The Spread of a Pathogenic and an Apathogenic Strain of Newcastle Disease Virus in the Chick Embryo as Depending on the Protease Sensitivity of the Virus Glycoproteins

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SUMMARY

The pathogenic strain Italien and the apathogenic strain Ulster of Newcastle disease virus have been compared with respect to organ tropism and spread of infection in 11-day-old chick embryos. After infection of the endodermal layer of the chorioallantoic membrane by intra-allantoic inoculation with strain Italien, high virus titres are found in all extra-embryonic membranes and fluids and in the embryo itself. Infection results in early death of the embryo. In contrast, after infection with strain Ulster by the same route of inoculation, high virus titres are found only in the allantoic sac and embryos are not killed. Inoculation with strain Italien on to the ectodermal layer through an artificial air sac results in rapid spread of infection in the chorioallantoic membrane and the embryo dies before the virus invades other tissues including the embryo. Under the same conditions of infection, strain Ulster neither spreads within chorioallantoic membrane nor does it kill the embryo. Virus spread in each germinal layer of the chorioallantoic membrane was analysed by immune fluorescence. These studies showed that endoderm as well as mesoderm and ectoderm allowed the spread of strain Italien, whereas only the endoderm is permissive for strain Ulster. These differences in host range are based upon differential activation of the virus glycoproteins by proteolytic cleavage. The glycoproteins of strain Italien are cleaved in each germinal layer, whereas those of strain Ulster are cleaved only in endoderm. These studies demonstrate that, in the system analysed here, spread of infection and organ tropism are important factors for pathogenicity and both of these factors are determined by the susceptibility of the virus glycoproteins to proteolytic cleavage.

INTRODUCTION

Newcastle disease virus (NDV) comprises a wide range of naturally occurring strains which differ in pathogenicity for their hosts, the chicken and the chick embryo (Waterson et al. 1967). Previous reports have shown that (1) precursors exist to both glycoproteins of NDV which are converted by proteolysis into the biologically active form, (2) NDV strains differ from each other with respect to the susceptibility of their glycoproteins to proteolytic cleavage which is believed to be carried out by host specific enzymes and (3) these differences are an important in vitro marker for the variations in pathogenicity (Nagai et al. 1976a; Nagai & Klenk, 1977). Thus, a concept has been proposed that infection with a pathogenic strain which produces virions with cleaved glycoproteins in a wide variety of cultured cells...
in vitro may spread more rapidly in the organism than infection with an apathogenic strain which produces virions with uncleaved glycoproteins in most cells. Similar conclusions have been made by Scheid & Choppin (1976) who showed that an altered protease susceptibility in glycoprotein F of Sendai virus also resulted in a change in host range.

The present study carried out in an intact organism further substantiates this concept. A pathogenic and an apathogenic strain were selected which widely differ in pathogenicity and their mode of spread in ovo has been compared. The data obtained indicate that the pathogenic strain has the capacity to spread in a wide variety of the embryonic tissues whereas the apathogenic strain shows a narrow tissue specificity. These differences were found to be due to differential activation of the virus glycoproteins by proteolytic cleavage.

METHODS

Virus strains. The pathogenic strain Italien and the apathogenic strain Ulster of NDV were used. Seed stocks were grown in the allantoic cavity of 11-day-old chick embryos inoculated with $10^{3.5}$ TCID$_{50}$. Allantoic fluid was harvested at 48 h p.i. with the pathogenic strain and at 72 h p.i. with the apathogenic strain and stored at $-80^\circ$C. The pathogenicity of both virus strains was re-examined by determining the mean death time (MDT) for chick embryos after intra-allantoic inoculation. Strain Italien showed a MDT of 50 h, whereas embryos infected with strain Ulster survived. These data are in good agreement with those of Waterson et al. (1967), indicating that the pathogenic properties of these strains have been maintained unchanged over more than 10 years.

Virus inoculation of chick embryos. Intra-allantoic inoculation was carried out through a hole drilled in the shell above the air sac, about 3 mm from the edge of the chorioallantois and the air sac. Chorioallantoic inoculation was carried out through an artificial air sac. An area of chorioallantoic membrane (CAM) which is well developed but free of blood vessels and usually located in the centre of the CAM, i.e. near the embryo, was separated from the shell membrane and dropped by formation of an artificial air sac. Inocula in a volume of 0.3 ml were deposited on to the dropped CAM using a 1 ml syringe. Details of these two techniques of inoculation were based upon the description by Blaškovič & Styk (1967).

Sampling and processing of the materials from infected eggs. In order to determine the virus titre in various sites of the infected egg, allantoic fluid, CAM, embryo, amniotic fluid and yolk sac were harvested separately from the individual eggs. Usually 10 or more eggs were used for each determination. At appropriate times after inoculation, a circular opening was made in the shell above the air sac or the artificial air sac, depending on the inoculation method, and 1 ml of allantoic fluid was taken by syringe and pooled. A circular opening was then cut with scissors in the shell membrane and the CAM below it to a width of about 20 to 30 mm. The egg was tilted and the contents were allowed to drain out slowly through the opening in the shell membrane and the CAM, taking care that the CAM remained adhered to the whole inner side of the shell. While the contents were draining out, the remaining allantoic fluid was discarded and the amniotic fluid (usually 0.5 to 3 ml) was aspirated with a syringe. The contents (minus the CAM) were then put into a Petri dish, and embryo and yolk sac were collected in separate dishes after cutting the connections between embryo and extra-embryonic membranes. The CAM was then harvested by removing it from the shell with a pincette. After separation, embryos, yolk sacs and CAMs were washed repeatedly with PBS, cut into pieces and homogenized with a glass homogenizer in PBS to give suspensions of 30, 20 and 10%, respectively. The samples were then sonicated for 30 s with an ultrasonic oscillator (Tominage type UR 168 W, 20 kHz) and centrifuged at 850 g for 15 min. The supernatants were assayed for their haemagglutinating activity and infectivity.
Pathogenicity of NDV for chick embryo

Virus assays. The method for haemagglutination titrations was described previously (Maeno et al. 1970). Infectivity titrations were carried out by determining the infectivity for MDBK cells incubated in the presence of trypsin (Nagai et al. 1976a). MDBK cells grown in microplates with reinforced Eagle's medium (REM) containing 10% foetal calf serum were infected with serial virus dilutions and after washing once with PBS they were incubated for 72 to 96 h in the presence of trypsin (1 μg/ml). Infectivity titres were determined by 50% infectivity end points (TCID$_{50}$/ml).

Antiserum and fluorescent antibody staining. Antiserum against strain Ulster grown in LLCMK$_2$ cells was obtained from immunized rabbits. The serum was adsorbed extensively with the powder of normal CAM of 11-day-old chick embryo. It exhibited 640 haemagglutination inhibition units against 4 haemagglutinin units of both strains Ulster and Italien. For immunofluorescent studies, pieces of CAM with adjacent shell membrane were obtained from the area below the air sac in the case of intra-allantoic inoculation. In the case of chorioallantoic membrane inoculation, CAM was taken from the site of inoculation below the artificial air sac and from an area opposite the site of inoculation. After rinsing with PBS, the CAMs were folded and frozen sections of 10 μm thickness were cut on a Lipshaw cryostat. The sections were then dried, fixed in cold acetone and reacted with the antiviral serum overnight at 4 °C. They were then washed with PBS, stained with fluorescein-conjugated anti-rabbit-γ-globulin and observed under a fluorescent microscope (Olympus model FLM).

Analysis of virus grown in each of the three germinal layers of the CAM. After cleaning the shell, the egg was cut in half transversely round the shell with scissors. The contents of the egg were discarded, taking care that the CAM remained adhered to the upper half of the shell. This part of the de-embryonated egg was then rinsed with PBS and the endodermal epithelium exposed at the inner side was used for virus growth. To grow virus in the ectodermal epithelium, the inner side was covered with 2% agar in minimal essential medium. After solidification of the agar, a circular opening was made in the shell in the area above the air sac. Three to four of these CAM on shell preparations were transferred into a 10 cm Petri dish and fixed with 2% agar in such a way that the area of the shell membrane below the air sac was exposed. The shell membrane was then carefully detached and removed from the outer surface of the CAM which was now accessible to virus inoculation. The principal cell found in the mesoderm is the fibroblast, although this layer also contains collagen bundles and variable numbers of blood vessels (Donnelly & Yunis, 1971). Cultures of fibroblasts were prepared by cutting CAMs into pieces with scissors followed by digestion with trypsin (Difco; 1:250, 0.2%/o) in the presence of EDTA (0.2%). The cells obtained were seeded in Petri dishes. They were mixed populations of fibroblasts and epithelial cells. However, after two subsequent passages with MEM containing 10% foetal calf serum, the epithelial cells were eliminated and cultures consisting mainly of fibroblasts could be obtained.

Endoderm, mesoderm and ectoderm preparations were inoculated with 1 ml stock virus with an infectivity of $10^{6.5}$ TCID$_{50}$. After washing with PBS, cultures were incubated in MEM containing 20 μCi/ml of 6-³H-glucosamine (10 to 30 mCi/mmol, New England Nuclear Corp., Boston, Mass.). The MEM contained 10 mM-fructose instead of glucose (Nagai et al. 1976b). After incubation for 15 to 18 h, the virions were collected from the culture medium and purified as described previously (Nagai et al. 1976a).

Polyacrylamide gel electrophoresis. High resolution polyacrylamide slab gel electrophoresis in tris-glycine buffer with sodium dodecyl sulphate (SDS; Laemmli, 1970) was used for analysis of the virus glycoproteins. For detection of ³H-glucosamine, gels were treated as described by Bonner & Laskey (1974) with dimethyl sulphoxide and 2, 5-diphenyl oxazole prior to drying and were exposed to Kodak RP Royal 'X-Omat' film at −70 °C, followed by scanning of the film in a Densitron, model-PAN (Jookoo Sangyo Co. Ltd.).
RESULTS

Strain-dependent differences in virus spread in ovo

Fig. 1. shows the kinetics of virus growth in various sites of chick embryo. The input dose ($10^{5.5}$ TCID$_{50}$) was inoculated into the allantoic cavity. The growth of the pathogenic strain Italien was first detectable in the allantoic sac and also in the CAM. The virus titre in these sites had reached a maximum by 30 h. Later on, the titre increased in the embryo as well as in the amnion and yolk sac. The increase of the virus titre appeared to be paralleled by the development of the pathological changes of the embryo ultimately resulting in death (Fig. 1c). It should be noted that the most prominent pathological symptom, severe subcutaneous haemorrhage on the whole body, could be detected at 35 h when virus titres were high in the CAM but had not yet reached their maxima in the embryo and other tissues.

After inoculation by the same route, the growth characteristics of the apathogenic strain Ulster are in some sites similar to and in others completely different from those of Italien. In the allantoic sac the virus titre increased as rapidly as that of Italien and reached a comparable level (Fig. 1a). In the CAM, Ulster has almost the same growth rate as Italien during
The spread of both strains in 11-day-old chick embryos was also analysed when infection was initiated at the ectodermal side of the CAM. Viruses were inoculated with either $10^{7.5}$ or $10^{8.5}$ TCID$_{50}$ in 0.3 ml. After appropriate periods of incubation at 37 °C, virus titres were determined in various parts of the egg. As shown in Table I, infection with strain Italien gave a high virus yield in the CAM. However, the virus titre in the allantoic sac was significantly lower than after infection via the allantoic sac (cf. Fig. 1a). Furthermore, there was no detectable haemagglutinin in the embryo and in the amniotic sac. The yolk sac showed virus growth to some extent, although the titre was much lower than after intra-allantoic inoculation (cf. Fig. 1e). Nevertheless, all the embryos were found to be dead and showed severe bleeding over the entire skin. Thus, the pathogenic strain inoculated on to the ectodermal side of the CAM appears to cause the death of the embryo when it has spread extensively in the CAM but before it has invaded other tissues including the embryo.

In contrast, after infection with strain Ulster under the same conditions there was little virus production in any part of the embryo including the CAM, indicating that this virus cannot spread readily from the chorionic side of the CAM (Table I). The embryos usually survive. Occasional death appears to be the result of traumatic damage during the inoculation procedure as suggested by the absence of detectable amounts of virus in these eggs.

**Virus growth in the different germinal layers of the CAM**

As described in the preceding section virus inoculation at the ectodermal side of the CAM and inoculation at the endodermal side result in different modes of spread of infection in ovo. It was therefore of interest to analyse virus growth in the individual germinal layers of the CAM. Fig. 2 shows the development of virus specific antigens in these layers as detected by staining with fluorescent antibodies. A relatively high input dose ($10^{7.5}$ TCID$_{50}$) was used to secure synchronous infection and the CAMs were harvested with the shell membrane adjacent to the ectoderm in order to facilitate identification of the individual cell layers. The shell membranes (labelled S) are easily recognized since they show strong non-specific fluorescence (Fig. 2a, b) which is also observed in samples from non-infected eggs (not shown).

Forty-eight h after endodermal infection with strain Italien, specific fluorescence is detectable in the entire CAM, i.e. in the endodermal allantoic epithelium (En) which consists predominantly of a single cell layer, in the ectodermal chorionic epithelium (Ec) beneath the shell membrane and in the mesodermal layer (M) which occupies a broad area between the early incubation period. However, at about 20 h, spread of infection with strain Ulster appears to cease. The increase in virus titre in the CAM that is observed with strain Italien at later times has not been found with strain Ulster. Further, the titres in the embryo, amniotic fluid and yolk sac were all found to remain at very low levels, and neither pathological changes nor the death of the embryo have been observed throughout the observation period.
Fig. 2. Fluorescent antibody-staining of virus antigens in sections of CAM. Virus \((10^{5.5} \text{ TCID}_{50})\) was inoculated into the allantoic cavity or on to the outer surface of the CAM. (a) The area of the CAM below the air sac after intra-allantoic inoculation with strain Italien. CAM was harvested together with the shell membrane at 48 h. (b) The area below the air sac harvested together with the shell membrane at 72 h after intra-allantoic inoculation with strain Ulster. (c) The area below the artificial air sac (site of inoculation) harvested at 40 h after chorioallantoic inoculation with strain Italien. (d) The same CAM as shown in (c), but an area distant from the site of inoculation. (e) The area below the artificial air sac harvested at 40 h after chorioallantoic inoculation with strain Ulster. (f) The same CAM as shown in (e), but an area distant from the site of inoculation. S, shell membrane; En, endoderm; M, mesoderm; Ec, ectoderm. Magnification \(\times 100\).

the inner and the outer epithelial layers (Fig. 2a). Samples obtained at 24 h p.i. were also examined (data not shown) and the results indicated that the endoderm already shows full fluorescence. The mesoderm and ectoderm also displayed specific fluorescence at this stage but its intensity was significantly lower than at 48 h. These findings indicate that the
pathogenic strain inoculated into the allantoic sac multiplies initially in the endoderm and spreads rapidly from there to the entire CAM.

In contrast, after endodermal infection with the apathogenic strain Ulster, specific fluorescence was observed in the endodermal layer but not in the mesodermal and ectodermal layers throughout the observation period until 72 h (Fig. 2b). It was further found that in the endoderm fluorescence developed and attained strong intensity as rapidly as after infection with strain Italien. Similar results were obtained when a low virus dose ($10^{3.5}$ TCID$_{50}$) was used for inoculation. These data indicate that the apathogenic strain can grow well in the endoderm but not spread from there to other germinal layers. This would explain the observation that replication of the apathogenic strain ceases in the CAM after a rapid increase in the early incubation period without reaching the titre of the pathogenic strain (cf. Fig. 1b). However, virus replication in the endoderm appears to be sufficient to produce virus in the allantoic fluid in amounts that are as high as after infection with the pathogenic strain (cf. Fig. 1a).

Striking differences in tissue specificity between the strains have been also demonstrated after inoculation of the chorionic epithelium of the CAM. At 40 h p.i. with strain Italien, the membrane region at the site of inoculation was harvested and examined by immunofluorescence. The CAM was harvested without the shell membrane, since both membranes had been separated from each other prior to inoculation. However, it is possible to distinguish the two different epithelial layers from each other since the endoderm is formed predominantly by a single cell layer whereas the ectoderm consists of several layers (Romanoff, 1960). As shown in Fig. 2(c), the specific fluorescence induced by strain Italien is most distinct in the chorionic epithelium, but it is also detectable in the mesoderm, indicating again a rapid spread of this strain from one CAM layer to another. However, the endodermal cells showed little specific fluorescence under this condition of infection. This is apparently due to the rapid death of the embryo before the virus is able to arrive and grow at the endoderm. Thus, the allantoic fluid of the egg used in this experiment showed no detectable haemagglutinating activity, although the embryo was dead. Areas of the CAM located at a far distance from the site of inoculation were also examined. As shown in Fig. 2(d), such areas show distinct fluorescence in the entire ectodermal layer and in the cells surrounding the blood vessels present in the mesoderm. This interesting observation indicates that the virus infection did proceed not only by cell spread but also through the blood stream.

Fig. 2(e, f) show the CAM after ectodermal inoculation with strain Ulster. Specific fluorescence is present only in ectodermal cells of the site of inoculation (Fig. 2e). The ectodermal layer is many cells thick as described above and it is interesting to note that only the outermost cells of this layer exhibit fluorescence, whereas underlying cells of the chorionic epithelium do not. It is reasonable to assume that this fluorescence pattern is the result of single cycle replication of the input virus. This view has been supported by the observation that antigen-positive cells are rarely seen in CAM areas distant from the site of inoculation (Fig. 2f). Thus, after ectodermal inoculation, infection with the apathogenic strain cannot spread into the mesoderm and the endoderm and it is confined to the site of inoculation.

**Glycoprotein structure of virions grown in different CAM layers**

Previous studies on NDV have shown that glycoprotein F acquires biological activity by proteolytic cleavage of a precursor F$_0$. With strain Ulster a precursor HN$_0$ has also been identified which is again converted by proteolytic cleavage into the biologically active HN glycoprotein. Furthermore, studies in various types of cultured cells have shown that it depends on cleavage whether or not the virus is able to undergo multiple replication cycles (Nagai et al. 1976a; Nagai & Klenk, 1977). It was therefore of interest to examine whether
Fig. 3. The glycoproteins of virions of strain Italian and Ulster grown in (a) ectodermal chorionic epithelium, (b) mesodermal fibroblasts, and (c) endodermal allantoic epithelium. Virus was labelled with \( ^3\)H-glucosamine and analysed by polyacrylamide gel electrophoresis. Migration is from the left to the right. The radioactivity was detected by scintillation autography followed by scanning of the film in a densitometer. Under the electrophoresis conditions used, glycoproteins HN\(_0\), HN, F\(_0\), and F can be recognized. The smaller cleavage fragment of F\(_0\) (Scheid & Choppin, 1977) does not appear.

such a principle could account for the striking differences between the pathogenic and the apathogenic strain in their ability to spread in the CAM. Virus was grown in preparations of ectoderm, mesoderm and endoderm as described in Methods. Cultures were infected with a high input dose (\(10^{6.5}\) TCID\(_{50}\)) and were maintained in MEM containing \( ^3\)H-glucosamine for 15 h. Fig. 3 shows the glycoprotein patterns of virions purified from the culture medium. The pathogenic strain Italian produces virions with cleaved glycoproteins in each layer of the CAM. In contrast, after infection with the apathogenic strain Ulster such virions are formed only in endodermal cells. Mesodermal and ectodermal cells form virions containing predominantly the uncleaved glycoproteins HN\(_0\) and F\(_0\). These results together with those of the preceding section demonstrate that infection with the pathogenic strain can spread in the entire CAM, because the glycoproteins of this virus are cleaved in all CAM layers. In contrast, the glycoproteins of the apathogenic strain are activated only in the endoderm. Thus, this virus cannot spread in the mesoderm and the ectoderm.
DISCUSSION

The data show that the pathogenic strain Italian undergoes multiple replication cycles and thus readily spreads in all germinal layers of the CAM, whereas the apathogenic strain Ulster does so only in the endoderm. Thus, as previously demonstrated under *in vitro* conditions (Nagai *et al.* 1976a), the pathogenic strain also has a broader host range *in vivo* than the apathogenic strain. These results are largely in agreement with observations made over 25 years ago by Bang and his colleagues who also found that both types of viruses grew to high titres in the allantoic sac, whereas only pathogenic viruses were able to penetrate into the mesoderm, thus gaining entrance to the blood vessels and spreading from there (Bang, 1953; Liu & Bang, 1953; Bang & Luttrell, 1961). This concept is also confirmed by more recent electron microscopic studies demonstrating that morphogenesis of an apathogenic strain was confined to the endoderm (Feller *et al.* 1969), whereas after infection with a pathogenic strain all CAM layers showed virus maturation (Yunis & Donnelly, 1969). However, the cause for the differences in the virus growth was not understood at that time.

The data presented here clearly demonstrate that it is based upon the differential activation of the virus glycoproteins by proteolytic cleavage. The glycoproteins of the pathogenic strain are cleaved in each of the CAM layers, whereas those of the apathogenic strain are cleaved only in endoderm. As suggested previously by an experiment involving coinfection with the pathogenic and the apathogenic strains in certain types of cell, the relative resistance of the glycoproteins of the apathogenic strain to cleavage is a genuine structural property of these molecules rather than a result of a lower level of proteolysis in the infected cells with the apathogenic strain (Nagai *et al.* 1976a).

The CAM is the site through which the virus enters the embryo and our studies showed that proteolytic activation in this organ is of prime importance for the further development of the infection. Depending on the virus strain and the inoculation site on CAM, four patterns of virus spread could be discriminated. (1) Inoculation at the endodermal site of the CAM with the pathogenic strain resulted in generalized virus spread into all extra- and intra-embryonic tissues analysed and in the death of the embryo. Compared to the CAM, most other embryonic organs have a complex fine structure and are difficult to manipulate. This has prevented us from analysing cleavage of the virus glycoproteins in these organs. However, since the glycoproteins of pathogenic strains were found to be cleaved in all cell types analysed to date, it is reasonable to assume that efficient cleavage is an important factor for the rapid spread of this type of virus in the embryo. (2) After endodermal inoculation with the apathogenic strain high virus titres are produced only in the allantoic sac, because infection is not able to penetrate the barrier of the non-permissive mesoderm. For reasons not fully understood infection of the endoderm apparently does not interfere with the vital functions of the embryo which therefore survives. (3) However, confinement of infection to a single organ can also be lethal. Thus, after ectodermal inoculation with the pathogenic strain, extensive virus replication is observed only in the respiratory apparatus of the embryo located in the mesodermal and ectodermal layer of the CAM (Romanoff, 1960). Infection of this organ appears to be sufficient to kill the embryo before the virus has a chance to spread into other tissues. (4) Finally, ectodermal infection with the apathogenic strain produces, directly at the inoculation site, non-infectious virus which cannot undergo further replication cycles. Infection is therefore limited to a small area of the ectoderm and the embryo survives. These observations demonstrate that the susceptibility of the virus glycoproteins to proteolytic cleavage is of high importance for organ tropism and spread of infection and thus for pathogenesis.

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