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Bile immunoglobulin of the duck (*Anas platyrhynchos*)

II. ANTIBODY RESPONSE IN INFLUENZA A VIRUS INFECTIONS

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SUMMARY

The capacity of the IgM-like bile immunoglobulin (IgX) of the duck (*Anas platyrhynchos*) to express antibody activity to H3N2 influenza A viruses, and the dependence of this activity on the co-existence of serum IgM antibodies were investigated. Ducklings infected orally and intranasally at 15–29 days of age with viruses isolated from different host species were examined for haemagglutination-inhibiting (HI) antibodies in biles and sera 16–29 days after infection (p.i.). All biles had antibodies associated with IgX; all sera had antibodies associated only with the 7.8S IgG. Following oral infection of birds 42-days-old with influenza A/duck/HK/7/75 virus, serum HI antibodies were an initial IgM response occurring from 5–12 days p.i., followed by the appearance of 7.8S IgG antibodies. Virus-neutralizing (VN) antibodies in serum were also biphasic; isotype classification was not attempted. Bile IgX developed HI and VN activity. HI antibodies reached peak titres 12 days p.i. and fell to low levels by 24 days p.i. VN antibodies also reached peak titres 12 days p.i., but thereafter persisted at quite high levels throughout the experiment. Development of high titres of antibody in bile coincided with the termination of virus excretion in faeces. These experiments confirm that bile IgX of the duck can function as antibody in response to influenza A viruses, and that its activity appears to be independent of serum IgM. Its possible relevance in determining survival of virus in the intestine is discussed.

INTRODUCTION

Bile of the duck (*Anas platyrhynchos*) contains immunoglobulin (Ig) of a single (IgX) class resembling, but not identical to, serum IgM, and with an ontogeny distinct from serum Igs (Ng & Higgins, 1986). Some fish and amphibians also possess a secretory Ig resembling serum IgM (Lobb & Clem, 1981; Hsu, Flajnik & Du Pasquier, 1985; Hart *et al.*, 1987). These molecules might represent an evolutionary link between IgM and IgA. IgM has remained a component of bile in chickens (Mockett, 1986), rats (Peppard, Jackson & Hall, 1983) and humans (Mullock *et al.*, 1985). The bile IgM in these species is capable of transient antibody response to pathogens (Jackson & Walker, 1983; Mockett & Rose, 1986) prior to a more prolonged IgA response.

In the more primitive species which possess only IgM-like secretory Ig, important issues are the capacity of these molecules for antibody activity and mucosal protection, and the dependence of this activity on the existence of serum IgM antibodies. It was therefore relevant to compare bile and serum antibody responses in ducks infected with epitheliotropic viruses.

Abbreviations: HI, haemagglutination inhibition; Ig, immunoglobulin; NI, neutralization index; p.i., after infection; RID, radial immunodiffusion; VN, virus neutralization.

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MATERIALS AND METHODS

Ducks

Ducks were hatched and reared under laboratory conditions and were free of detectable virus in oral and cloacal swabs taken prior to infection.

Viruses and infection technique

These were all H3N2 influenza A viruses isolated from ducks, pigs and humans in surveillance studies and now held in a Hong Kong reference laboratory. The history and properties of representative viruses have been recorded elsewhere (Scholtissek *et al.*, 1985; Shortridge, King & Webster, 1987). Viruses were propagated in the allantoic sac of fertile hens' eggs (Palmer *et al.*, 1975). Infection was by oral (1·0 ml) and nasal (0·5 ml) administration of freshly harvested allantoic fluid with a haemagglutination (HA) titre of 1:64–1:128.

Virus detection

Fluids from swabs and homogenized organs were each inoculated into the allantoic sacs of two fertile hens' eggs (Palmer *et al.*, 1975). Virus was detected by HA test in allantoic fluid after 3

days of culture; the HA subtype of random isolates was confirmed against a panel of monospecific antisera. Negative samples were inoculated into a further two eggs to confirm the result.

Purification of bile IgX

Biles were dialysed extensively against 0·1 M Tris-HCl, pH 8·7, containing 1 M NaCl and 1 mM EDTA, then fractionated through Sephacryl S-300 (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer. The second peak, monitored at 280 nm, contained pure IgX (Ng & Higgins, 1986) which was concentrated by dialysis against polyvinylpyrrolidone. Concentrations of IgX in the resulting solutions were determined by applying the extinction coefficient $E^{1\%}_{1\text{ cm }280\text{ nm}} = 14{\cdot}2$.

Preparative ultracentrifugation

Sera were fractionated by centrifugation of 0·1-ml samples through 5·3 ml 10–40% sucrose gradients at 50,000 r.p.m. for 18 hr at 4° in the SW 50.1 rotor of a Beckman L5-65 centrifuge. Nine fractions of 0·6 ml were collected. Ig isotype distribution in the gradient was determined by micro-immunodiffusion against rabbit antisera to duck IgM and IgG heavy chains (Ng & Higgins, 1986); 7.8S and 5.7S IgG were distinguished by their location within the gradient.

Radial immunodiffusion (RID)

The concentrations of IgM and IgG in sera and IgX in samples of bile were determined by RID against isotype-specific rabbit antisera (Ng & Higgins, 1986).

Antibody assays

Haemagglutination-inhibition (HI) tests were performed in microtitre trays (Palmer *et al.*, 1975) against four haemagglutinating (HA) units of an ether-disrupted preparation of virus (Berlin *et al.*, 1963) containing free haemagglutinin for increased sensitivity (Lu, Webster & Hinshaw, 1982). Virus neutralization (VN) tests were performed using 10-fold dilutions of virus against constant antibody. Each dilution was inoculated into three fertile hens' eggs. Presence of virus in the allantoic fluid was detected by HA test after 3 days of incubation. Fifty percent end-points of virus–antibody and control (virus–buffer) titrations were calculated by the method of Reed & Muench (1938); the neutralization index (NI) was the difference between these $\log_{10}$ values. When the concentration of Ig in the solution tested had been determined by RID or spectrophotometry, antibody titres were expressed per mg of Ig. Whole bile was not examined for anti-viral antibodies, non-specific factors in bile would have given erroneous results in VN, while HI could not be performed since bile caused haemolysis which could not be reduced by extensive dialysis or by standard methods of heat treatment or agglutinin absorption.

Experimental design

Experiment 1. For this initial study, material was obtained from an experiment primarily designed to investigate virological aspects of the host–virus interaction in ducks infected with a range of H3N2 influenza A viruses (Scholtissek *et al.*, 1985). Ducklings of the outbred Cantonese breed were infected with virus at 15–29 days of age. Oral and cloacal swabs were taken daily for virus isolation. The ducks were killed by exsanguination 16–29 days after infection (p.i.) and sera and biles were stored at −20°. Concentrations of IgM and IgG in sera and IgX in samples of bile were determined by RID. Sera were fractionated by ultracentrifugation and IgX was purified from samples of bile by chromatography. HI tests were performed on sera, fractions collected from ultracentrifugation of sera, and purified bile IgX.

Experiment 2. A flock of 12 hybrid Pekin ducks (Super M, Cherry Valley Farms Ltd, Lincoln, U.K.), 42 days of age, was infected with the A/duck/HK/7/75 virus. Blood was collected from four birds 1 day before and 1, 3, 5, 7, 9, 12, 15, 18 and 21 days p.i.; 3, 2 and 1 remaining birds were bled on Days 24, 28

Table 1. Influenza A (H3N2) virus shedding, HI antibodies and immunoglobulin concentrations in serum and bile of ducklings 16–29 days after infection

Virus	Bird no.	Duration of isolation (days p.i.) from swabs oral/cloacal	Serum HI titre	Antibody activity (HI units/mg) of			Concentration (mg/ml) of		
				Serum IgM	Serum IgG	Bile IgX*	Serum IgM	Serum IgG	Bile IgX†
Duck/HK/7/75	1	7/9	1:160	0	2329	65	6·00	1·37	4·02
	2	9/7	1:320	0	5416	3832	1·73	1·18	2·34
Duck/HK/24/76	3	9/7	1:80	0	1502	47	3·16	1·06	7·65
	4	4/11	1:80	0	1384	126	3·30	1·16	5·99
Duck/HK/315/78	5	11/18	1:60	0	1180	571	2·29	1·02	6·83
	6	6/11	1:80	0	1139	126	2·25	1·40	7·20
Human/HK/14/83	7	—	1:60	0	1198	747	2·25	1·00	0·46
	8	—	1:40	0	625	2667	2·35	1·28	0·92
Human/HK/26/83	9	—	1:160	0	2605	5714	4·20	1·23	0·92
	10	—	1:160	0	3466	787	1·61	0·92	0·61
Swine/HK/3/76	11	—	1:480	0	5411	154	2·32	1·77	7·60
Swine/HK/127/82	12	—	1:120	0	2396	4571	1·40	1·52	6·05
	13	—	1:20	0	433	6095	2·80	0·92	1·41

* HI titre of purified IgX.
† Concentration of IgX in bile.

Table 2. Isolation of virus from oral and cloacal swabs and from organs following infection of ducks at 42 days of age with influenza A/duck/HK/7/75 virus

Day of slaughter (p.i.)	Bird no.	Duration of isolation (days p.i.) from swabs oral/cloacal	Organs infected at time of slaughter
1	12	1/1	Oesophagus, jejunum, ileum, bursa of Fabricius
3	3	0/3	Ileum, bursa of Fabricius
5	11	3/5	Jejunum, ileum
7	2	3/5	—
9	8	1/7	—
12	9	3/3	—
15	1	3/5	—
18	10	3/3	—
21	4	0/5	—
24	7	3/12	—
28	5	1/7	—
32	6	1/3	—

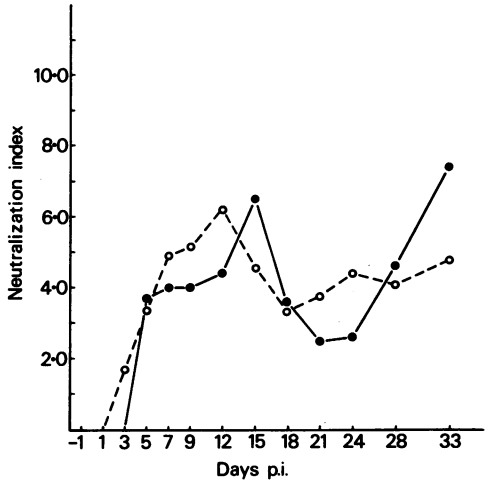

Figure 1. Log_{10} virus neutralization indexes (NI) of serum pools (●——●) and purified bile IgX (O- - -O) in ducks following infection with influenza A/duck/HK/7/75. Serum pools contained equal volumes of sera from four birds, except on Days 24 (three birds), 28 (two birds) and 33 (one bird). NI of bile IgX samples were adjusted arithmetically to an IgX concentration of 1 mg/ml.

and 33 p.i., respectively. At each bleeding p.i., one of the birds was exsanguinated. From this bird, bile and a range of organs (heparinized whole blood, bursa of Fabricius, thymus, spleen, cervical lymph node, pancreas, liver, gall bladder, kidney, gonad, ovarian duct, muscle, brain, trachea, lung, oesophagus, a piece of jejunum adjacent to the duodenum and a piece of ileum 2 inches anterior to the ileocaecal–colic junction) were collected. Oral and cloacal swabs were taken from all available birds on each occasion of bleeding. Swabs and organs were

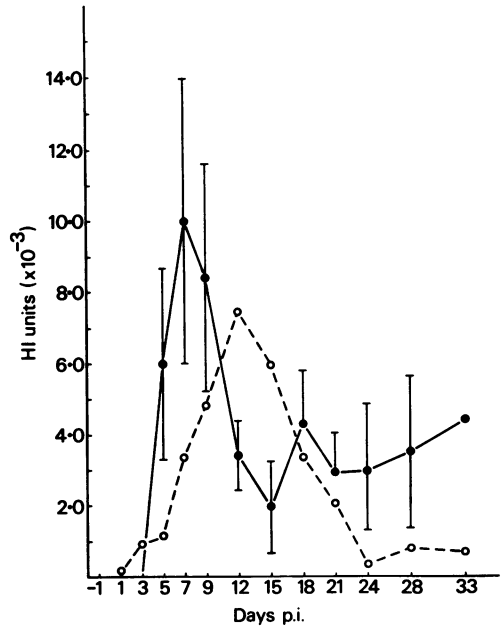

Figure 2. Serum (●——●) and bile IgX (O- - -O) haemagglutination inhibition (HI) antibody responses of ducks following infection with influenza A/duck/HK/7/75. Serum antibodies are expressed as HI units/ml and are given as the mean ± SD of four birds, except on Days 24 (three birds), 28 (two birds) and 33 (one bird). Bile IgX antibodies represent single purified samples and are expressed as HI units/mg IgX.

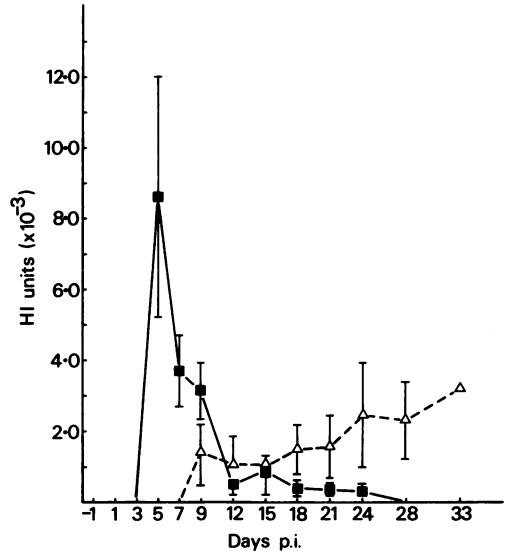

Figure 3. Association of serum HI antibodies with immunoglobulin classes in ducks following infection with influenza A/duck/HK/7/75. Sera were fractionated by ultracentrifugation through sucrose gradients. Presence of IgM and IgG was determined by agar-gel diffusion against isotype-specific antisera. IgM HI titres (■——■) were the sum of the titres of fractions containing IgM; 7.8S IgG titres (Δ- - -Δ) were the sum of the titres of fractions containing 7.8S IgG. Antibody activity was not present in fractions containing 5.7S IgG. Each point is the mean ± SD of four birds except on Days 24 (three birds), 28 (two birds) and 33 (one bird).

Table 3. Antibody activities and immunoglobulin concentrations in sera and biles of six individual ducks killed at various times after infection (p.i.) with influenza A/duck/HK/7/75 virus at 42 days of age

		Serum				Purified bile IgX			Concentration of IgX in bile (mg/ml)*
Day p.i.	Bird no.	HI titre	NI	IgM mg/ml	IgG mg/ml	HI titre	NI	Concentration (mg/ml)	
1	12	1:120	1·60	3·93	2·95	1:2·5	0	0·62	5·55
5	11	1:320	1·40	4·22	4·51	1:24	3·30	0·84	8·26
9	8	1:640	4·00	2·41	5·43	1:96	5·20	0·80	7·55
12	9	1:160	3·00	2·90	4·03	1:192	6·22	1·03	6·18
15	1	1:160	2·75	3·54	2·82	1:80	4·59	1·05	11·60
21	4	1:120	3·30	4·10	2·27	1:24	3·40	0·46	7·72

* Antibody assays not performed on whole bile.

cultured for virus. Individual sera, serum fractions obtained by ultracentrifugation, and purified bile IgX were tested for HI antibody, and pools of sera from each occasion of bleeding and the purified bile IgX samples were examined for VN antibody. To further clarify the relationship between VN antibody in serum and bile IgX, some individual sera, corresponding to the birds from which bile was obtained, were also tested.

RESULTS

Experiment 1

The results are shown in Table 1. All ducklings had HI antibodies in serum and bile at the time of sampling. Comparison of the distribution of HI activity and Ig isotypes in sucrose density gradients indicated in all cases that serum antibody was associated with 7.8S IgG; fractions containing IgM or 5.7S IgG did not have HI activity. Viruses of duck origin were isolated from oral and cloacal swabs for up to 18 days p.i. Generally, low levels of bile IgX antibody activity were detected in these birds. Conversely, viruses originating from pigs or humans were not shed, but in some (4/7) ducks stimulated high levels of antibody associated with the bile IgX; it was also noteworthy that birds receiving viruses of human origin had unusually low concentrations of IgX in their bile. Levels of serum IgG antibodies were similar in response to infection with all viruses. Sera and bile IgX from un-inoculated ducks did not have antibody activity to influenza viruses (data not shown).

Experiment 2

The results of virus-isolation attempts on swabs and organs (Table 2) indicated that the major sites of virus replication were the intestine and bursa of Fabricius. Bile IgX showed an initial peak of VN antibody at 12 days p.i., and strong VN activity persisted throughout the experiment (Fig. 1). Serum VN antibodies appeared in a biphasic pattern, achieving an initial peak NI of 6·5 at Day 15 p.i. and later increasing again on Days 28 and 32 p.i. (Fig. 1). The pattern of HI antibody production (Fig. 2) was distinct from VN antibody both in bile IgX and sera. HI antibody activity of bile IgX reached a peak of 7418 HI units/mg 12 days p.i. and fell to low titres by 24 days p.i. Serum HI antibody reached an initial peak on Day 7 p.i. HI tests on fractions from ultracentrifugation showed that this early serum HI activity was exclusively IgM (Fig. 3). IgM antibodies diminished by Day 12 p.i. and were replaced by antibodies of the 7.8S IgG class. HI antibodies were not found in fractions containing 5.7S IgG. Comparison of Ig concentrations and antibody activities in sera and biles of six individual birds (Table 3) showed that bile IgX expressed more antibody activity per mg than could be attributed to serum IgM or IgG.

DISCUSSION

These experiments show that the IgX of duck bile is capable of expressing HI- and VN-antibody activity to influenza A viruses. The following observations suggest that the IgM and IgX responses were distinct.

Firstly, about 4 weeks p.i. with a variety of H3N2 viruses, HI antibodies were associated with bile IgX and serum 7.8S IgG, but not with IgM. This could, however, reflect the low concentration (~2–4 mg/ml) of IgM in most duck sera and its further dilution in sucrose gradients, in contrast to the high concentration of IgX in bile and in the purified samples tested.

Secondly, the temporal patterns of synthesis of IgM and IgX antibodies to the A/duck/HK/7/75 virus, although similar, were not identical. These differences could, however, reflect the involvement of a transport mechanism requiring hours or days to concentrate Ig from serum into bile.

Thirdly, the antibody activity of bile IgX in response to influenza A/duck/HK/7/75 could not have been accounted for by transfer of IgM or IgG from serum. It remains possible that transfer of Ig to bile is selective for antibody populations of certain specificities.

Other studies in this laboratory (D. A. Higgins, unpublished observations) have shown that after intravenous administration of the inert dinitrophenyl (DNP) hapten–human IgG conjugate, ducks mount a strong serum IgM response to DNP but antibody does not appear in the bile. Mucosal exposure to antigen might be an important requirement either in stimulating the production of bile antibodies or in attracting their transportation from serum precursors. It is relevant, therefore, that virus isolation confirmed previous reports (Webster *et al.*, 1978) that the bursa of Fabricius and ileum are the main sites of virus replication.

The involvement of serum Ig isotypes in the antibody response to A/duck/HK/7/75 virus followed expected patterns.

The IgM response was transient, as it is in the chicken to some viruses (Higgins & Calnek, 1975) and protozoa (Mockett & Rose, 1986). The failure to demonstrate HI antibodies in serum fractions containing 5.7S IgG was not surprising. Physically and antigenically this molecule resembles the 7.8S IgG minus the Fc portion of the heavy chain (Grey, 1967a; Zimmerman, Shalatin & Grey, 1971). Although this is the predominant isotype with antigen-binding activity in the serum of hyperimmunized ducks (Grey, 1967b), it is unable to express secondary properties such as agglutination, precipitation and, presumably, HI. Maternally derived passive immunity in ducklings is exclusively 5.7S IgG (Toth & Norcross, 1981; P. L. K. Ng & D. A. Higgins, unpublished observations). Ducklings from vaccinated mothers are passively immune to virus infections, indicating that the 5.7S IgG can neutralize virus. Involvement of the 5.7S IgG might explain why the VN titre of ducks rose toward the end of the experiment while HI titres in sera and fractions did not rise. As the priority of this work was to investigate the properties of bile IgX, serum fractions were not examined by VN test.

These new observations, along with our previous data concerning the antigenic structure and ontogeny of bile IgX (Ng & Higgins, 1986), indicate that this molecule is part of an independent secretory immune system in the duck. It will be particularly informative to study the cell and tissue origins of serum IgM and bile IgX. In most mammalian mucosal immune systems bile IgA antibodies are produced by plasma cells located in the submucosa of the small intestine (Manning *et al.*, 1984), and transported by liver from plasma to bile (Jackson *et al.*, 1978; Orlans *et al.*, 1978). In man, some bile IgA appears to be produced by the gall bladder (Vuitton *et al.*, 1985). In the rat, bile IgM is the product of a subpopulation of spleen cells that migrate from spleen to liver after antigenic stimulation (Jackson *et al.*, 1985). Duck liver contains many lymphocytes, and their origins as well as their role in production of IgX require investigation.

The duck is the major host of avian influenza viruses (Hinshaw & Webster, 1982; Shortridge, 1982). Genetic reassortment of viruses (Webster & Laver, 1975) in this species has been proposed as a mechanism for the emergence of new subtypes pathogenic for man (Shortridge, 1983). Since the avian viruses are predominantly enterotropic (Webster *et al.*, 1978; Kida, Yanagawa & Matsuoka, 1980), secretory antibodies would be likely to influence the outcome of infection. The situation is comparable to enteric infection with Newcastle disease virus in the chicken, where bile IgA almost certainly acts as the predominant local defence mechanism (Lee & Hanson, 1975). It may not be coincidental that cloacal shedding of the A/duck/HK/7/75 virus ceased at about the same time that bile IgX antibody reached peak activity. Also, bile IgX is the first component of the immunological system of ducks that appears to respond differently to viruses varying in ability to replicate. Thus, viruses of duck origin could be isolated from oral and cloacal swabs for several days, and generally stimulated lower antibody activity of bile IgX than did viruses originating from humans or pigs, which were not recovered from oral or cloacal swabs. Furthermore, the ontogeny of bile IgX (Ng & Higgins, 1986) appears to parallel the development, with age, of resistance to infection (or cessation of virus shedding) seen in farm birds (Markwell & Shortridge, 1982). Whether the bile IgX plays a determinative role in the host–virus interaction will require further prospective studies for clarification.

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REFERENCES

BERLIN B.S., MCQUEEN J.C., MINUSE E. & DAVENPORT F.M. (1963) A method for increasing the sensitivity of the hemagglutination-inhibition test with equine influenza virus. *Virology*, **121,** 665.

GREY H.M. (1967a) Duck immunoglobulins. I. Structural studies on a 5.7S and 7.8S γ-globulin. *J. Immunol.*, **98,** 811.

GREY H.M. (1967b) Duck immunoglobulins. II. Biologic and immunochemical studies. *J. Immunol.*, **98,** 820.

HART S., WRATHNALL A.B., DOGGETT T.A. & HARRIS J.E. (1987) An investigation of the biliary and intestinal immunoglobulin and plasma cell distribution in the gall bladder and liver of the common dogfish, *Scyliorhinus canicula* L. *Aquaculture* (in press).

HIGGINS D.A. & CALNEK B.W. (1975) Fowl immunoglobulins: quantitation and antibody activity during Marek's disease in genetically resistant and susceptible birds. *Infect. Immun.* **11,** 33.

HINSHAW V.S. & WEBSTER R.G. (1982) The natural history of influenza A viruses. In: *Basic and Applied Influenza Research.* (ed. A. S. Beare), pp. 79–104. CRC Press Inc., Boca Raton.

HSU E., FLAJNIK M.F. & DU PASQUIER L. (1985) A third immunoglobulin class in amphibians. *J. Immunol.* **135,** 1998.

JACKSON G.D.F., LEMAITRE-COELHO I., VAERMAN J.-P., BAZIN H. & BECKERS A. (1978) Rapid disappearance from serum of intravenously injected rat myeloma IgA and its secretion into bile. *Eur. J. Immunol.* **8,** 123.

JACKSON G.D.F. & WALKER P.G. (1983) The transient appearance of IgM antibodies in the bile of rats infected with *Salmonella enteriditis*. *Immunol. Letters*, **7,** 41.

JACKSON G.D.F., WALKER P.G., SCHIFF J.M., BARRINGTON P.J., FISHER M.M. & UNDERDOWN B.J. (1985) A role for the spleen in the appearance of IgM in the bile of rats injected intravenously with horse erythrocytes. *J. Immunol.* **135,** 152.

KIDA H., YANAGAWA R. & MATSUOKA Y. (1980) Duck influenza lacking evidence of disease signs and immune response. *Infect. Immun.* **30,** 547.

LEE J.S. & HANSON R.P. (1975) Effects of bile and gastrointestinal secretions on the infectivity of Newcastle disease virus. *Infect. Immun.* **11,** 692.

LOBB C.J. & CLEM L.W. (1981) Phylogeny of immunoglobulin structure and function. XII. Secretory immunoglobulins in the bile of the marine teleost *Archosargus probatocephalus*. *Mol. Immunol.* **18,** 615.

LU B.-L., WEBSTER R.G. & HINSHAW V.S. (1982) Failure to detect hemagglutination-inhibiting antibodies with intact avian influenza virions. *Infect. Immun.* **38,** 530.

MANNING R.J., WALKER P.G., CARTER L., BARRINGTON P.J. & JACKSON G.D.F. (1984) Studies on the origins of biliary immunoglobulins in rats. *Gastroenterology*, **87,** 173.

MARKWELL D.D. & SHORTRIDGE K.F. (1982) Possible waterborne transmission and maintenance of influenza viruses in domestic ducks. *Appl. Envir. Microbiol.* **43,** 110.

MOCKETT A.P.A. (1986) Monoclonal antibodies used to isolate IgM from chicken bile and avian sera and to detect specific IgM in chicken sera. *Avian Path.* **15,** 337.

MOCKETT A.P.A. & ROSE M.E. (1986) Immune responses to eimeria: quantitation of antibody isotypes to *Eimeria tenella* in chicken serum and bile by means of the ELISA. *Parasite Immunol.* **8,** 481.

MULLOCK B.M., SHAW L.J., FITZHARRIS B., PEPPARD J.V., HAMILTON M.J.R., SIMPSON M.T., HUNT T.M. & HINTON R.H. (1985) Sources of proteins in human bile. *Gut*, **26**, 500.

NG P.L.K. & HIGGINS D.A. (1986) Bile immunoglobulin of the duck (*Anas platyrhynchos*). I. Preliminary characterization and ontogeny. *Immunology*, **58**, 323.

ORLANS E., PEPPARD J., REYNOLDS J. & HALL J. (1978) Rapid active transport of IgA from blood to bile. *J. exp. Med.* **147**, 588.

PALMER D.F., COLEMAN M.T., DOWDLE W.R. & SCHILD G.C. (1975) *Advanced laboratory techniques for influenza diagnosis.* Immunology Series, No. 6. U.S. Department of Health, Education and Welfare, Washington, DC.

PEPPARD J.V., JACKSON E. & HALL J.G. (1983) The occurrence of secretory IgM in the bile of rats. *Clin. exp. Immunol.* **53**, 623.

REED L.J. & MUENCH H. (1938) A simple method of estimating 50 per cent end points. *Amer. J. Hyg.* **27**, 493.

SCHOLTISSEK C., BURGER H., KISTNER O. & SHORTRIDGE K.F. (1985) The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. *Virology*, **147**, 287.

SHORTRIDGE K.F. (1982) Avian influenza A viruses of southern China including Hong Kong: ecological aspects and implications for man. *Bull. WHO*, **60**, 129.

SHORTRIDGE K.F. (1983) Pandemic influenza: application of epidemiology and ecology in the region of southern China to prospective studies. In: *Origin of Pandemic Influenza Virus.* (ed. W. G. Laver), pp. 191–200. Elsevier Science Publishing Co., New York.

SHORTRIDGE K.F., KING A.P. & WEBSTER R.G. (1987) Monoclonal antibodies for characterizing H3N2 influenza viruses that persist in pigs in China. *J. infect. Dis.* **155**, 577.

TOTH T.E. & NORCROSS N.L. (1981) Immunoelectrophoresis of duck sera and immunoglobulins. *Avian Dis.* **25**, 1.

VUITTON D.A., SEILLES E., CLAUDE P., SAVA P. & DELACROIX D.L. (1985) Gall bladder: the predominant source of bile IgA in man. *Clin. exp. Immunol.* **62**, 185.

WEBSTER R.G. & LAVER W.G. (1975) Antigenic variation of influenza viruses. In: *The Influenza Viruses and Influenza.* (ed. E. D. Kilbourne), pp. 269–314. Academic Press, New York.

WEBSTER R.G., YAKHNO M., HINSHAW V.S., BEAN W.J. & MURTI K.G. (1978) Intestinal influenza: replication and characterization of influenza viruses in ducks. *Virology*, **84**, 268.

ZIMMERMAN B., SHALATIN N. & GREY H.M. (1971) Structural studies on the duck 5.7S and 7.8S immunoglobulins. *Biochemistry*, **10**, 482.