Pathogenesis of avian encephalomyelitis viruses

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The pathogenesis of a field strain, a vaccine strain and the egg-adapted Van Roekel strain of avian encephalomyelitis virus in susceptible chicken embryos and day-old chickens was investigated using enzyme-linked immunosorbent assays for the detection of virus-specific antibody and antigen. The Van Roekel strain was shown to be highly neurotropic whereas the field and vaccine strains were enterotropic. Radioimmuno-precipitation studies using Na$^{125}$I-labelled purified virus failed to detect any differences in the composition of the structural viral proteins of each strain that could account for these differences. As expected, the field and vaccine strains were more efficient than the Van Roekel strain at inducing antibody following oral administration. Primary cultures of chicken embryo brain cells supported the growth of the Van Roekel strain to a much greater extent than the field and vaccine strains.

Introduction

Avian encephalomyelitis viruses (AEVs) are the aetiological agents of a widespread disease of poultry that is transmitted by the oral–faecal route (Calnek et al., 1961). In young chickens typical symptoms include paralysis, ataxia and muscular dystrophy (Jungherr & Minard, 1942), whereas in older chickens infection is usually subclinical, resulting in declines in egg production and hatchability (Taylor et al., 1955). Based upon their fine structure and physical properties, AEVs probably belong to the family Picornaviridae within the genus enterovirus (Butterfield et al., 1969; Gostling et al., 1980), although details of their genome structure are required for a definitive classification.

There have been few studies on the pathogenesis of AEVs, due largely to their inability to grow efficiently in cell cultures and to the unsatisfactory nature of most methods previously used for detecting virus. Most AEVs grow in chick embryos without visible signs of infection; infectious virus has been usually detected according to the capacity of a virus to induce neurological symptoms in hatched chickens after inoculation of 6- to 7-day embryos via the yolk sac. Some strains, most notably the Van Roekel strain, have been adapted to grow in chick embryos and induce signs of infection before hatching, which include the development of splayed appendages, dwarfing, paralysis and an overall haemorrhagic appearance (Jungherr & Minard, 1942).

Previous studies revealed that field isolates of AEV grow to a higher titre in the intestine and pancreas than the egg-adapted Van Roekel strain. Serial passage of field isolates in eggs appears to select out a virus population that is neurotropic (Butterfield et al., 1969).

Embryo adaptation has also been reported to decrease the capacity of the virus to grow in the gut and be excreted in the faeces of infected chickens (Butterfield et al., 1969; Calnek et al., 1961; Miyamae, 1976, 1977). Most vaccines consist of non-egg-adapted viruses and are prepared by inoculating chick embryos via the yolk sac and obtaining suspensions of brains from birds showing neurological signs of infection. Vaccines are administered to 14- to 16-week-old chickens by the drinking water.

All of these studies were carried out using relatively crude and insensitive techniques. In recent years sensitive ELISAs have been developed for the detection of AEV antigen and antibody (Shafren & Tannock, 1988), and in this study we report the application of ELISAs to a study of the pathogenesis of three strains of AEV of differing neurovirulence.

Methods

Viruses. A preparation of the embryo-adapted Van Roekel strain of AEV was obtained from Dr Gary Cross of the Veterinary Research Station, Glenfield, New South Wales, Australia. Stocks were prepared by inoculating 7-day-old specific pathogen-free (SPF) chick embryos with 0·2 ml of the preparation via the yolk sac. After 10 to 12 days incubation at 37 °C, the brains, intestines and pancreases of the infected embryos were harvested and suspended to 10% (w/v) in PBS, pH 7·4, containing antibiotics (100 units penicillin/ml, 100 μg streptomycin/ml) and were homogenized using an Ultra Turrax disperser (Janke and Kunkel IKA). The titre of the stock was $10^{14}$ 50% egg infectious doses (EID$_{50}$/ml) when assayed in 6- to 7-day-old SPF embryos (Jungherr et al., 1956).

A field isolate (IC 79100), consisting of a 20% (w/v) pancreatic suspension was obtained from Mr G. A. Firth, Australian Poultry Ltd, Blackhill, New South Wales, Australia. Seven-day-old SPF embryos were inoculated via the yolk sac with 0·2 ml of a $10^{-1}$ dilution of
The embryos were allowed to hatch and chickens that displayed neurological signs of ataxia, paralysis or depression after 7 to 28 days were killed by decapitation and their brains removed, suspended in PBS and homogenized. The titre of the stock was $10^{6.3} \text{ID}_{50}/\text{ml}$ when assayed in SPF chickens by the hatchout assay (Hockstra, 1964). A vaccine consisting of the I strain was supplied as a glycerol suspension by Dr Richard Bevan, Arthur Webster Pty Ltd, Glenorie, New South Wales, Australia. Its titre was $10^{6.5} \text{ID}_{50}/\text{ml}$, as determined by the hatchout assay.

**Chicken embryo brain (CEB) cultures.** These were prepared using a modification of the method of Adams (1965). Brains from 14-day-old chicken embryos were removed by squeezing the cranium with blunt forceps and were washed three times with 100 ml cold PBS. They were then chopped finely with a scalpel and resuspended in cold citrate saline containing 0.125% (w/v) trypsin, using 10 ml per brain. The suspension was incubated at 4 °C for 4 h, then shaken vigorously for 15 s and filtered through a coarse conical gauze filter. An aliquot of the cell filtrate was centrifuged at 200g at 4 °C for 10 min and the packed cell volume was then measured. A final dilution of 1:100 of packed cells in medium 199 containing 20% (w/v) foetal calf serum (FCS) was used for cell growth. Ten millilitres of the CEB suspension was seeded to 25 cm² tissue culture flasks. Confluent monolayers, consisting mainly of neuroglial cells, appeared after 5 to 7 days incubation.

**AEV antigen and antibody ELISAs.** These were carried out as described by Shafren & Tannock (1988). For the antigen ELISA, test samples were added to plates coated with a dilution of rabbit immunoglobulins prepared from serum from an animal hyper-immunized with a purified preparation of the Van Roekel strain. After incubation and washing, a dilution of chicken antiserum was added, followed, after further incubation and washing, by a dilution of horseradish peroxidase-conjugated goat anti-chicken immune globulin and a substrate. For the antibody ELISA a standard amount of a partly purified preparation of the Van Roekel strain was added to the coated plates, followed by a 1:100 dilution of test chicken antiserum, conjugate and substrate. The antigen ELISA was capable of detecting 4 ng of AEV antigen in tissues. Antigen concentrations were determined against a standard curve for absorbance for a purified preparation of the Van Roekel strain (see below). Antibody was tested at 1:100 dilution; absorbances of 0.2 or greater have been shown to indicate the presence of antibody (Shafren & Tannock, 1988).

** Infectivity assays.** The brain antigen ELISA method was used for all AEV strains (Shafren & Tannock, 1990). Seven-day-old embryos were infected with dilutions of virus and incubated for 11 days. Virus growth was detected according to the presence of antigen, as described above and titres are expressed as EID$_{50}$.

**Iodination of viral polypeptides.** Purified preparations of each AEV strain (25 µg each) were labelled with Na$^{125}$I by the method of MacGregor et al. (1983).

**PAGE.** Labelled virus preparations were diluted in electrophoresis buffer to a final radioactivity of 100 000 c.p.m./60 µl. The iodinated samples were electrophoresed in 15% (w/v) slab acrylamide gels containing 0.1% (w/v) SDS (Sigma) at 60 to 70 V for 16 h at 22 °C by the method of Laemmli (1970). Urea was not included in the gels. Each gel was then stained in a solution consisting of 2.5% (w/v) Coomassie blue in 10% (v/v) acetic acid and 50% (v/v) ethanol to stain the marker proteins. Destaining was accomplished with two changes of a 10% ethanol and 7% acetic acid solution. Virion proteins were undetectable by staining but could be demonstrated by autoradiography. The gels were dried under vacuum on Whatman 3MM chromatography paper and autoradiographs were prepared by exposing the dried gels to Omat X-ray film (Kodak) in the dark at −70 °C.

**Radioimmunoprecipitation.** Preparations of $^{125}$I-labelled virus were examined using modifications to the methods of Kessler (1975) and Stephenson & ter Meulen (1979). Labelled virus (250 µl) was mixed with 25 µl rabbit antiserum and incubated overnight at 4 °C. Sepharose CL2B–Protein A, reconstituted in PBS, was diluted 1:1 with PBS to form a thick slurry and 50 µl was added to the virus–antiserum mixture and incubated at 4°C for 4 h with intermittent mixing. The virus–antiserum–Sepharose complex was pelleted in a Model B Microfuge (Beckman Instruments) at 8700 g for 5 min at 22 °C. The supernatant was decanted and the pellets resuspended in 1 ml of a buffer consisting of 0.15 M-NaCl, 1 mM-EDTA, 0.01% (w/v) sodium azide, 1% Nonidet P40 and 10 mM-Tris–HCl pH 7.4, and pelleted as before. This cycle was repeated three times, after which the pellet was dissolved in 50 µl sample buffer and heated at 100 °C for 5 min in a water bath. The sample was stored at −80 °C before analysis by PAGE.

**Preparation of purified AEV.** Purified AEV antigen was prepared by a modification of a method previously described by Shafren et al. (1989). For the Van Roekel strain, embryos showing typical signs of AEV infection were removed after 12 days and a 25% (w/v) homogenate of brains was prepared in PBS. For the vaccine strain and the field isolate, 6- to 7-day embryos were inoculated via the yolk sac and, after incubation for 13 days, their livers were removed and processed by the same method as for embryos infected with the Van Roekel strain. The homogenates were frozen and thawed three times to disrupt cells and release virus, and were then clarified by centrifuging at 2500 g for 15 min at 4 °C, and at 12000 g in a Beckman SW28 rotor for 30 min at 4 °C. Aliquots consisting of 3.0 ml supernatant were then layered onto 2 ml discontinuous CsCl gradients in PBS in 5 ml tubes which were centrifuged at 200000 g in a SW50.1 rotor at 12 °C for 2 h. The gradients were formed by placing 1.0 ml CsCl (density 1.34 g/ml) in the tube and gently layering 1.0 ml CsCl of density 1.29 g/ml on top. After centrifugation, the tube contents were fractionated from the bottom and those fractions containing virus (density 1.31 to 1.34 g/ml) were pooled, diluted 1:4 in PBS, extracted with an equal volume of Freon TF and centrifuged at 2500 g for 25 min at 4 °C. The upper aqueous phases containing virus were clarified at 12000 g in an SW28 rotor at 12 °C for 20 min and the virus present was pelleted at 200000 g in a SW50.1 rotor for 2 h. The pellet was washed in PBS and disaggregated by sonication at 6 to 10 cycles/min for three periods of 15 s, using a Soniprep 150 ultrasonic generator (MSE Scientific Instruments). The protein concentration of each preparation was determined using the Bio-Rad protein estimation kit.

**Preparation of purified poliovirus.** Monolayers of BSC-1 cells in 75 cm² tissue culture flasks were infected with Sabin type 1 poliovirus. After incubation for 24 h at 37 °C, an extensive c.p.e. was apparent; the cultures were frozen and cell debris was removed by low-speed centrifugation for 15 min at 2500 g. Subsequent purification steps were performed by the method described for AEV.

**Results**

**Polypeptide composition of AEV**

Purified preparations of the Van Roekel strain and poliovirus type 1 were examined by PAGE and Fig. 1 (lane 1) reveals that the virion proteins of the Van Roekel strain were three polypeptides with $M_{r}$ values of 35K, 30K and 26K. These figures differed from previously reported values of 43K, 35K, 33K and 14K for a
Fig. 1. Autoradiogram of the structural proteins of AEV and poliovirus type 1, and of ovalbumin. Poliovirus proteins in lane 4 served as an internal control. All preparations were iodinated and submitted to discontinuous PAGE in a 15% slab gel. Lanes: 1, 125I-labelled Van Roekel AEV; 2, 125I-labelled Van Roekel AEV + 125I-labelled ovalbumin; 3, 125I-labelled ovalbumin; 4, 125I-labelled poliovirus type 1. Designation of the order of structural polypeptides is provisional.

preparation of the same strain that had been purified by rate-zonal centrifugation (Tannock & Shafren, 1985). As the Van Roekel strain was propagated in eggs, it was considered likely that ovalbumin (Mr 43K) could have been present as a contaminant. In order to test for this possibility, a solution of electrophoretic grade ovalbumin (10 μg/ml) was labelled with Na125I and electrophoresed in parallel (Fig. 1, lane 3).

Three polypeptides with Mr values of 35K, 29K and 24K were observed for a similarly prepared preparation of poliovirus type 1 run in parallel (Fig. 1, lane 4), which were similar to those reported elsewhere for poliovirus (Cooper et al., 1978). However, VP4 could not be detected for either AEV or poliovirus, due possibly to the low sensitivity of the method or the low number of tyrosine residues in these proteins. These results suggest that the 43K protein described previously for AEV was indeed contaminating ovalbumin which could be removed by the CsCl purification and that VP1 to VP3 of both AEV and poliovirus type 1 are similar in size.

Immunoprecipitation of polypeptides of different AEV strains

Radiolabelled polypeptides prepared from purified preparations of the Van Roekel, field and vaccine strains were immunoprecipitated with homologous and heterologous rabbit antisera raised against each strain, and then separated by PAGE. Fig. 2 indicates that the polypeptides of each virus were precipitated by each antiserum and that the viruses were antigenically indistinguishable.

Growth of AEV in CEB cell cultures

The antigen ELISA was used to detect the growth in CEB cell cultures of the Van Roekel strain, the field strain and the vaccine strain. Each strain (100 μl) containing 10^4 EID50 of virus, as determined by the brain antigen ELISA, was inoculated to confluent cultures of CEB cells in 25 cm² flasks and allowed to adsorb for 1 h at 37 °C. The inoculum was then decanted and the cell monolayer washed twice with PBS, and 5.0 ml medium 199 containing 5.0% FCS was added to each flask which was then incubated at 37 °C. Flasks were removed daily for 1 week and were examined for the presence of antigen in the culture fluid and cell monolayers. Fig. 3 indicates that the Van Rockel strain
Fig. 3. Kinetics of growth of the Van Roekel strain (●), a field strain (○) and a vaccine strain (▲) of AEV in CEB monolayer cultures. Viral antigen was detected in association with cell monolayers (a) and, in the supernatant (b), by an ELISA. For cell-associated antigen, medium was removed and the cell sheet washed twice and suspended in 1.0 ml PBS containing 2% (w/v) SDS. It was frozen and thawed three times and then sonicated three times for 15 s at 6 to 10 cycles/min. The virus in the maintenance medium was pelleted by centrifugation at 50,000 r.p.m. in an SW50.1 rotor for 3 h and the pellet resuspended by sonication in 1.0 ml PBS. Fifty microlitre samples of media and cell suspensions were tested for AEV antigen by the ELISA.

Fig. 4. Mean levels of antigen in the brain, heart, liver and intestine (a to d) of chicken embryos administered with 100 EID₅₀ of the Van Roekel strain (●), the field strain (○) or the vaccine strain (▲) via the yolk sac at 7 days. The bars represent the s.e.m. for three replicates.

Replication of different strains of AEV in chicken embryos

Six-day-old embryos were inoculated via the yolk sac with 100 EID₅₀ of the Van Roekel, field or vaccine strains. Antigen levels in the brain, heart, liver and intestines at 17, 18, 19 and 20 days [i.e. 10, 11, 12 and 13 days post-infection (p.i.)] were determined and the results are shown in Fig. 4. The data indicate that similar levels of antigen were observed in suspensions of heart and intestine of embryos inoculated with any of the strains. However, the highest concentration of antigen in brain tissues was induced by the Van Roekel strain and those embryos showed typical signs of infection. Embryos inoculated with the field and vaccine strains were indistinguishable from the uninfected controls and possessed approximately 50 times less antigen in the brain than was present in the brains of embryos infected with the neurotropic Van Roekel strain. The hepatotropic nature of the field and vaccine strains was indicated by the presence of higher concentrations of antigen in the liver of embryos infected with these strains than others infected by the Van Roekel strain.

Replication of different strains of AEV in susceptible day-old chickens infected by the oral route

Seventy-two day-old SPF chickens were divided into four groups of 18 and each was administered 10⁶ EID₅₀ of the Van Roekel, field or vaccine strains directly into
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Fig. 5. Mean concentration of antigen in the brain, heart, liver, pancreas and small intestine (a to e) of chickens administered with 10^4 EID_{50} of the Van Roekel strain (○), the field strain (□) or the vaccine strain (▲) of AEV by the oral route within 24 h of hatching. The bars represent the S.E.M. for three replicates.

Fig. 6. Levels of serum antibody (○) and faecal antigen (©) in chickens administered the Van Roekel strain, the field strain or the vaccine strain (a to c) by the oral route, as described for Fig. 5. The mean ± S.E.M. for three replicates is shown.

Discussion

These studies clearly demonstrate that avian encephalomyelitis is a multi-organ disease of chickens. The three strains of AEV used in this study appear to be antigenically indistinguishable but exhibit differing tissue tropisms. Of these, the embryo-adapted Van Roekel strain was highly neurotropic, as shown by its capacity to replicate efficiently in primary neural cultures (Fig. 3) and in neural tissue (Fig. 4). The field and vaccine strains were enterotropic because of their predilection for the small intestine, pancreas and liver (Fig. 5). Immunoprecipitation studies revealed no...
differences in the serological reactivity of viral structural proteins that might account for this variation (Fig. 2). However, differences in antigenicity may be detected by other procedures, perhaps involving the use of panels of monoclonal antibodies.

Based upon indirect evidence that growth of the Van Roekel strain is not inhibited in CEB cultures by inhibitory concentrations of 5-bromo-2'-deoxyuridine (D. R. Shafren & G. A. Tannock, unpublished observations), its genetic material appears to be RNA, which is consistent with the biophysical properties of AEVs closely resembling those of the Picornaviridae. Small differences in the nucleotide sequence of AEV genomes may alter their tissue tropism. In the case of poliovirus type 3, two nucleotide changes at positions 472 in the 5' non-coding region and 2034 in VP3 are sufficient to cause reversion of the attenuated Sabin type 3 vaccine strain to neurovirulence (Racaniello, 1988). Such detailed analysis would be possible if full-length infectious cDNA clones of the different AEV strains were available. However, it may be possible to use the polymerase chain reaction (PCR) to amplify the 5' non-coding region of different strains for sequencing using specific oligonucleotide primers that bind to conserved blocks of amino acids within the 5' non-coding regions of other picornaviruses. Alternatively, differences may be detected from oligonucleotide fingerprints of their RNA genomes. Because many AEV strains do not replicate to a high enough titre in cell culture to allow efficient radiolabelling (Fig. 3; unpublished observations) the PCR approach appears to be the most feasible.

In contrast to the field and vaccine strains, the Van Roekel strain is unsuitable for use in vaccines because it is relatively inefficient at inducing antibody following oral administration (Shafren & Tannock, 1988; Fig. 6). Ideally, AEV vaccine strains should be highly enterotropic and poorly neurotropic. Neither the field nor the vaccine strain grew efficiently in CEB cultures (Fig. 3) which are, therefore, unsuitable for use as substrates in the propagation of vaccine viruses. However, the capacity of AEVs to grow in CEB cultures may serve as a useful marker for determining their neurovirulence. The slow, highly cell-associated and persistent infection of cell cultures by AEVs is reminiscent of hepatitis A viruses (Gust et al., 1985). However, their capacity to grow in myocardial tissues (Fig. 4 and 5) is a clear point of difference. Further classification may be possible from tests to determine whether a stretch of poly(C) is present in the RNA genomes, which is a feature of cardioviruses (Cooper et al., 1978), some of which also grow in myocardial tissues. The genome of hepatitis A virus is thought to allow a biased packaging of RNA into capsid coats, thereby decreasing potential for rapid RNA translation, replication and subsequent cell lysis (Anderson et al., 1988). This situation may exist in AEVs but will remain unknown until detailed studies into the structure of their viral genomes have been carried out.

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References


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