SOME IMMUNOLOGICAL ASPECTS OF A RECENT AUSTRALIAN ISOLATE OF INFECTIOUS BRONCHITIS VIRUS

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Introduction

Gilchrist and Sinkovic (1964, 1967) showed that an egg-adapted isolate of an Australian infectious bronchitis (IB) virus could be used to induce resistance to IB nephritis in young chicks. Using this principle, infection of chicks with a variety of commercially available live virus vaccines has dramatically reduced the major losses from nephritis in Australia (Cumming 1969a). Nevertheless, there have been persistent reports of 'outbreaks' of nephritis occurring in vaccinated flocks (Gilchrist and Sinkovic 1967; Hungerford 1969; P. Gilchrist and Wells 1974, personal communication). Undoubtedly, some of these outbreaks have been the result of faulty vaccination techniques and immunological immaturity has also been suggested as a factor (Chubb 1973).

These 'outbreaks' of nephritis could also be the result of infection with an IB virus against which the currently available commercial vaccines are ineffective or, at best, induce only a partial resistance. This communication describes the isolation, from a previously vaccinated flock, of an IB virus which is distinct from previously described Australian IB viruses.

Materials and Methods

Viruses

The isolates of IB virus used were the A virus [titre 107.6 50% embryo infective doses (EID_{50})/ml] and the T virus ($10^{6.4}EID_{50}$ /ml) in their twenty-ninth and thirteenth egg passages respectively (Cumming 1963, 1967), two commercially available vaccines, and the G48 Virus ($107.6EID_{50}$ /ml) in its fifth egg passage. The G48 virus was isolated from the kidneys of some birds in a flock of broilers near Sydney, New South Wales, which had been vaccinated with commercial vaccine 2 at 4 days of age and experienced an outbreak of nephritis between 4 and 6 weeks of age. After 4 egg passages by the allantoic route, G48 induced typical IB-like dwarfing and curling of 9-day-old embryos. Electron microscopy of samples of infected allantoic fluid, negatively stained with 3% phosphotungstic acid (pH 6.4) after Spradbrow and Francis (1969) revealed coronavirus particles. The virus induced nephritis in 6 out of 10 4-week-end susceptible cockerels between 6 and 10 days after infection.

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The above results led us to believe that we had isolated an avian IB virus (Cumming 1967, 1969b). As this virus was isolated from a vaccinated flock, it could have been the original vaccine virus or another virus able to induce nephritis, despite vaccination.

Vaccination

The commercial vaccine viruses were made up for use by adding 10 ml of sterile distilled water to a 1000 dose vial of freeze-dried vaccine (titre approximately 10^6EID_{50} /ml).

The other viruses were used as a 1/10 dilution of allantoic fluid in phosphate buffered saline. Birds were vaccinated or challenged, individually, by instilling 1 drop of the diluted virus onto the conjunctiva. The procedures used for virus isolation, experimental chickens, fertile eggs and isolation facilities have been described by Cumming (1967, 1969a). Neutralising antibodies were produced against each virus by vaccinating 3 four-week-old cockerels housed in isolation pens. A second vaccination was given 3 weeks later. The birds were bled at weekly intervals.

Virus Neutralisation

Virus neutralisation tests were carried out by the method of Fontaine *et al* (1963) using undiluted serum or serum diluted 1/4. A positive serum, diluted 1/4, was expected to neutralise 100 EID_{50} of the virus under test. Serums from each group of 3 vaccinated birds were pooled for testing, except at the final bleeding, taken 5 weeks after the second vaccination, when individual serums were tested. Each serum, or pool of serums, was tested at least twice against the various viruses tested.

Challenge Experiments

These experiments were designed to show whether the G48 virus could overcome the resistance induced in 4-week-old cockerels by 2 commercial vaccines or the A virus. This resistance was compared with the resistance these viruses induced against the T virus.

Approximately 35 3-week-old cockerels, reared in isolation, were placed in an isolation pen and vaccinated with the A virus. Two similar pens of birds were vaccinated with each of the 2 commercial vaccines. A fourth pen of birds was left as untreated controls. One week later, 2 birds from each pen were killed to provide kidney specimens for virus isolation. As the weather was mild, electrolyte replacers (Cumming 1967, 1969a; Cumming and Heath 1969) were given to prevent losses from the nephritis syndrome.

Three weeks after vaccination the birds in each pen were divided into 2 groups. One of the groups in each pen was replaced by a group from another pen in such a way that 2 pens contained a mixture of birds vaccinated with commercial vaccines 1 and 2 and 2 pens contained mixtures of control birds and birds vaccinated with the A virus. The birds in 1 pen of each mixture of birds were challenged with either the T virus or the G48 virus. Five days after challenge, all the birds were

TABLE 1

Mortality with Nephritis of Vaccinated Birds after Challenge with either T or G48 Isolates of IB Virus

Challenge Virus		Vaccine Virus							
		Commercial Vaccine 1		Commercial Vaccine 2		A		Control (unvaccinated)	
		Deaths		Deaths		Deaths		Deaths	
Exp. 1	G48	$\frac{17^*}{35}$	49%	$\frac{11}{27}$	41%	$\frac{3}{36}$	8%	$\frac{8}{30}$	27%
	T	$\frac{6}{32}$	18%	$\frac{13}{43}$	30%	$\frac{2}{21}$	7%	$\frac{16}{27}$	59%
Exp. 2	G48	$\frac{9}{31}$	29%	$\frac{23}{35}$	65%	$\frac{1}{33}$	3%	$\frac{20}{36}$	56%
	T	$\frac{0}{32}$	0%	$\frac{5}{34}$	15%	$\frac{1}{35}$	3%	$\frac{14}{31}$	45%

* Numerator = no. of birds dead from nephritis
Denominator = no. of birds vaccinated

removed to a cold room held at 13°C. Susceptibility to challenge was determined by death from kidney failure (Cumming 1963, 1967).

A second experiment (2) was carried out in a similar manner to experiment 1.

Results

Vaccination and Challenge of Birds

Infectious bronchitis virus was isolated from the kidneys of the 2 vaccinated birds examined from each group in both experiments, with the exception that virus was isolated from only 1 of the 2 birds vaccinated with commercial vaccine 2 in experiment 1. Virus was not isolated from any of the unvaccinated birds examined.

In experiment 1 (Table 1), in birds vaccinated with either of the 2 commercial vaccines, mortality from nephritis, after challenge with G48 virus was not significantly greater than the mortality experienced in the unvaccinated group. The low mortality after challenge with G48 virus in the group of birds vaccinated with the A virus was not significantly less than the mortality in the unvaccinated group of birds.

In experiment 2, Table 1, after challenge with G48 virus, mortality in the birds vaccinated with commercial vaccine 2 was similar to the mortality in the unvaccinated group whereas the lower mortality in birds vaccinated with commercial vaccine 1 approached significance at the 5% level* compared with the mortality in the unvaccinated group of birds. The mortality in the group of birds vaccinated with the A virus after challenge with G48 virus was significantly lower ($P < 0.001$) than the mortality in the unvaccinated group of birds.

In both experiments, most vaccinated birds were more resistant to challenge with the T virus

than the birds of the unvaccinated group (significance levels for commercial vaccine 1 are 10% and 1%, for commercial vaccine 2, n.s. and 10%, and for the A virus, 5% and 1%, for experiments 1 and 2 respectively).

Cross-Neutralisation Tests

Table 2 summarises the results of all the cross-neutralisation tests carried out. No real difference in this pattern was observed between the pooled sera of the 3 birds used for antibody production of each virus and the individually tested sera of the final bleeding. There was no difference in this pattern when undiluted sera were used.

The results show that the A antiserum neutralised all of the viruses tested (commercial vaccines 1 and 2, A virus, T virus, and G48 virus) whereas the G48 antiserum neutralised only G48 virus. The T virus has a limited antigenic spectrum, having full reciprocal cross neutralisation with the A virus and only doubtful relationships with the 2 commercial vaccine viruses and no antigenic relationship with the G48 virus.

Similarly, the 2 commercial vaccine viruses have full reciprocal cross neutralisation with each

TABLE 2
Cross Neutralisation Tests between Isolates of
IB Virus and Hyperimmune Serums

Antiserum	Viruses				
	Virus G48	Virus T	Virus A	Commercial V ₁	Vaccine V ₂
G48	+	—	—	—	—
T	—	+	+	±	±
A	+	+	+	+	+
V ₁	±	—	±	+	+
V ₂	—	—	±	+	+

* + denotes neutralisation of 100 EID₅₀ of virus
± denotes doubtful neutralisation
— denotes no neutralisation

* using chi-square test corrected for continuity.

other, but only doubtful relationships with the A and T viruses. Commercial vaccine 1 virus has a doubtful one-way cross neutralisation with the G48 virus. It should be noted, however, that doubtful relationships ($\pm$) are shown when on at least one occasion positive neutralisation has been seen. This has usually occurred when the virus dose to be neutralised has been between 80 and 100 EID₅₀/ml.

Discussion

The results of the challenge experiments show that the birds vaccinated with the 2 commercial vaccines did not have a significantly greater resistance to challenge with the G48 virus than the unvaccinated control birds. Nevertheless, birds similarly vaccinated with virus A withstood a challenge from both the T and G48 viruses.

The results of the cross-neutralisation tests show that the G48 virus is antigenically different from the other viruses. The A virus serum has a good one-way cross neutralisation with the G48 virus, whereas antiserum to the G48 virus only neutralises its homologous virus. None of the antisera to the other virus isolates neutralised the G48 virus. It is of interest that Westbury (1970) found that the A virus serum showed the widest antigenic relationship in cross neutralisation tests with the 12 Australian IB viruses he tested.

While varying degrees of antigenic difference were found in 12 isolates of IB virus tested, Westbury (1970) suggested that these differences were insufficient for evidence of separate antigenic types. Antigenic differences have also been clearly demonstrated in the present work with 5 isolates, and these differences were of a sufficient magnitude to be able to differentiate between the isolates. It suggests that using one IB virus isolate for the detection of IB antibodies from sera from the field could give false negative results. There have been reports, for example, of a lack of neutralising antibodies after vaccination (Smith *et al* 1970; Chubb and Cumming 1971) which could be explained by a lack of serological identity between IB virus isolates.

As has been reported by a number of American workers (Hofstad 1961; Hitchner *et al* 1964; Raggi and Lee 1965), the relationships shown by cross neutralisation between isolates do not necessarily correlate with cross challenge results. The T virus has a limited serological relationship with the 2 commercial vaccines tested, but chickens vaccinated with these vaccine viruses successfully resisted its challenge. Further,

chickens vaccinated with virus A, which was antigenically related to all the viruses used, exhibited considerable resistance on challenge to both viruses T and G48.

The results presented in this communication suggest that the G48 isolate of IB virus is different from the hitherto described Australian IB virus isolates as reviewed by Cumming (1969a). The presence of such differing viruses in Australia may help to explain the 'outbreaks' of nephritis reported after vaccination (Gilchrist and Sinkovic 1967; Hungerford 1969) and also the continual mortality from nephritis which has been recorded throughout the life history of many Australian poultry flocks (Jackson *et al* 1972; Cumming, unpublished).

Summary

An infectious bronchitis virus, designated G48, isolated from birds during an outbreak of nephritis in a previously vaccinated broiler flock, overcame the resistance induced in birds vaccinated with 2 commercially available vaccines. Birds vaccinated with the A isolate of infectious bronchitis resisted challenge with this new virus.

Cross neutralisation studies revealed that the new virus was serologically distinct from the 4 viruses tested. Homologous antiserum to G48 did not neutralise the other viruses and only antiserum to the A virus completely neutralised the new virus.

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EDITORIAL

EPHEMERAL FEVER VACCINATION

The apparent increase in frequency of outbreaks of Ephemeral Fever in recent years, together with reports from practitioners of an increasing number of severe cases, suggest the need for the development of an effective vaccine. Research workers in the Department of Veterinary Preventive Medicine, University of Queensland, and the Division of Animal Health, CSIRO, have anticipated this need and, with the support of the Australian Meat Research Committee, have been attempting to develop respectively a live and a killed virus vaccine.

Recent studies at the Division of Animal Health seem to indicate that a killed vaccine is unlikely to be protective, even though vaccination with large doses of killed virus in bland adjuvant stimulates extremely high levels of neutralising antibody (Della-Porta and Snowdon 1975). While disappointing, the result is not entirely surprising as CSIRO workers had realised at the outset that the chances of developing an effective killed vaccine were somewhat slim and probably significantly less than the chances of developing a protective live vaccine. In this connection, Snowdon (1970) reported that vaccination with passaged strains of Ephemeral Fever virus effectively protected cows against challenge with virulent virus. Nevertheless, CSIRO workers decided to attempt to develop a killed vaccine for reasons of safety. An important consideration in the original rejection of a live vaccine by these workers was the perceived danger of its use in the field being associated with the emergence of virulent virus after passage through the vector. In this connection, virus has been isolated recently from *Anopheles bancrofti* (St. George *et al* 1976) but in view of the limited geographical distribution of this insect, it is clear that it is not the only vector involved; indeed, it may be of relatively minor importance in the southward spread of the disease from the endemic area in the north. Nevertheless, the isolation of virus from this insect allows experiments to be undertaken to

determine whether passage of laboratory-manipulated virus through the insect is associated with a reversion to virulence.

It seems likely that Chief Veterinary Officers in Queensland and New South Wales will, in due course, be required to make a decision about the use of a live virus vaccine in their States should this vaccine be demonstrated to be protective. At the present time a number of live vaccines are under test (Spradbrow 1975). The question of safety inevitably arises, and the subject ought to be discussed and debated by those who feel they have a contribution to make. I am hoping that this will stimulate a useful correspondence involving not only those directly concerned with the experimental work on vaccine production, but particularly others with a general knowledge of the subject. Interesting field observations which may help to throw light on the pathogenesis of the disease or the persistence of immunity would be welcomed.

The issue raises numerous questions and I hesitate to specify any for fear of limiting the scope of discussion. However, it is important that some attention is directed to these 3 questions:

What is the likelihood of passaged virus reverting to virulence?

If reversion to virulence does occur, then what are the possible sequelae?

In general terms, what standards would need to be adopted in order to establish the purity, efficiency and safety of a live vaccine?

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