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Growth in chick chorioallantoic membranes of strains of Newcastle disease virus of differing virulence

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SUMMARY

The growth of eight strains of Newcastle disease virus in chick embryo chorioallantoic membranes was studied by comparing, at different times after infection, the amounts of haemagglutinin released into the allantoic fluid (extracellular haemagglutinin) with that associated with the membrane (cell-associated haemagglutinin). The virulence of the strains examined differed in that some killed chick embryos more rapidly than others. All strains released similar amounts of extracellular haemagglutinin and maximum titres were achieved about 12 hr. after infection. With virulent strains cell-associated haemagglutinin titres increased exponentially until the death of the host and maximum titres were much higher than those of extracellular haemagglutinin. With avirulent strains cell-associated haemagglutinin titres increased exponentially for only a limited time and titres were always lower than the titres of extracellular haemagglutinin.

Similar results were obtained when the titres of neuraminidase and viral ribonucleoprotein were measured during the growth of two virulent and two avirulent strains. Virulence appears to be associated with the continued intracellular accumulation of viral antigens.

INTRODUCTION

Newcastle disease virus (NDV) has been suggested as a suitable virus for the study of virulence (Waterson, Pennington & Allan, 1967). Many strains are available and their virulence, defined here as the ability to cause disease or death in a host, ranges from highly virulent strains, which are extremely pathogenic for chickens, kill chick embryos rapidly and destroy infected chick embryo fibroblast cells in culture, to strains isolated from apparently normal chickens which are not cytopathic and kill chick embryos infrequently or not at all. Virulence can be measured accurately and reproducibly in chick embryos and young chickens, and all strains grow readily in the chick embryo chorioallantoic membrane and release high titres of infectious virus into the allantoic fluid. They can, therefore, be cultured in quantity. Virus titres can be accurately and easily measured in terms of infectivity, haemagglutinin and neuraminidase activity. Besides this, NDV is not only an important animal pathogen in its own right, but is biologically akin to some important pathogens of man and other vertebrates, including mumps, the

para-influenzas, measles and distemper viruses: studies with NDV are thus relevant in a wide context.

Studies on the isolated virion show a surprising uniformity among strains: all those so far examined under the electron microscope look alike (Waterson & Cruickshank, 1963) and no major serological differences have been seen using serum neutralization or haemagglutination-inhibition (HAI) tests (W. H. Allan, unpublished observations) or in gel diffusion studies (Pennington, 1967).

Strains which differ in virulence for the chick embryo also differ, in parallel with this, in cytopathogenicity. Schloer & Hanson (1968) have shown, for example, that the most virulent strains for chick embryos produce the largest plaques on chick embryo fibroblast monolayers. This suggests that it is in the intracellular stages of virus development that strain differences might be found.

T. H. Pennington (unpublished observations) has reported differences in the quantity of haemagglutinin found in the chorioallantoic membranes of chick embryos infected with different strains of NDV, although with all strains substantially similar quantities of haemagglutinin (HA) were released into the allantoic fluid. However, these findings were based on samples taken, for all strains, 40 hr. after inoculation of the chick embryos: thus no information was available on the rates at which the haemagglutinin was produced. We have, therefore, measured the production of haemagglutinin in chorioallantoic membranes of chick embryos throughout one cycle of growth of several strains of NDV. We have compared these results with the titres of haemagglutinin released into the allantoic fluid and we have also measured the rates at which neuraminidase (Mucopolysaccharide *N*-acetylneuraminylhydrolase) and viral ribonucleoprotein are produced.

METHODS

Viruses

The following strains of NDV were used:

Herts 33 (Herts), received from W. H. Allan, Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, Weybridge, Surrey. Isolated at Weybridge in 1933 by Dobson (1939).

Italien, received from Professor R. Rott, Institute of Virology, Giessen, Germany. Isolated in Italy at some date before 1949 (Schäfer, Schramm & Traub, 1949).

Texas GB, (Texas), received from W. H. Allan; isolated in 1948 near Austin, Texas, U.S.A.

Field Pheasant, isolated at Weybridge in 1962 from pheasants; clinically more virulent than Herts 33 (W. H. Allan, personal communication).

Beaudette C, received from Professor R. Rott. This is a heat stable variant isolated from the Beaudette strain by Granoff (1959).

Strain F, received from W. H. Allan, isolated in England by Asplin in 1949 (Asplin, 1952).

Queensland V4 (Queensland), received from W. H. Allan, isolated in 1966 by Rylie, in Brisbane, Australia (W. H. Allan, personal communication).

Ulster, received from J. B. McFerran, Ministry of Agriculture for Ireland, Belfast. Isolated in 1966 in Northern Ireland from the faeces passed by healthy chickens.

The viruses were grown in the allantois of 10-day-old chick embryos incubated at 37° C., and virus-infected allantoic fluids were stored at −70° C.

Growth cycles in chick embryo chorioallantoic membranes

For each experiment about 50 10-day-old chick embryos were inoculated with 0·5 ml. of infected allantoic fluid containing about 10^9 Egg $_{ID50}$, and incubated at 37° C. At intervals thereafter three chick embryos were harvested and the allantoic fluid pooled. The chorioallantoic membranes were pooled after three washes with ice cold saline (0·15 M). All samples were stored at −70° C. until assayed.

Allantoic membranes were thawed and blended in homogenizers with motor-driven Teflon pestles to give 50 % (w/v) suspensions in phosphate buffered saline, pH 7·2 (PBS).

Haemagglutination titrations

Plastic plates were used with 0·2 ml. volumes and 1 % (v/v) chicken erythrocytes; titres are expressed as the reciprocal of the last dilution giving complete agglutination.

Neuraminidase assays

Sample volumes of 0·1 ml. were incubated at 37° C. for 10 min. with 0·1 ml. fetuin and 0·3 ml. 0·2 M-KH_2PO_4-Na_2HPO_4 buffer (pH 6·0). Fetuin was prepared from foetal calf serum by the method of Graham (1961). The *N*-acetyl neuraminic acid (NANA) released was assayed by the method of Aminoff (1961). Results are expressed as μg. NANA liberated per ml. of sample.

Incorporation of ^3H-uridine

The allantoic fluid of each egg at the start of the growth cycle was inoculated with 100 μc. ^{3}H-uridine 5-T (purchased from the Radiochemical Centre, Amersham).

The estimation of ^{3}H-uridine incorporated into ribonuclease-insensitive acid-insoluble material was based on the method of Plagemann (1968). For each sample, 0·1 ml. was diluted with 0·5 ml. 0·01 M sodium deoxycholate, 0·1 ml. ribonuclease (100 μg. per ml. in PBS) and incubated at 37° C. for 30 min.; 2·0 ml. 1 N perchloric acid was added and, after centrifugation at 1000 *g* for 10 min., the precipitate was washed three times with ice cold 5 % (w/v) trichloracetic acid. The precipitate was dissolved by adding 0·5 ml. 1 N sodium hydroxide and incubating at 60° C. for 30 min.: 0·2 ml. of this solution was added to 10 ml. of scintillation fluid containing 8·0 g. butyl PBD (Ciba Ltd), 80·0 g. naphthalene, 400 ml. oxitol (ICI Ltd) and 600 ml. toluene. Radioactivity was measured using an ABAC SL 40 Liquid Scintillation Spectrometer (Inter-technique Ltd) with an efficiency of about 35 %.

RESULTS

The growth of seven strains of NDV in chick embryo chorioallantoic membranes was examined by measuring the amount of haemagglutinin released into the allantoic fluid (extracellular haemagglutinin) and that still associated with the allantoic membrane (cell-associated haemagglutinin). The strains examined differed in virulence in that some killed chick embryos more quickly than others; Table 1 shows the mean death time (MDT) of chick embryos inoculated with one minimum lethal dose. Herts was the most virulent strain examined, with an MDT of 49 hr. Neither Queensland nor Ulster consistently killed chick embryos, many of which survived even high inocula of these strains. With all strains an increase in haemagglutinin was first detected in the allantoic fluid about 4 hr. after inoculation, and thereafter titres increased exponentially until 12–14 hr. after inoculation (Fig. 1).

Table 1. *Relative virulence of Newcastle disease virus strains* (*from Waterson, Pennington & Allan,* 1967)

Strain	Mean death time (hr.)	Intracerebral index
Herts	49	1·88
Italien	50	1·86
Texas	50	1·80
Beaudette C	62	1·48
F	168	0·25
Queensland	∞	0·25
Ulster	∞	0·00

Mean death time is the average time in which eggs inoculated with one minimum lethal dose of virus are killed. The intracerebral index is estimated from the time taken for chicks to die after intracerebral inoculation. The results are based on a scoring system in which the maximum index is 2 (100 % mortality in one day) and the minimum is 0 (no recorded symptoms after 8 days).

All strains produced approximately the same levels of extracellular haemagglutinin but marked differences were apparent when haemagglutinin associated with the allantoic membranes was assayed. With strains Herts, Italien and Beaudette C the production of cell-associated haemagglutinin continued exponentially until at least 20 hr. after inoculation and only diminished when at least 50 % of the chick embryos were already dead (Table 2). Cell-associated haemagglutinin titres of these virulent strains greatly exceeded those measured in the allantoic fluid at the end of the growth cycle.

Strains Queensland, Ulster and F, on the other hand, produced only low levels of cell-associated haemagglutinin and the rate of production appeared to diminish after approximately 12 hr. The titres of cell-associated haemagglutinin in these avirulent strains never exceeded those measured in the allantoic fluid.

All chick embryos inoculated with the strains Herts, Italien and Beaudette C had died after approximately 30 hr.; of the less virulent strains only F consistently killed the majority of chick embryos (90 % dead within 96 hr.) whereas

50 % of chick embryos inoculated with Queensland and Ulster survived for seven days after inoculation (Table 2).

Four strains (Herts, Texas, Queensland and Ulster) were examined in greater detail to see if other structural antigens making up the NDV virions, viz. neuraminidase and ribonucleoprotein, also accumulated in the allantoic membranes infected with virulent virus but not in those infected with avirulent strains. The results obtained supported this possibility: ^{3}H-uridine incorporated into ribonuclease-resistant, acid-insoluble material and neuraminidase titres both increased exponentially in allantoic membranes infected with the virulent strains Herts and

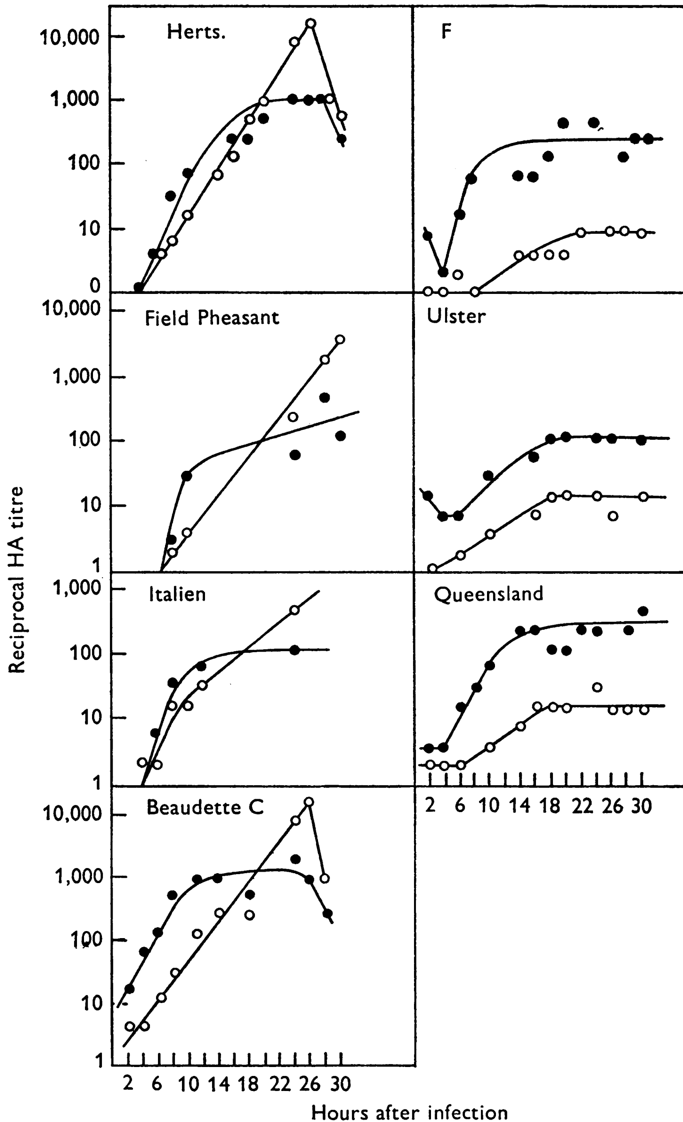

Fig. 1. The comparison of cell-associated (○—○) and extracellular (●—●) haemagglutination titres during the growth of seven different strains of NDV in eggs.

Texas until the embryos died (Fig. 2). We have assumed that the ^{3}H-labelled product is either viral RNA or ribonucleoprotein: both double-stranded RNA (Plagemann, 1968) and ribonucleoprotein (Kingsbury & Darlington, 1968) resist digestion with ribonuclease whereas single-stranded (cellular) RNA would be destroyed. In allantoic membranes infected with the avirulent strain Queensland, titres of neuraminidase and ribonuclease-resistant, acid-insoluble ^{3}H-labelled material increased exponentially only for the first 14–16 hr. after infection and then levelled off; with strain Ulster the exponential phase extended rather longer but levelled off within 24 hr. after infection (Fig. 3).

Table 2. *Percentage death of chick embryos in eggs inoculated with different strains of Newcastle disease virus*

Time after inoculation (hr.)	Strains of NDV					
	Herts	Italien	Beaudette C	F	Queensland	Ulster
22	0	0	0	0	0	0
24	7·5	—	0	0	0	0
26	15	59	9	0	0	0
28	34	78	56	0	0	0
29	—	100	—	0	0	0
30	44	.	100	0	0	0
32	100	.	.	0	0	0
48	.	.	.	0	0	0
69	.	.	.	57	7	20
74	.	.	.	70	7	20
89	.	.	.	97	20	27
96	.	.	.	100	23	30
114	.	.	.	.	36	43
138	.	.	.	.	40	53
142	.	.	.	.	57	53

DISCUSSION

The results obtained show that some strains of Newcastle disease virus had higher titres of viral components associated with the chorioallantoic membrane than with the allantoic fluid, suggesting two discrete groups of strains; (1) Virulent strains which kill chick embryos rapidly and accumulate all the major viral components in the chorioallantoic membrane to titres higher than in the allantoic fluid, and (2) avirulent strains which kill embryos slowly or not at all and produce similar extracellular antigen titres to virulent strains but in which the intracellular accumulation of virus antigen is limited.

Our results show that virulence is associated with the continued exponential production of cell-associated viral antigens without concomitant release of mature virions, a process terminated only by the death of the chick embryo.

Newcastle disease virus, like other myxoviruses, is assembled at the cell surface and the envelope may consist of or contain cell membrane (Waterson, 1968). The modification of the cell membrane presumably has a drastic effect on the cell

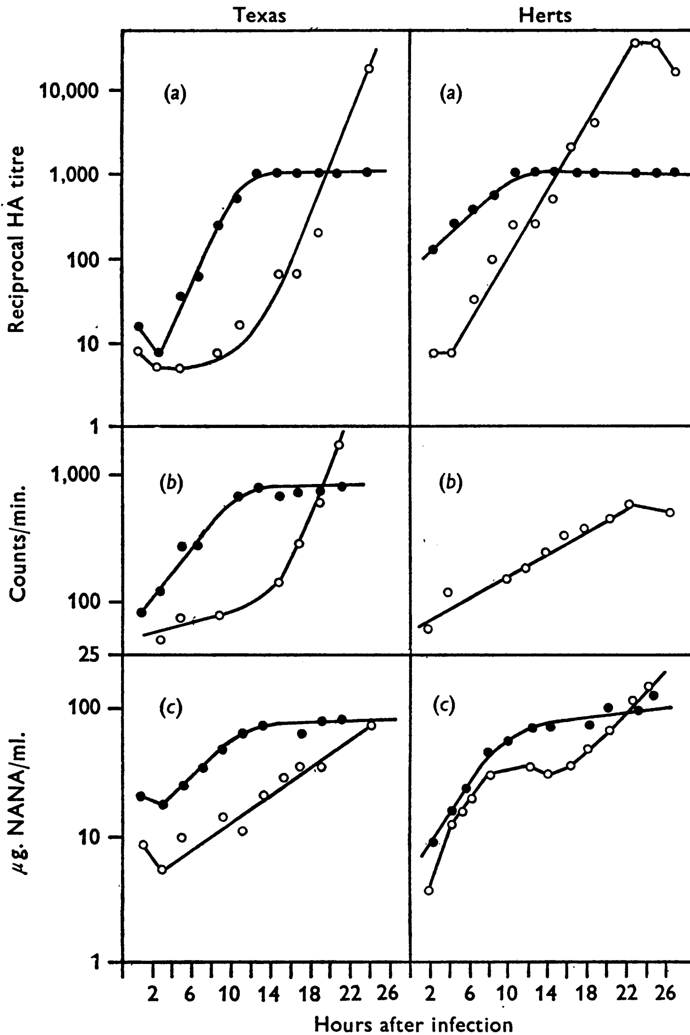

Fig. 2. Comparison of the extracellular (●—●) and cell-associated (○—○) major antigens of two virulent strains of NDV during growth in eggs. (*a*) Haemagglutinin. (*b*) Material incorporating ^{3}H-uridine, in an acid-insoluble RNase-resistant fraction. (*c*) Neuraminidase, as μg. NANA released.

permeability and metabolism. It could therefore be postulated that the strains of virus producing the most intracellular haemagglutinin would be the most virulent. The loss of ability to carry out cellular repair would enhance this effect and Wilson (1968) has shown that infection with the strain Texas GB, which is highly virulent, inhibits host cell protein synthesis in chick embryo fibroblasts.

Compans *et al.* (1966) in a morphological study showed that BHK21-F cells infected with SV5 virus accumulated viral ribonucleoprotein and disintegrated after extensive fusion although little mature virus was released. In contrast, primary rhesus monkey kidney (MK) cells infected with SV5 did not accumulate RNP, released infective virus, but were not destroyed. Virulence in their system

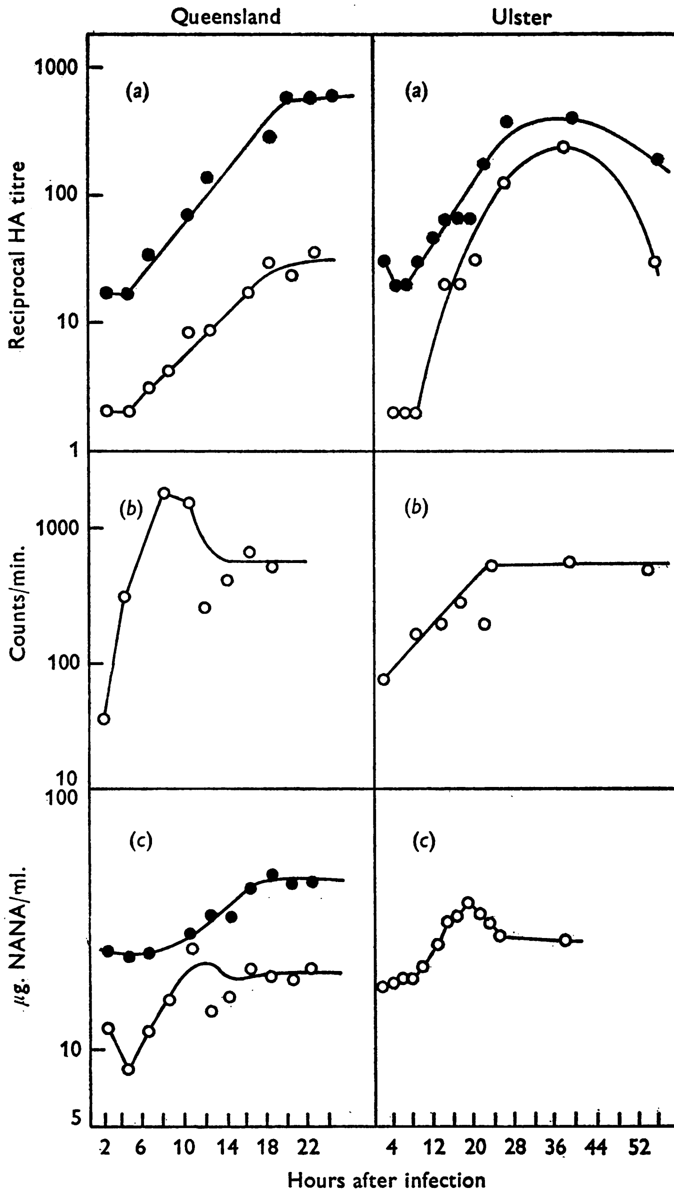

Fig. 3. Comparison of the extracellular (●—●) and cell-associated (○—○) major antigens of two avirulent strains of NDV during growth in eggs. (*a*) Haemagglutinin. (*b*) Material incorporating ^{3}H-uridine in an acid-insoluble RNase-resistant fraction. (*c*) Neuraminidase, μg. NANA released.

was determined by host cell differences but was also mediated by changes in the cellular surface and accumulation of viral antigen. In our system, unlike that studied by Compans *et al.*, virulence is determined by differences in the virus strain, the same host system being used throughout. Although avirulent strains did not appear to accumulate viral antigens in the chorioallantoic membrane we do not

know if all the cells were infected. Fewer cells may have been infected by avirulent strains than by virulent strains, but since extracellular virus titres are similar with all strains this would require cells infected with avirulent viruses to release relatively more virus into the allantoic fluid than cells infected with virulent virus.

If the same number of cells is infected the difference between strains may be that the intracellular production of viral antigen is limited in cells infected with avirulent strains either by some host cell response or some limiting factor in the virus antigen production mechanism.

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REFERENCES

AMINOFF, D. (1961). Methods for quantitative estimation of *N*-acetylneuraminic acid and their application to hydrolysates of sialomucoids. *Biochemical Journal* **81**, 384.

ASPLIN, F. D. (1952). Immunisation against Newcastle disease with a virus of low virulence (strain F) and observations on sub-clinical infection in partially resistant fowls. *Veterinary Record* **64**, 245.

COMPANS, R. W., HOLMES, KATHRYN V., DALES, S. & CHOPPIN, P. W. (1966). An electron microscopic study of moderate and virulent virus-cell interactions of the parainfluenza virus SV5. *Virology* **30**, 411.

DOBSON, N. (1939). Newcastle Disease. *Proceedings of the 7th World's Poultry Congress, Cleveland*, p. 250.

GRAHAM, E. R. B. (1961). Some aspects of the structure of the carbohydrate moiety of fetuin. *Australian Journal of Science* **24**, 140.

GRANOFF, A. (1959). Studies on mixed infection with Newcastle disease virus. *Virology* **9**, 636.

KINGSBURY, D. W. & DARLINGTON, R. W. (1968). Isolation and properties of Newcastle disease virus nucleocapsid. *Journal of Virology* **2**, 248.

PENNINGTON, T. H. (1967). Studies with Newcastle disease virus. Ph.D. Thesis, University of London.

PLAGEMANN, P. W. (1968). Mengovirus replication in Novikoff rat hepatoma and mouse L cells: Effects on synthesis of host-cell macromolecules and virus specific synthesis of ribonucleic acid. *Journal of Virology* **2**, 461.

SCHÄFER, W., SCHRAMM, G. & TRAUB, E. (1949). Untersuchungen über das Virus der atypischen Geflügelpest. *Zeitschrift für Naturforschung* **46**, 157.

SCHLOER, G. M. & HANSON, R. P. (1968). Relationship of plaque size and virulence for chickens of 14 representative Newcastle disease virus strains. *Journal of Virology* **2**, 40.

WATERSON, A. P. (1968). *Introduction to Animal Virology*. 2nd ed., Cambridge University Press.

WATERSON, A. P. & CRUICKSHANK, J. G. (1963). The effect of ether on Newcastle disease virus: a morphological study of eight strains. *Zeitschrift für Naturforschung* **18B**, 114.

WATERSON, A. P., PENNINGTON, T. H. & ALLAN, W. H. (1967). Virulence in Newcastle disease virus. A preliminary study. *British Medical Bulletin* **23** (2), 138.

WILSON, D. E. (1968). Inhibition of host-cell protein and RNA synthesis by Newcastle disease virus. *Journal of Virology* **2**, 1.