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Immunodeficiency in the chicken

IV. AN IMMUNOLOGICAL STUDY OF INFECTIOUS BURSAL DISEASE

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SUMMARY

Chickens inoculated orally with infectious bursal disease virus (IBDV) 1 day after hatching subsequently showed a 50% incidence of immunodeficiency but little mortality. Antibody responses against IBDV and to immunization with sheep red blood cells (SRBC) or human serum albumin (HSA) were suppressed. Serum IgG concentration was decreased while IgM occurred exclusively in its 7S monomeric form (mIgM). An allotypic marker of chicken IgM ($M1^a$) was lacking in mIgM derived from IBDV-infected birds. The loss of $M1^a$ occurred gradually in several birds between 3 and 12 weeks after perinatal infection. Inoculation of IBDV into chickens 3 weeks after hatching resulted in a 50% mortality level but little immunodeficiency. Paradoxically, the serum IgG concentration was elevated, in comparison with normal birds. Histology of the bursa showed permanent hypo- or aplasia of follicles irrespective of the age of infection. The results suggest that bursal but not peripheral B cells are targets for IBDV, and immunodeficiency results from impaired peripheral seeding of B cells in infected juvenile chickens.

INTRODUCTION

An acute infectious disease of young chickens which is characterized by diarrhoea, nephrosis and an enlarged bursa of Fabricius occurred originally in an area of Delaware known as Gumboro (hence the eponym 'Gumboro disease') (Cosgrove, 1962). Subsequently, the viral aetiology was discovered and two viruses responsible for the nephrotic and bursal manifestations respectively were isolated (Winterfield & Hitchner, 1962). Recently, two morphologically distinct but antigenically related viruses of Infectious bursal disease (IBDV) were shown in preparations producing the Infectious bursal disease (IBD) (Almeida & Morris, 1973). The histology early after infection is characterized by acute necrosis of cells within the bursal lymphoid follicles followed by reticular cell hyperplasia and occurrence in the spleen of blast and plasma cells (Helmboldt & Garner, 1964; Cheville, 1967). In view of the mandatory role of the bursa of Fabricius for the ontogeny of avian B lymphocytes, it is feasible to assume that the humoral responsiveness of chickens infected with IBD may be impaired. Indeed, this was established in antibody responses to Newcastle disease vaccine, although the reports were controversial regarding the age (1 day and 6 weeks respectively) when immunodepression was most pronounced (Faragher, Allan & Cullen, 1972; Hirai *et al.*, 1974).

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In view of the suspicion that IBD is a widespread inapparent infection among broiler flocks (Report, 1969) it has become of obvious interest to analyse the nature and extent of immunological damage. The data reported here suggest that the disease is also of considerable theoretical interest, particularly for the general understanding of mechanisms which may play a role in the pathogenesis of immunodeficiencies.

MATERIALS AND METHODS

Virus. Isolate 12/69 (Central Veterinary Laboratory, Weybridge) was used at its fifth bursal passage in 3-week-old IBD-susceptible birds. The clarified bursal homogenate prepared by a method reported previously (Almeida & Morris, 1973) had an egg infective titre of $10^{5\cdot7}EID_{50}$/ml as assayed on the chorioallantoic membrane of 11-day embryonated eggs; endpoints (deaths and membrane lesions) were read after 6 days of incubation at 37°C (Hitchner, 1970).

Chickens. Fertile eggs of a specific pathogen-free flock of White Leghorn IBD-susceptible chickens were received from Wickham Laboratories (SPF Farms) Ltd. They were inoculated orally with $10^{3\cdot7}EID_{50}$ per bird at various days after hatching.

Serum IgG concentration. The single diffusion method of Mancini, Carbonara and Heremans (1965) was employed using a goat anti-chicken IgG serum.

Immunization and serology. Chickens were injected with 1 ml of 10% SRBC or 20 mg of HSA/kg body weight. Sera were obtained 4, 7 and 12 days after immunization, and peak serological titres were determined. Agglutination reactions were performed within 1 week of bleeding with sera stored at 4°C. Haemagglutination of SRBC or of SRBC sensitized with HSA by the carbodiimide method was performed using the Takatsy microdilution technique, and recording $\log_2$ values of end point dilutions. For 2-mercaptoethanol (ME) treatment sera were diluted serially into 0·2 M ME and incubated 1 hr at 37°C before red cells were added. The ME-sensitive titre (representing IgM antibodies) was calculated by subtracting the titre in the presence of ME (representing IgG antibodies) from the titre in saline. Previous experiments demonstrated that this criterion is adequate for distinguishing the antibody class of the majority of antibodies (Ivanyi, 1973, 1975a).

Radial diffusion test for IBD antibodies. The gel consisted of 1% agarose (Induboise A37, L'Industrie Biologique Francais SA) containing 0·4% phenol and 8% NaCl in distilled water. Antigen (Bursal Homogenate clarified with Arcton from the 6th passage of the virus) was incorporated into the gel at a final dilution of 1:6 by mixing with an equal volume of 2 X-gel. Test sera (10 μl) were placed into wells of 3 mm diameter and allowed to diffuse for 24 hr. Gels were stained with amido black to facilitate the measurement of precipitin zones. A standard convalescent serum from infected birds was used as a control in each test and the diameter of the precipitin zone formed by this serum was taken as the arbitrary value to which all test sera were related. Thus, the antibody titre against IBDV is expressed as 'ratio' = (test serum diameter)/(standard diameter).

Histology. Organs were fixed in neutral 10% formol–saline and embedded in paraffin. Transverse 5-μm sections of the bursa were cut across the equatorial plane and stained with haematoxylin and eosin.

Analysis of serum proteins. Serum was fractionated on a column of Sephadex G-200 and eluted with 0·15 M NaCl containing 0·002% Hibitane as a preservative. Pooled fractions were concentrated by negative pressure dialysis and tested by immunoelectrophoresis using 1% agar in barbitone buffer, pH 8·6, $\mu = 0{\cdot}03$. The antigens were precipitated by polyspecific sheep anti-chicken immunoglobulin (SaIg) and monospecific goat anti-chicken IgG (GaG) serum.

Detection of allotypes. Sera were examined by the double-diffusion precipitin assay in 1% agar gels containing 8% NaCl and results were scored after 2 days of incubation at room temperature. Allotypic markers of IgM ($M1^a$) and IgG ($G1^a$) were detected by specific typing reagents (antisera nos 773, 561 and 414) which were described fully elsewhere (Ivanyi, 1975c).

RESULTS

Age-dependence of mortality

All infected chickens showed diarrhoea at 3–5 days post infection, but mortality was distinctly related to the age at which birds were injected (Fig. 1).

A 50% mortality occurred within 10 days of infection in birds which were infected at the age of 3 weeks whereas only 10–20% died in groups infected 1 or 11 days after hatching.

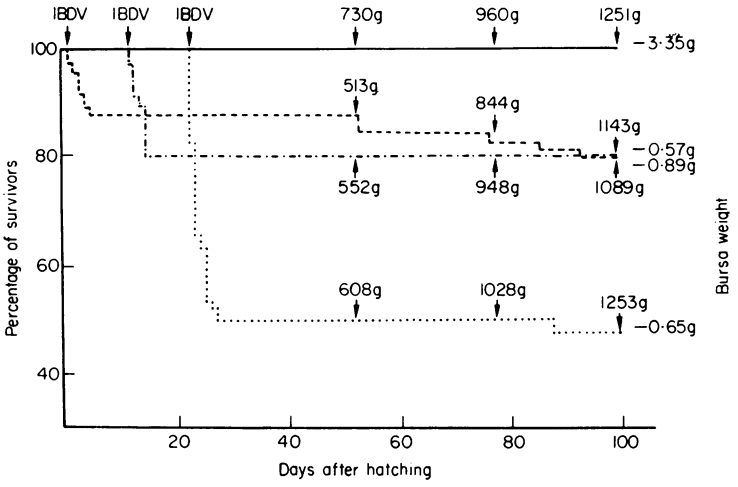

FIG. 1. Cumulative mortality of chickens infected with IBDV at various ages. IBDV inoculated to sixty-six chickens on day 1 (– – –), thirty-five chickens on day 11 (– · – ·) and fifty chickens on day 21 (· · · ·); twenty-two controls (——). Mean body weights were determined at time intervals indicated by arrows. Bursa weights (wet tissue) were obtained at 100 days.

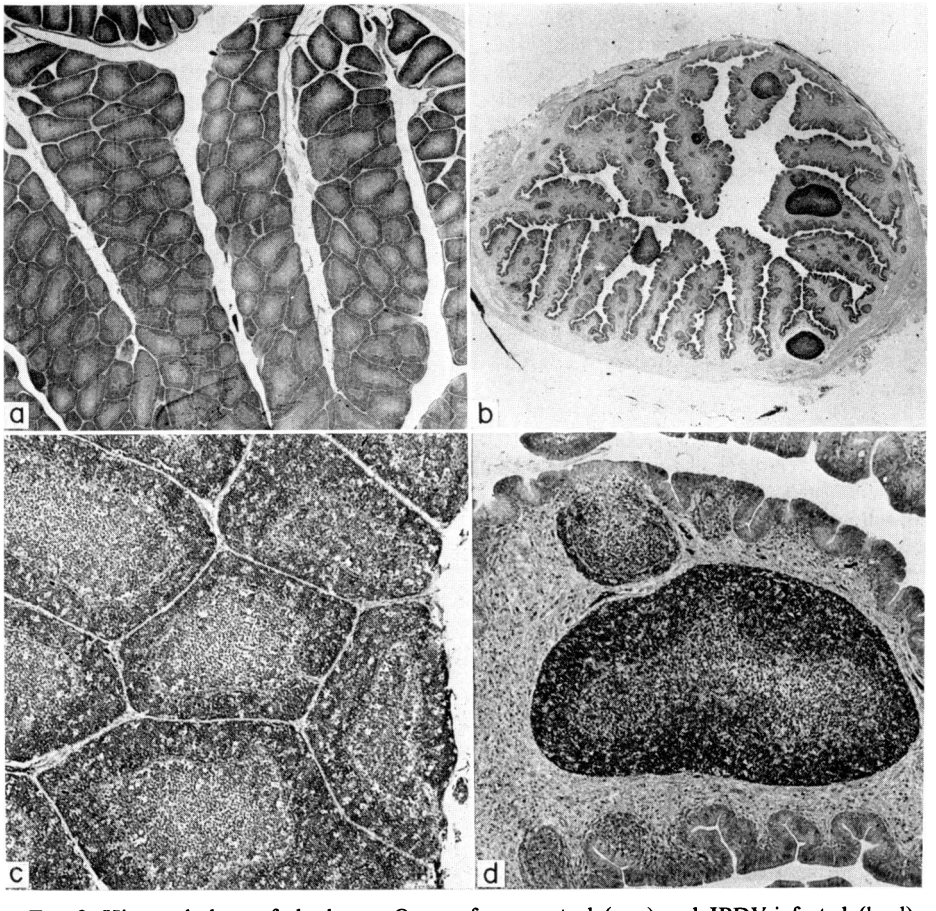

FIG. 2. Histopathology of the bursa. Organs from control (a, c) and IBDV-infected (b, d) chickens were harvested at the age of 6 weeks. Transverse sections (5 μm) were stained with haematoxylin and eosin. (Magnification: ×10 (a, b); ×70 (c, d).)

The body weights of surviving infected chickens were marginally decreased at 56 days but in comparison with controls no significant differences were apparent at the age of 100 days.

Histopathology of the bursa

The weight of the bursa showed an approximate 5-fold reduction, irrespective of the age when birds were infected (Fig. 1). This is apparent also by comparing the histological sections which represent a fragment (approximately 1/5) of the normal bursa (Fig. 2a) with the full equatorial section of the bursa from an infected bird (Fig. 2b). The bursal folds were reduced in size and almost free of developed follicles which had regressed into 'ghost-like' structures of rudimentary size. However, a few large fully developed follicles which occupied almost the whole area of a fold were also observed (Fig. 2d). These follicles were heavily stained with haematoxylin and eosin and their medullary and cortical boundaries were demarcated in a similar manner to bursal follicles from normal chickens of the same age (Fig. 2c).

Serum IgG level and humoral responsiveness

Serum IgG concentrations are presented for individual chickens to demonstrate the large variation within experimental groups (Fig. 3). Fifty per cent of 1-day infected birds

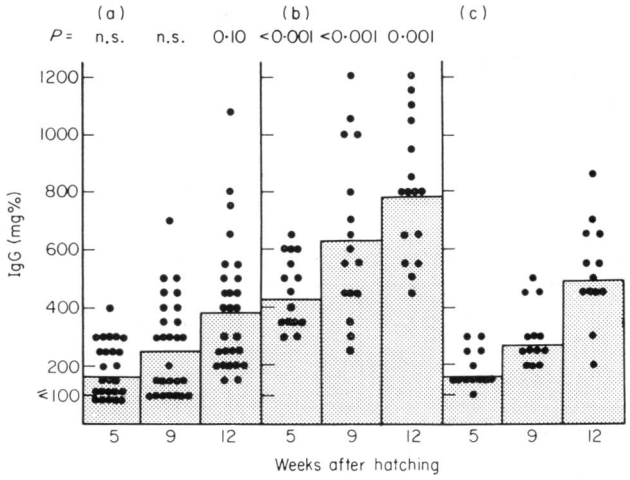

FIG. 3. Serum IgG concentration in IBDV-infected chickens. Stippled area = mean values; symbols = individual chickens. (a) 1 day—IBDV; (b) 21 day—IBDV; (c) controls. P = group mean values compared with corresponding control groups.

had decreased IgG levels, whereas almost all 21-day infected birds manifested an increased IgG concentration between 5 and 12 weeks of age.

The primary and secondary antibody response of the same experimental groups was measured following immunization with SRBC (Fig. 4). About one half of 1-day infected birds failed to show a primary or secondary haemagglutinin response; the remaining birds of this group showed suppressed IgG but enhanced IgM primary responses and normal secondary responses. The latter pattern of responsiveness was found also in the 21-day infected group where the suppressed primary IgG response contrasted with the elevated concentration of serum IgG. Similar results were obtained when HSA was used for immunization (Table 1); furthermore, an intermediary type of reactivity (when compared with 1- and 21-day infection) was demonstrated in the group infected at the age of 11 days (Table 1).

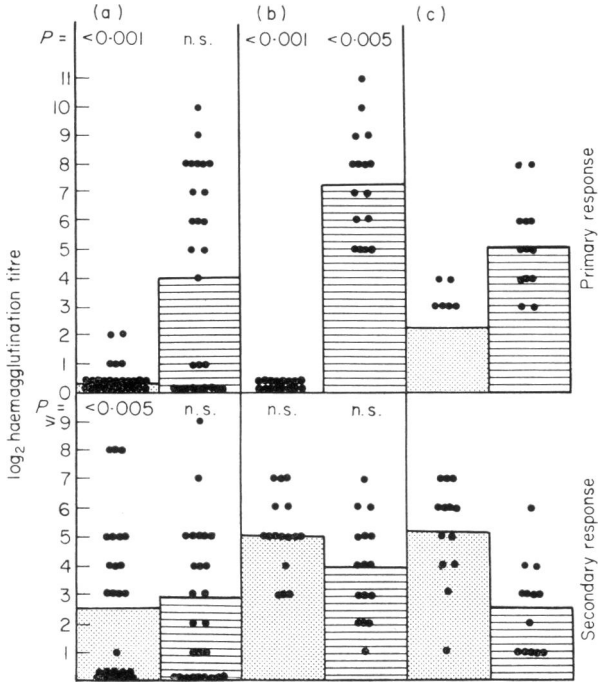

FIG. 4. Antibody response to SRBC in IBDV-infected chickens. (a) 1 day—IBDV; (b) 21 day—IBDV; (c) control. Shaded areas = mean values; symbols = individual chickens. Hatched area = IgM titre; stippled area = IgG titre. *P* = group mean values compared with controls. Immunization with 10^9 SRBC at the age of 8 and 11 weeks.

TABLE 1. Serum IgG and antibody responses in IBDV-infected chickens

Assay at 12 weeks		Inoculation of IBDV at: 1 day		11 days		21 days		None	
	Serum IgG (mg%)								
	601–800	2*		5		2		0	
	501–600	4		7		3		1	
	401–500	3		10		1		3	
	301–400	6		3		2		4	
	201–300	5		2		0		2	
	< 200	6		1		0		0	
	Mean (mg%)	350		450		550		390	
	$\log_2$ HA titre	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
7-day response to 20 mg HSA/kg	8–10	1	1	5	1	0	0	0	1
	6–7	9	3	13	2	2	1	1	6
	4–5	9	2	10	4	4	1	5	3
	2–3	3	6	0	12	2	3	4	0
	< 2	4	14	0	10	0	4	0	0
	Mean HA titre	4·4	2·1	6·4	2·3	4·8	2·1	4·0	6·1

* Denotes number of chickens.

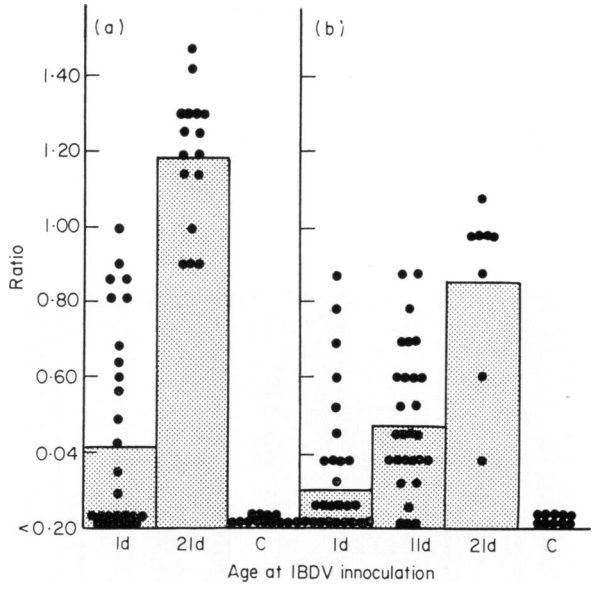

FIG. 5. Antibody response against IBDV. (a) Sera examined at the age of 9 weeks (1st experiment), or (b) sera examined at age of 13 weeks (2nd experiment). Ratio = arbitrary value, see the Materials and Methods section. C = non-infected controls.

Serum antibodies against IBDV were determined quantitatively in both these experiments (Fig. 5). There was a 'split' result in the 1-day infected group in which some chickens failed to respond while others produced antibodies of moderate titre. Chickens infected 21 days post-hatching manifested a relatively high antibody response against the virus, particularly in the first experiment in which they were bled at the age of 9 weeks. The negative values in chickens of control groups may serve as evidence that cross-infection did not occur.

Since in the experiments described above the chickens infected at the age of '1 day' were obtained from the site where the parental flock was housed, we considered the possibility that the 'split' immunodeficiency in one half of the chickens may have resulted from some variations in age at the time of inoculation. Consequently, the time of hatching was closely observed and IBDV was inoculated at intervals listed in Table 2. The results showed that inoculation of the virus within 6 hr after hatching increased the incidence of chickens with

TABLE 2. Antibody response after IBDV inoculation at closely defined time intervals after hatching

IBDV innoculation hours after hatching	Number of birds per group	Anti-SRBC response at 8 weeks		
		Negative		Responder mean titre total/MER
		No.	%	
< 6	15	12	80	7·2/0·0
6–18	16	8	50	6·0/0·0
18–42	19	6	32	5·2/0·0
None	13	0	0	8·0/4·7

suppressed anti-SRBC response to 80%; conversely a delay of inoculation of up to 42 hr resulted in only 32% of immunosuppressed birds.

Occurrence of monomeric IgM in serum

Immunoelectrophoretic analysis of sera showed variable patterns which were classified into three categories (Fig. 6): (1) a complete lack of IgG but a strong IgM precipitin line (G1, G2); (2) a weak and/or less heterogeneous IgG but a strong IgM line (G3, G4); and (3) a normal pattern (see N1, N2).

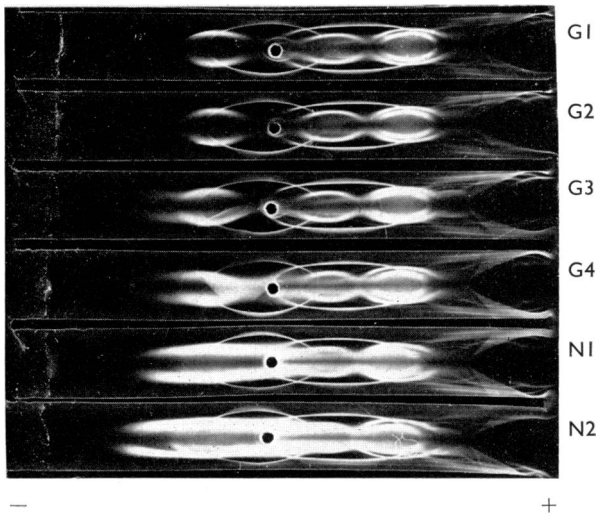

FIG. 6. Immunoelectrophoresis of representative sera. G = IBDV-infected; N = normal chickens. Antiserum = sheep anti-chicken serum.

Sera from category A were pooled and fractionated on a column of Sephadex G-200 (Fig. 7). The resulting fractions (A–D) were concentrated and tested by immunoelectrophoresis (Fig. 8). IgM was localized in fraction B which corresponds to the ascending portion of the 7S peak of normal serum. The separation of fraction B and C resulted apparently from the absence of IgG which is the major 7S component of normal serum. Fraction A corresponding to 19S contained alpha$_2$-macroglobulin but no IgM. (The precipitin arc developed with GaG in the anodic portion of precipitin A is an artefact in the agar gel.)

Loss of M1^a (IgM) allotype

The White Leghorn SPF chickens used in this study were positive for an IgM allotypic marker which was designated previously as M1^a (Ivanyi, 1975c). M1^a was detectable in the sera of all normal chickens at the age of 3 weeks (Table 3). One half of perinatally infected chickens manifested a loss of M1^a from serum. Surprisingly, M1^a was detected in 30% of infected birds at 3 weeks but was lost gradually during the period of observation between 3 and 14 weeks. This loss of M1^a allotype occurred only in chickens which manifested monomeric IgM, and a lack of immune responsiveness to SRBC. Although these birds also manifested a deficiency of IgG which was determined by the loss of G1^a allotype, the loss of the latter occurred at a rate which was not in parallel with the rate of loss of M1^a (Table 4).

DISCUSSION

We have demonstrated that the occurrence of immunodeficiency in IBD is determined by the stage of ontogeny when chickens become infected. The data suggest that B-cell precur-

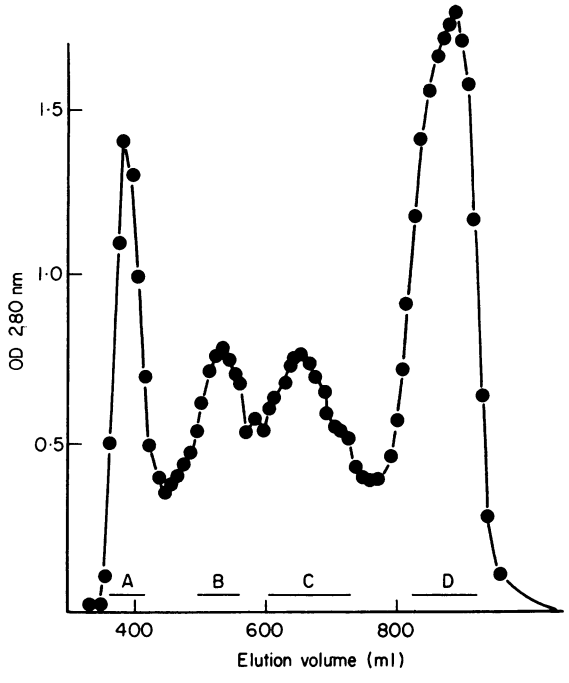

FIG. 7. Sephadex G-200 fractionation of IgG-free serum from IBDV-infected chickens. Twenty millilitres of serum was separated on a 5 × 100 cm column at a flow rate of 20 ml/hr. Pools A–D were concentrated and tested by immunoelectrophoresis (see Fig. 8).

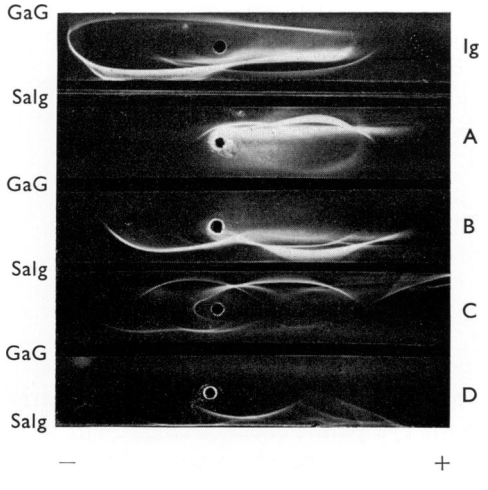

FIG. 8. Immunoelectrophoresis of Sephadex G-200 fractions (A–D, see Fig. 7). Antisera: GaG = goat anti-IgG; SaIg = sheep anti-Ig (polyspecific); Ig = crude immunoglobulin from normal chicken serum.

TABLE 3. Gradual loss of M1[a] allotype

Time of IBDV infection	Number of chickens	Presence in serum of M1[a] allotype Weeks after hatching						Percentage from total in group
		3	4/5	6/7	8/10	11/12	14	
18 hr	5	−	−	−	−	−	−	17
	3	+	−	−	−	−	−	
	1	+	+	−	−	−	−	
	1	+	±	±	−	−	−	30
	1	+	+	±	±	−	−	
	3	+	+	+	±	±	−	
	1	+	+	+	±	±	±	3
	15	+	+	+	+	+	+	50
18–48 hr	2	−	−	−	−	−	−	4
	4	+	+	−	−	−	−	
	4	+	+	±	−	−	−	
	3	+	+	+	+	−	−	30
	2	+	+	+	±	±	−	
	4	+	+	+	+	±	±	9
	25	+	+	+	+	+	+	57
3 weeks	16	+	+	+	+	+	+	100
None	30	+	+	+	+	+	+	100

Sera tested by double-diffusion precipitation in agar gel.

TABLE 4. Lack of correlation between the loss of IgM and IgG allotypes

Last week when allotype detected*	
M1[a]	G1[a]
3	3,6,11
4	5,6,8
5	7
6	4
8	3,5,7,8

* Individual birds.

sors in the bursa are the primary target for the virus, while peripheralized mature B cells are probably not prone to the cytopathic effect of IBDV. The seeding of B cell clones from the bursa to peripheral lymphoid organs, which normally occurs within the first days after hatching (Ivanyi, 1973, 1975a) is obviously obliterated by the acute necrosis of bursal follicles. Since a proportion of B cells is seeded from the bursa prior to hatching (Cooper *et al.*, 1969) it is not surprising that IBDV inoculation 1 day post-hatching produced little immunosuppression in 50% of chickens. Selective suppression of IgG responses in these birds is in agreement with previous demonstrations of late seeding of IgG compared with IgM precursors (Cooper *et al.*, 1969; Ivanyi, 1973). The increased incidence of suppressed birds

which was obtained by inoculating the virus within 6 hr of hatching suggests a high rate of B-cell peripheralization within that relatively short period. On the other hand, IBD infection of 3-week-old chickens resulted in normal IgM but inhibited primary IgG responsiveness. The latter finding is a paradox in view of elevated levels of serum IgG concentration when compared with normal chickens of equal age. Since the natural seeding of B cells is completed at the age of 3 weeks, it is possible that the partial suppression of IgG responses occurred from an impairment of the splenic reticular stroma which is essential for the expression of precursors of IgG antibody formation (Hanna, Nettesheim & Peters, 1971). Although the immunodeficiency which results from IBD infection in many aspects resembles a viral 'bursectomy', it also appears that the severity of immunodeficiency in some of the neonatally infected chickens is higher than would be expected from surgical ablation of the bursa (Ivanyi, 1975a). Thus we cannot exclude the possibility that some peripheralized though still immature B cells might be prone to IBDV-induced damage. Alternatively, some impairment of splenic reticular stroma may have persisted from the early acute stage of the disease which was characterized by cell necrosis and reticular cell hyperplasia (Helmboldt & Garner, 1964; Cheville, 1967).

It was demonstrated previously that IBDV is localizing selectively in the bursa where it has been demonstrated by immunofluorescence and where the virus reaches the highest concentration a few days after infection (Cheville, 1967). Despite the lack of apparent damage in the epithelium of bursal folds, the damage of follicular development was irreversible except in the case of a very few hypertrophic follicles (Fig. 2e). It is not clear whether this was due to some other undetected changes in the bursal environment or to the inadequacy of post-embryonic stem cells to colonize the bursa.

The presence of mIgM in sera from IBD chickens suggests that the virus may cause either a deletion of, or an impairment in, the capacity of B cells to respond to antigenic stimulation. The exact nature of impairment within the mIgM synthesizing cells is not understood although the demonstration of a chain with unique size characteristics (Ivanyi, 1975b) and the loss of the $M1^a$ allotypic marker suggest the synthesis of an immunoglobulin with abnormal structure rather than of a normal monomeric IgM which failed to polymerize as a result of an impaired intracellular assembly mechanism.

Several infected birds lost the $M1^a$ allotype, thereby signalling the change from polymeric to monomeric IgM production between the 3rd and 14th week after hatching. The simplest explanation of this observation would be that a population of normal IgM synthesizing cells (which peripheralized prior to IBDV inoculation) was replaced gradually by pathological mIgM producing cells. This is supported by the previous observation of a decline between 2 and 6 weeks post hatching in the IgM antibody responsiveness of surgically bursectomized chickens (Ivanyi, 1973). Although the decline may be a reflection of the relatively short life span of IgM producing B cells together with a lack of normal B-cell replenishment, it is surprising that IBDV-infected B cells which synthesize mIgM would be seeded as late as 2–3 months post hatching from severely hypoplastic bursal folds which furthermore undergo already their normal involution at this stage of ontogeny. It seems more likely that the change to mIgM production results from an active suppressor mechanism which acts on peripheralized B cells. Suppressor T cells were demonstrated recently in bursectomized-irradiated, agammaglobulinaemic chickens (Blaese, Weiden & Dooley, 1974), but the mechanism by which the suppressor cells are generated is not understood. Although this 'autoimmune' concept of immunodeficiency seems an attractive hypothesis for IBD, further experimental work is needed to substantiate it.

The mechanism of hyperglobulinaemia in juvenile-infected chickens is not clear. In view of its persistence for several weeks after the acute stage of the disease was over, it is unlikely that the increased serum level of IgG represents merely a 'nonspecific' reaction towards the virus-induced acute inflammatory disease. In view of our finding of high antibody

titres against IBDV, it is probable that at least at some stage of the disease circulating complexes of IBDV–antibody occurred. It was suggested that adherence of complexes of antigen–antibody–complement to 'innocent bystander' B lymphocytes via their C3 receptor may stimulate a nonspecific increase in immunoglobulin synthesis (Nussenzweig & Pincus, 1972). Although this attractive hypothesis is supported by evidence of *in vitro* lymphocyte stimulation by antigen–antibody complexes (Bloch-Stacher, Hirschorn & Uhr, 1968; Möller, 1969; Oppenheim, 1972), its validity for IBD remains to be elucidated. Alternatively, it would be feasible to assume that B cells were stimulated by lymphokine-like factors similar to those which are now released *in vitro* by antigen-stimulated lymphocytes (Gorczynski, Miller & Phillips, 1972; Rosenthal, Stastny & Ziff, 1973).

We have considered also the possible role of immune complexes in the pathogenesis of acute mortality of juvenile-infected IBD chickens. Since the size of the bursa relative to body weight has its peak in White Leghorn chickens at the age of 3 weeks (Glick, 1956), a massive increase in virus concentration and consequently more severe clinical manifestations might result from the formation of large amounts of immune complexes. The importance of quantitative factors in the pathogenesis of immune complex-type glomerulonephritis has been stressed recently (Yoshiki *et al.*, 1974).

It is not known whether the loss of susceptibility of peripheralized B cells towards the cytopathic effect of IBDV results from the loss of a membrane receptor or a change in histological architecture. It was reported that bursal but not peripheral B cells are targets for the lymphomagenic effect of avian leukosis virus (ALV) (Peterson, Burmester & Fredrickson, 1964; Peterson *et al.*, 1966). Thus it is tempting to speculate that the marked predisposition of bursal lymphoid elements towards both lymphoid leukosis and IBDV may have a common denominator. These viruses probably interfere at a precisely defined stage of B-cell differentiation. It has been demonstrated that the lymphoma cells secrete both polymeric and monomeric IgM and that lymphomatous birds had elevated or normal titres of antibodies against various antigens (Cooper *et al.*, 1974). These authors suggested that ALV may preclude the natural sequence of gene expression by integrating its RNA-directed DNA into the genome of B cells at the point of intraclonal switch from IgM to IgG expression. It is possible that in those cells which produce monomeric IgM, the arrest in physiological differentiation and impaired immunoglobulin biosynthesis may occur by a similar mechanism. This view is supported by the demonstration that IBDV is an RNA-virus (Kösters, Becht & Rudolph, 1972) although additional morphological characteristics have precluded its classification into any of the conventional categories (Almeida & Morris, 1973).

Although IBD is a unique virus-induced immunodeficiency of the chicken without an analogue in mammalian species, it offers an attractive model to study further the various mechanisms which are of general interest in immunopathology.

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