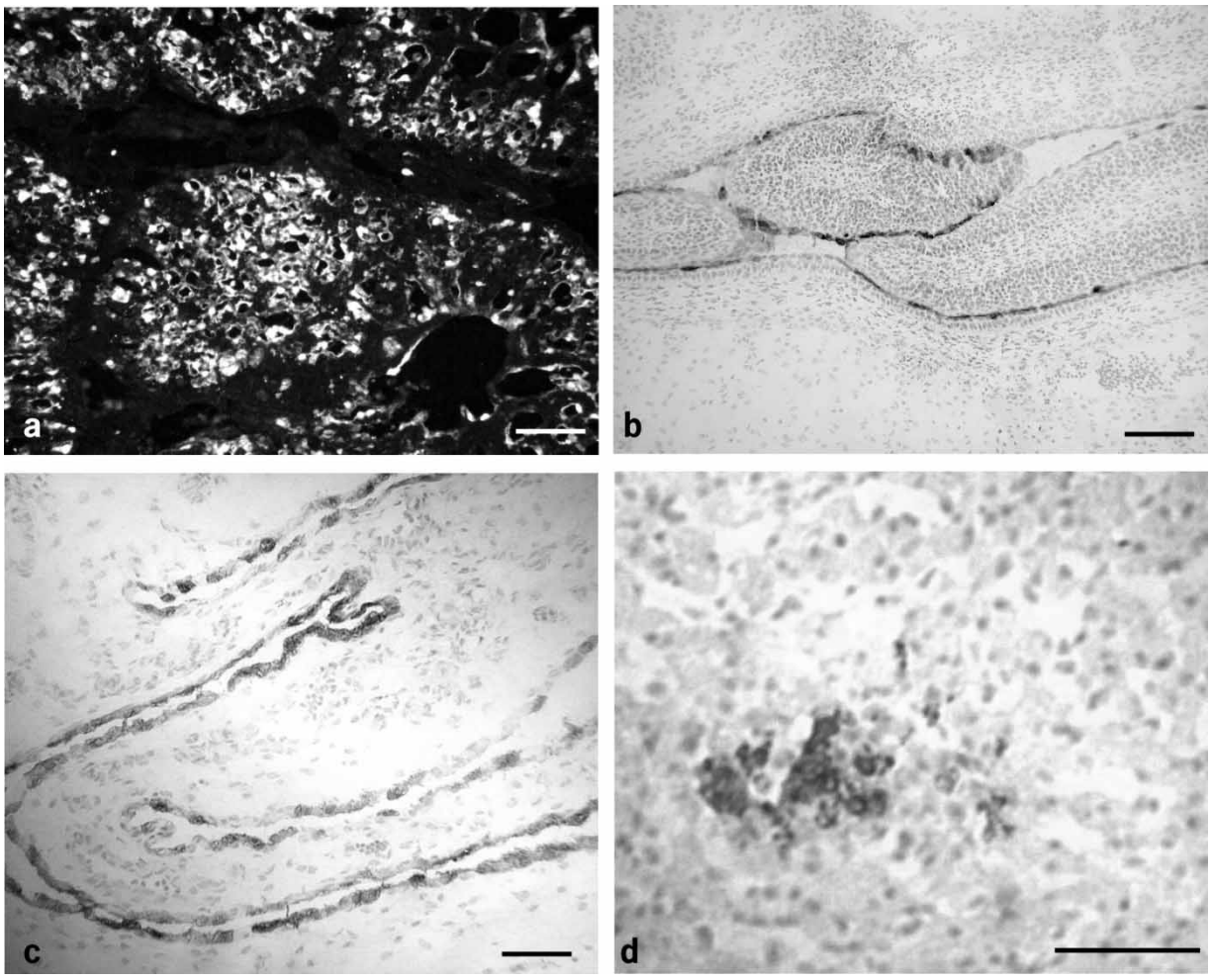

Figure 1. *Tissue sections examined without treatment using fluorescence microscopy to visualise the expression of GFP in lung (1a). Viral antigen was also detected in feather follicles (1b) of 8-day-old ECE and the CAM (1c) and spleen (1d) of 14-day-old ECE infected with a virulent NDFLtag-GFP strain of NDV by immunohistochemical staining. Bar = 40 μm (1a, 1b), 50 μm (1c) and 20 μm (1d).*

Table 1. *Tissue tropism in 8-day-old chicken embryos demonstrated by immunohistochemical staining after inoculation with the non-virulent NDFL-GFP and the virulent NDFLtag-GFP strains of NDV*

	Organ score[a]	
Time (h) after infection	CAM	Rest of the embryo
NDFL-GFP		
24	+++	–
48	+++	–
72	+++	+ skin
96	+++	+ skin
NDFLtag-GFP		
24	+++	+ skin[b]
48	+++	+ skin[b]
72	+++	+ skin[b]
96	+++	+ skin[b]

[a] Viral staining was categorized semiquantitatively: negative, –; dispersed positive cells or spots, +; medium positive areas, + +; and various groups of positive-stained cells, +++.
[b] Infection was detected in the epithelium of coelomic cavity and the capsules of the visceral organs.

peritoneum (coelomic cavity), skin and in the feather follicles of the wing region.

In the 14-day-old ECE, viral antigen was detected in various cell populations. In the lung, both strains were observed mainly in the epithelial cells of alveoli, macrophages and fibroblasts. In the spleen, viral antigen of the virulent strain was found in cells with features of dendritic cells, macrophages, and lymphocytes. In the heart, viral antigen was detected in myocardial cells and some fibroblasts.

The kinetics of virus replication in the CAM was examined at various time-points after infection. Generally, the rate of viral antigen detection by immunohistochemical staining was different for the NDFL-GFP and NDFLtag-GFP strains, but closely resembled each other in the 8-day-old and 14-day-old ECE (Table 3). Staining of the allantoic epithelium of the CAM was observed from 6 h after inoculation with the NDFLtag-GFP strain for both embryo groups. Only a few spots were detected in the CAM of embryos infected with the NDFL-GFP strain at 10 h after inoculation. Various groups of positively stained cells were observed in embryos from 14 h after inoculation with either the NDFL-GFP or NDFLtag-GFP. Moreover, in agreement with earlier data (Liu & Bang, 1953), higher virus titres were measured by haemagglutination test in the allantoic fluid of the 8-day-old embryo compared with the 14-day-old embryos at all time-points. Remarkably, the difference in virus titre was not reflected by the immunohistochemical data.

Table 2. *Tissue tropism in 14-day-old chicken embryos demonstrated by immunohistochemical staining after inoculation with the non-virulent NDFL-GFP and the virulent NDFLtag-GFP strains of NDV*

	Organ score[a]			
Time (h) after inoculation	CAM	Lung	Spleen	Heart
NDFL-GFP				
24	+++[b]	–	–	–
48	+++	–	–	–
72	+++	+	–	–
96	+++	+	–	–
NDFLtag-GFP				
24	+++	–	–	–
48	+++	+	+	+
72	+++	++	+	+
96	+++	+++	+++	+++

[a] Viral staining was categorized semiquantitatively: negative, –; dispersed positive cells or spots, +; medium positive areas, + +; and various groups of positive-stained cells, +++.

Table 3. *Viral titration (haemagglutination (HA) test) of the allantoic fluid and antigen detection in the CAM of 8-day-old and 14-day-old ECE inoculated with the non-virulent NDFL-GFP and the virulent NDFLtag-GFP strains of NDV*

	HA titre and histochemistry staining[a] in embryos inoculated at:			
	Day 8		Day 10	
Time (h) after inoculation	HA titre[b]	IHC	HA titre[b]	IHC
NDFL-GFP				
4	0	–	0	–
6	0	–	0	–
10	1	++	0	+
14	3	+++	0	+++
24	6	+++	2	+++
NDFLtag-GFP				
4	0	–	0	–
6	0	++	0	+
10	2^1	+++	0	++
14	2^2	+++	2^1	+++
24	2^5	+++	2^3	+++

IHC, immunohistochemical staining.
[a] Viral staining was categorized semiquantitatively: negative, –; dispersed positive cells or spots, +; medium positive areas, + +; and various groups of positive-stained cells, +++.
[b] Expressed as log (base 2).

Discussion

These data reveal that two viruses, NDFL-GFP and NDFLtag-GFP, which only differed in the amino acid sequence at the cleavage site of the fusion protein, had different tissue tropisms. Although, the non-virulent strain NDFL-GFP replicated in epithelial cells of the CAM and capsules of visceral organs, the virulent strain NDFLtag-GFP readily spread from the CAM to many internal organs. These results are largely in agreement with observations made by Nagai *et al.* (1979) who compared infection of the lentogenic enterotropic Ulster strain with the velogenic Italian strain of NDV in embryos. They found that the Ulster strain only replicated in the endoderm layer of the CAM, whereas the Italian strain was able to

penetrate the mesoderm (Nagai *et al*., 1979). The slight differences in spreading between the lentogenic NDFL-GFP strain and the lentogenic, asymptomatic Ulster strain might be due to sequence differences between the viruses, particularly in F and HN (Nagy *et al*., 1991). The Ulster strain has a slightly longer HN, that similar to F_0 has to be cleaved. When HN_0 is not cleaved the virus is even unable to attach to the cell receptor. Thus, from our results and those in the literature we can conclude that the sequence around the cleavage site is the main determinant of virulence for NDV (Peeters *et al*., 1999). Strains with multibasic residues in the F_0 cleavage site are virulent and infect a wide range of tissues, whereas strains that have single basic residues are non-virulent (Choppin & Scheid, 1980; Collins *et al*., 1993) and are able to infect some tissues only.

Cleavage of F proteins containing multibasic residues at the cleavage site occurs during transport of the protein through the *trans* Golgi network by a cellular protease, 'Furin-like' (Klenk & Garten, 1994). Purification of protease from the allantoic fluid of ECE has indicated that, for Sendai virus, activation is mediated by endoprotease that is homologous to blood-clotting factor X, a member of the prothrombin family (Gotoh *et al*., 1990, 1992). Clara cells of the bronchial epithelium in rats and mice secrete a protease with similar substrate specificity and this enzyme is probably responsible for activating paramyxoviruses in these species. Similar proteases that are responsible for cleavage of the NDFL-GFP fusion protein might be present in the allantoic fluid or in epithelial cells lining the cavity. As a result, viral antigen of the non-virulent NDFL-GFP virus was detected only in these tissues, whereas the fusion protein of the virulent strain NDFLtag-GFP is cleaved in various other tissues of the embryonated egg also, and therefore virus antigen could be demonstrated in spleen and heart sections.

Intracytoplasmic inclusion bodies have been observed in the epithelial cells of CAM of chick embryos infected with either velogenic, mesogenic or lentogenic strains of NDV (Menenedez & Martines, 1983). In the present study inclusion bodies were not encountered. Although our procedure could identify specific organs associated with viral replication, it was difficult to define the specific cells involved since only nuclei were counterstained. The histological location of positive staining suggested that many cell types, not only the epithelial cells, were infected. This finding fits with the data indicating that NDV has no strict selectivity for particular host cells in chicken embryos (Liu & Bang, 1953; Lam *et al*., 1995).

GFP-expressing recombinant NDV can be used to monitor infection of cells, embryos or chickens (Engel-Herbert *et al*., 2003). The advantage of GFP-expressing NDV is that large numbers of samples can easily be screened for virus replication using direct fluorescence microscopy without any pre-treatment. We show in this paper that it can also be used to study the effect on the tissue trop of small genome difference. Like Engel-Herbert *et al*. (2003), we did not observe any effect of the insertion site of the GFP gene on growth characteristics nor on the pathogenicity of the virus (data not shown), whereas the insertion site of the CAT or influenza haemagglutinin gene had an effect (Krishnamurthy *et al*., 2000; Nakaya *et al.*, 2001).

In conclusion, although autofluorescence using GFP appeared not to be more sensitive than immunohistochemical staining, it can be used to follow virus replication with a very simple methodology. Both 8-day-old and 14-day-old ECE were convenient experimental systems to investigate tissue tropism and pathogenicity of NDV strains. Using two viruses, which differ only in the F_0 cleavage site, we show that the cleavability of this protein is a major factor determining viral spread in chicken embryos.

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RÉSUMÉ

Etude du tropisme, au niveau des tissus embryonnaires, des souches de virus, virulentes et non virulentes, de la maladie de Newcastle qui expriment la protéine fluorescente verte.

L'étude du tropisme de virus, virulents et non virulents, de la maladie de Newcastle (NDV) a été réalisée en utilisant des œufs embryonnés de poule (ECE) âgés de 8 et 14 jours, inoculés avec un clone infectieux de la souche non virulente La Sota (NDFL-GFP) ou son clone virulent dérivé (NDFLtag-GFP). Les deux souches expriment le gène codant la protéine fluorescente verte de méduse (GFP) comme marqueur. La GFP a été facilement exprimée dans les cellules embryonnaires de poulet infectées par les souches de NDV prouvant ainsi la multiplication du virus. Alors que les deux souches se multiplient dans la membrane chorio-allantoïdienne (CAM) et infectent la peau des ECE inoculés à 8 jours, seule la souche virulente (NDFLtag-GFP) diffuse au niveau des organes internes (pleura/peritoneum). Chez les ECE inoculés à 14 jours, les organes cibles initiaux sont la CAM et les poumons pour les deux souches. La souche virulente (NDFLtag-GFP), 48 heures après inoculation, a diffusé au niveau de la rate du cœur et est détectée au niveau d'une grande variété de types de cellules. La cinétique de la multiplication du virus et la diffusion au niveau de la CAM sont semblables pour les deux souches inoculées aux ECE de 8 et 14 jours. L'inoculation aux ECE de 8 et 14 jours représente un modèle facile pour étudier le tropisme de tissu du NDV, ainsi que la cinétique de l'infection virale. L'avantage d'utiliser la GFP est que les échantillons peuvent être facilement screenés par microscopie à fluorescence directe sans aucun pré-traitement.

ZUSAMMENFASSUNG

Gewebetropismus im Hühnerembryo von avirulenten und virulenten Stämmen des Virus der Newcastle-Krankheit, die das Grünfluoreszenz-Protein exprimieren

Der Gewebetropismus von avirulenten und virulenten Viren der Newcastle-Krankheit (NDV) wurde unter Verwendung von 8- und 14-tägigen embryonierten Hühnereiern (ECE) untersucht, die mit einem infektiösen Klon des avirulenten La Sota-Stamms (NDFL-GFP) oder seines virulenten Derivats (NDFLtag-GFP) inokuliert wurden. Beide Stämme exprimierten das Gen, das für das Quallen-Grünfluoreszenz-Protein (GFP) kodiert als Marker. Das GFP wurde in den mit den NDV-Stämmen infizierten Hühnerembryozellen gebildet, was auf eine Virusvermehrung hinwies. Während sich beide Stämme in der Chorioallantoismembran (CAM) vermehrten und die Haut von achttägigen ECE infizierten, breitete sich nur der virulente Stamm (NDFLtag-GFP) auf innere Organe (Pleura/Peritonäum) aus. In 14-tägigen ECE schienen bei beiden Stämmen die ersten Zielorgane die CAM und die Lungen zu sein. 48 Stunden nach der Inokulation breitete sich der virulente Stamm (NDFLtag-GFP) auch auf die Milz und das Herz aus und wurde in einem breiten Spektrum von embryonalen Zelltypen nachgewiesen. Die Kinetik der Virusreplikation und –ausbreitung war in den 8- und 14tägigen ECE sehr ähnlich.

Die Infektion von 8- und 14tägigen ECE stellt ein geeignetes Modell dar, um den Gewebetropismus von NDV und die Kinetik der viralen Infektion zu studieren. Der Vorteil der Verwendung von GFP ist die leichte Untersuchung der Proben mittels direkter Fluoreszenzmikroskopie ohne irgendeine Vorbehandlung.

RESUMEN

Tropismo tisular en el embrión de pollo de cepas virulentas y no virulentas de virus de la enfermedad de Newcastle que expresan la proteína verde fluorescente

El tropismo tisular de virus virulentos y no virulentos de enfermedad de Newcastle (NDV) fue investigado mediante el uso de huevos embrionados de pollo (ECE) de 8 y 14 días de edad, inoculados con un clon infeccioso de la cepa no virulenta de La Sota (NDFL-GFP) o su derivado virulento (NDFLtag-GFP). Ambas cepas expresaban el gen que codifica para la proteína verde fluorescente de medusa (GFP) como marcador. Se detectó expresión de GFP en células de embrión de pollo infectadas con el NDV lo cual indicó replicación vírica. Mientras que ambas cepas se replicaban en la membrana corioalantoidea (CAM) e infectaron la piel de ECE de 8 días de edad, únicamente la cepa virulenta (NDFLtag-GFP) se diseminó a órganos internos (pleura/peritoneo). En ECE de 14 días de edad, los órganos diana iniciales fueron la CAM y los pulmones para ambas cepas. A las 48 h. tras la inoculación, la cepa virulenta (NDFLtag-GFP) se había también diseminado al bazo y corazón y fue detectada en un amplio rango de tipos celulares embrionarios. La cinética de la replicación vírica y la diseminación a la CAM fueron muy similares en los ECE de 8 y de 14 días de edad.

La infección de ECE de 8 y 14 días de edad representa un modelo conveniente para investigar el tropismo tisular del NDV, así como la cinética de la infección viral. La ventaja de usar las GFP es que las muestras pueden ser visualizadas fácilmente mediante el uso del microscopio de fluorescencia directa sin ningún tipo de pretratamiento.