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DETECTION IN CHICKEN AND HUMAN SERA OF ANTIBODY AGAINST HERPES TYPE VIRUS FROM A CHICKEN WITH MAREK'S DISEASE AND EB VIRUS DEMONSTRATED BY THE INDIRECT IMMUNOFLUORESCENCE TEST[1]

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SUMMARY Many sera from chickens with Marek's disease (MD) and apparently healthy chickens, as well as sera from healthy humans, were titrated by the indirect immunofluorescence tests for antibodies to duck embryo fibroblasts (DEF) infected with a herpes type virus, HTV-Biken C strain, isolated from a chicken with MD.

Sera from chickens with MD and chickens infected with HTV all showed high titers of antibody ($\geqq 1:640$).

The incidence and the titers of antibody in apparently healthy chickens raised under normal conditions depended upon the age of the birds. Sera of chicks of up to 10 days old showed a relatively high incidence and a moderate titer of antibody. Sera of chicks between 11 and 30 days old showed a low incidence and low titer of antibody. In older chicks the incidence and titer of antibody increased rapidly with age.

Sera from chick embryos (7–18 day old embryos) also showed a high incidence and high titer of antibody which seemed to be derived from maternal antibody.

Sera from chickens raised in vinyl isolators, contained no antibody throughout the experiment (165 days from hatching).

Sera from 64 healthy human subjects also all showed antibody activity against DEF passaged chick HTV except for sera from 4 young subjects.

The anti-EB virus titers in sera from chickens were also tested by the indirect immunofluorescent technique. Some sera of chickens showed antibody activity against EB virus, but the titers of antibody were not so high as against chick HTV.

INTRODUCTION

Marek's disease (MD), a disease causing proliferation of lymphoid tissue in chickens has been reported by several investigators (Churchill and Biggs, 1967; Solomon et al.,

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1968; Nazerian et al., 1968) to be caused by a herpes type virus (HTV) which is probably a herpes B group virus. Chubb and Churchill (1968) demonstrated a precipitating antigen of chick HTV by the agar gel double diffusion technique using sera from chickens infected with MD. Their results confirmed by Yuasa et al. (1969). Eidson and Schmitte (1969) described an indirect hemagglutination test with tannic acid for detecting MD antibodies in chicken serum.

The fluorescent antibody (FA) test for MD antigen was first described by Kottaridis and Luginbuhl (1968). They examined chick embryo fibroblasts inoculated with MD agent and bone marrow smears of birds with MD, using the indirect FA technique, and sera from a rabbit which had been hyperimmunized with extracts of infectious blood. Purchase (1969) also reported an indirect FA test for MD antigen in duck and chick embryo fibroblasts and chick kidney cells infected with MD agent. He used sera from 8 week old birds which had been immunized with fresh whole blood from a chicken with MD. The direct FA test for MD antigen was first described by Calnek and Hitchner (1969). They used γ-globulin obtained from a 20 week old bird which had been exposed to MD herpes virus. Independently Naito et al. (1969) also reported a direct FA test using fractionated immunoglobulin from sera of chickens with MD. A bright, specific fluorescence was obtained by DEAE cellulose chromatography eluting with 0.5 M sodium chloride, in duck embryo fibroblasts (DEF) infected with chick HTV.

The ubiquitous presence of MD-infection was demonstrated by Rispens et al. using an indirect FA technique (1969).

Human herpes type virus similar to chick HTV in cultures of Burkitt's lymphoma cells has been described by Epstein et al. (1964). The virus was subsequently discovered in lines of blastoid cells established from leukocytes of patients with various disorders and of healthy donors, and the virus is now named the Epstein-Barr (EB) virus after the cell lines in which it was first detected. Patients with Burkitt's lymphoma and nasopharyngeal carcinoma were regularly found to possess antibodies to EB virus, generally in high titers (Henle et al., 1968; Old et al., 1966). Most adults and healthy children also have some antibody activity against EB virus antigen.

The present report described the incidence of anti-chick HTV antibody in chickens with MD, apparently healthy chickens and healthy human subjects and the titration of the antibody by the indirect FA technique. The antibody activities against chick HTV and EB virus antigens in sera of chickens with MD and of humans were also examined.

MATERIALS AND METHODS

1. *Virus antigens*

The Biken C strain of chick HTV isolated in DEF cultures by Kato et al. (1970) was used throughout. The culture conditions used were described previously (Kato et al., 1970). DEF infected with HTV Biken C strain were dispersed in Leighton tubes with coverslips. When several typical foci were detected in the culture, the coverslips were taken out and fixed with cold acetone for 10 min.

To measure EB virus (EBV) antigenicity, the P3HR-1 subline of the P3J (Jijoye) Burkitt's cell line, kindly supplied by Dr. Y. Hinuma (Tohoku University) was used. EBV cells were grown as suspensions without shaking in Eagle's minimal essential medium supplemented with 20% calf serum at 37 C for 4 days and then at 35 C for one day. Cells were washed five times with phosphate buffered saline (PBS, pH 7.4) and suspended in PBS at a final concentration of approximately 10^6–10^7 cells/ml. A small drop of this suspension was smeared on a coverslip and allowed to dry at room temperature. Then the coverslip was fixed in cold acetone for 10 min.

2. *Chicken sera*

Sera were obtained from 20 chickens (De Kalb 161) of between 80–150 days old, with typical clinical and histopathological symptoms of MD. These samples were found to be free from COFAL agents and anti-RIF (RAV-1 and RAV-2) antibody. Attempts were made to isolate virus from 8 of the 20

chickens, in DEF culture and HTV, similar to HTV Biken C strain, was readily isolated from all 8 chickens.

Sera were obtained from 25 chickens (De Kalb 161) of 120 days old, which had been inoculated with DEF infected with HTV Biken C strain intraperitoneally 21–35 days after birth.

Sera were also taken from 33 so-called specific pathogen free (SPF) chickens showing negative reactions by the COFAL tests and anti-RIF antibody tests. Sera were also obtained from 11 SPF chickens raised in vinyl isolators for 165 days, after hatching.

Sera of 29 chick embryos (De Kalb 161) (1 of 7 days, 28 of 18 days) were obtained by cardiac puncture.

Sera of 154 apparently healthy chickens (De Kalb 161) were also obtained.

Blood samples were taken from 10 chicks (Hubbard strain) at 10 days intervals after hatching and from their parents and the sera were examined.

All these sera were stocked at −20 C before examination.

3. *Human sera*

Eleven human sera were obtained from 8 workers in a poultry farm and 3 laboratory workers and 53 sera were kindly supplied by Prof. K. Kawakami, Osaka Medical College. These sera were stocked at −20 C before use.

4. *Indirect immunofluorescent antibody technique to titrate the antibody level against virus antigens*

Chick and human sera were diluted 1 : 10, 1 : 40, 1 : 160, 1 : 640 and 1 : 2,560 with PBS (pH 7.4). These sera were used as primary antisera for the indirect FA technique.

As secondary antisera to primary chicken serum, anti-chicken globulin rabbit sera were prepared as follows. Serum which was free from COFAL agent, anti-RIF antibody and anti-chick HTV antibody was obtained from an SPF chicken. The standard ammonium sulfate method was used to separate the globulin from the serum. Rabbits were immunized against the globulin with Freund's adjuvant.

As secondary antisera to primary human serum, anti-human globulin sera were obtained from rabbits which had been immunized against human globulin with Freund's adjuvant.

These immune sera from rabbits were fractionated with ammonium sulfate and the globulin was conjugated with fluoresceine-isothiocyanate (FITC) by Kawamura's method (Kawamura, 1969). The fluoresent anti-chicken globulin rabbit globulin and anti-human globulin rabbit globulin showed single precipitin line in the agar gel diffusion test with chicken globulin and with human globulin, respectively.

Coverslips with the virus antigens were first exposed to the primary serum at a dilution of 1 : 10 or more for 30 min at 37 C. Then they were throughly washed with PBS, and overlaid with FITC-conjugated antibodies and incubated in a moist chamber for 1 hr at 37 C. Then they were washed twice for 10 min in petri-dishes containing PBS, agitating with a magnetic stirrer, and then dipped several times into two consecutive beakers of distilled water. Then they were mounted on silides with buffered glycerin and examined under ultraviolet illumination (Toshiba 200 W lamp) with a Nihon Kogaku microscope.

RESULTS

As shown in Table 1, high antibody level against chick HTV antigens were found in sera from chickens with viremia of chick HTV, chickens with MD and chickens inoculated with chick HTV (Fig. 1).

The age distribution of the antibody against chick HTV in apparently healthy chickens is shown in Table 2. A high incidence (100%) of antibody and high antibody levels were observed in sera of chicks of less than 10 days old, including chick embryos. A low frequency of positive sera and lower antibody levels were found in sera of chickens of 11 to 30 days old, but then both the incidence of antibody and the antibody levels increased rapidly until 60 days after birth and 100% of the sera of chickens of more than 61 days old had antibody. The antibody levels of these chickens were as high as those of the chickens shown in Table 1.

On the contrary, the sera of specific pathogen free (SPF) chickens which were found to be free from COFAL viruses showed a low frequency of antibody and low antibody levels (Table 3). Furthermore, the sera of 11 SPF

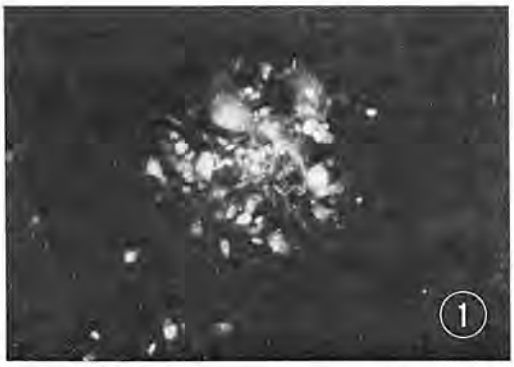

FIGURE 1. *Fluorescence photomicrograph of chick HTV-infected DEF treated with serum of an MD chicken.*

chickens which had been raised in vinyl isolators showed no antibody for up to 165 days after birth.

Blood samples were taken every 10 days from

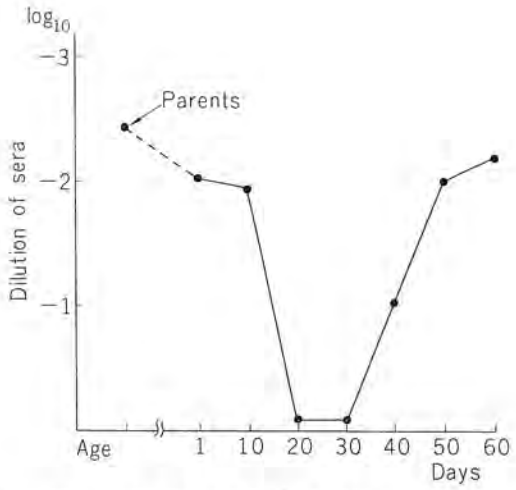

FIGURE 2. *Change in anti-HTV titers in chick sera with age. Blood samples were taken every 10 days from 10 Hubbard chickens raised under ordinary conditions.*

TABLE 1. *Distribution of Anti-HTV Titers in Chick Sera*

Group	Age (days)	No. of Sera	Anti-HTV titers				
			<1:10	1:10	1:40	1:160	1:640≦
HTV Isolated[a]	90~150	8	0	0	0	1	7
Signs of MD[b]	80~150	12	0	0	1	4	7
Infected with HTV[c]	21~35	10	0	0	1	2	7
	120	15	0	0	0	5	10

a These birds gave negative reactions by COFAL tests and anti-RIF tests, and viruses were isolated in DEF cultures.

b Typical MD lesions were identified in these birds clinically and histopathologically.

c These birds were inoculated with chick HTV intraperitoneally 14–18 days after birth.

TABLE 2. *Distribution of Anti-HTV Titers in Apparently Healthy Chick Sera*

Group	Age (days)	No. of Sera	Anti-HTV titers				
			<1:10	1:10	1:40	1:160	1:640≦
Embryos	(7, 18)	29	0	1	12	16	1
Apparently normal	1	22	0	0	8	14	0
	2~10	22	0	2	10	10	0
	11~30	44	19	20	5	0	0
	31~60	33	2	4	13	11	3
	61~280	33	0	2	2	15	14

10 Hubbard chicks raised under ordinary conditions. The antibody levels in these sera were measured by the indirect immunofluorescence technique. The sera of their parents were also examined. As shown in Fig. 2, the sera of chicks of under 10 days old, showed as high an antibody level as that of the parents. Sera of chickens of 20–30 days old showed no antibody or a low antibody level. However, the titers of antibody rapidly increased again in sera of chickens of over 40 days old.

The antibody titers in sera from healthy human subjects were also examined by the FA technique. As shown in Table 4, most of the sera contained antibody activity against chick HTV (Fig. 3).

The antibody activity of chicken serum against EB virus in P3HR-1 cells was examined. The antigenicity of EB virus in P3HR-1 cells were confirmed by the FA technique with a human serum which showed a high antibody level (1 : 640). In cell preparations, about 10% of the cells showed a specific fluorescence. As shown in Table 5, almost half the sera of MD chickens contained antibody, although the antibody levels were lower than those against chick HTV antigen (Fig. 4). Twenty one human sera tested all showed antibody against EB virus antigen.

The antibody activities of 6 human sera and

TABLE 3. *Distribution of Anti-HTV Titers in SPF Chick Sera*[a]

Group	Age (days)	No. of Sera	Anti-HTV titers				
			<1 : 10	1 : 10	1 : 40	1 : 160	1 : 640≦
SPF	40～100	33	23	10	0	0	0
SPF in vinyl isolator	165	11	11	0	0	0	0

a These birds gave negative reactions in the COFAL and anti-RIF antibody tests.

TABLE 4. *Distribution of Anti-HTV Titers in Human Sera*

Group	No. of Sera	Anti-HTV titers				
		<1 : 10	1 : 10	1 : 40	1 : 160	1 : 640≦
Workers in poultry farm	8	0	0	0	3	5
Laboratory workers	3	0	0	1	0	2
Children 1 year	4	1	1	2	0	0
Children 1～15 years	21	1	11	9	0	0
Adults 20～65 years	28	2[a]	16	8	2	0
Total	64	4	28	20	5	7

a 20 years old

TABLE 5. *Distribution of Anti-EB Virus Titers in Chick and Human Sera*

Group (age)	No. of Sera	Anti-EB titers				
		<1 : 10	1 : 10	1 : 40	1 : 160	1 : 640≦
MD Chickens (80–150 days)	11	3	6	2	0	0
Humans (20–65 years)	21	0	1	13	5	2

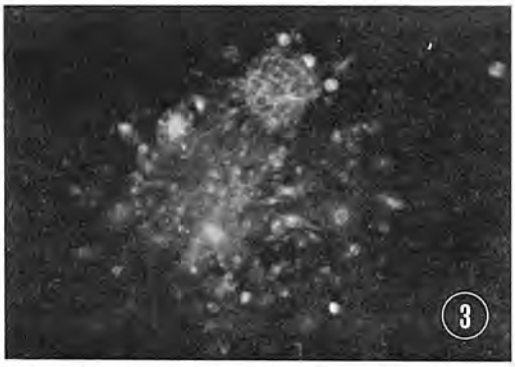

FIGURE 3. *Fluorescence photomicrograph of chick HTV-infected DEF treated with human serum of a worker in a poultry farm.*

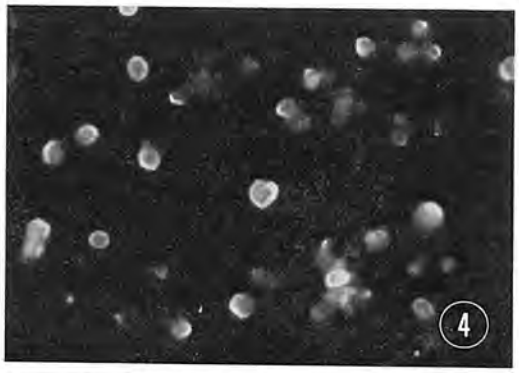

FIGURE 4. *Fluorescence photomicrograph of P3HR-1 cells treated with serum of an MD chicken.*

TABLE 6. *Anti-HTV and Anti-EBV Titers in Human and Chick Sera by the Indirect FAT*

	Sera	Antigen[a]	Dilution of Sera				
			1 : 10	1 : 40	1 : 160	1 : 640	1 : 2560
Human	Kaibara No. 4	HTV-Biken C	##	##	+	+	±
		P3HR-1	##	##	+	+	−
	Kaibara No. 7	HTV-Biken C	##	##	+	+	±
		P3HR-1	##	##	+	−	−
	Osaka No. 1	HTV-Biken C	##	##	+	+	−
		P3HR-1	##	##	+	−	−
	Osaka No. 2	HTV-Biken C	##	##	+	+	−
		P3HR-1	##	+	−	−	−
	Takatsuki No. 10	HTV-Biken C	−	−	−	−	−
		P3HR-1	##	##	−	−	−
	Takatsuki No. 127	HTV-Biken C	±	−	−	−	−
		P3HR-1	##	+	−	−	−
Chick	MD-51	HTV-Biken C	##	##	##	+	−
		P3HR-1	+	+	−	−	−
	T-76	HTV-Biken C	##	##	+	−	−
		P3HR-1	##	+	±	−	−
	S-4009	HTV-Biken C	−	−	−	−	−
		P3HR-1	+	−	−	−	−
	S-4014	HTV-Biken C	−	−	−	−	−
		P3HR-1	±	−	−	−	−

a HTV-Biken C: Chick herpes type virus P3HR-1: EB virus

4 chicken sera against the antigens of chick HTV and EBV were titrated simultaneously by the indirect FA technique. As shown in Table 6, the antibody titers of both sera against the two antigens seem to vary in parallel, although there were some exceptions such as one human serum (Takatsuki No. 10).

DISCUSSION

Previous investigators have shown that under natural conditions MD antibodies are ubiquitously found using agar gel diffusion (Chubb and Churchill, 1968; Yuasa et al., 1969) and the indirect fluorescent antibody technique (Rispens et al., 1969). The present quantitative analysis of the antibody activity against chick HTV using the indirect FA test also demonstrated that infection with chick HTV is widespread. Furthermore change in the titer of antibody in chickens with age was clearly demonstrated. The presence of antibody in chick embryos and chicks of less than 10 days old is probably due to passive transfer of the maternal antibody through the egg, as suggested by Chubb and Churchill (1968). The increase in antibody of chickens again from 30 days after birth is probably due to natural infection with the virus. Chick HTV has been isolated from apparently healthy chickens in high incidence (Kato et al., 1970). However, although there is a 100% incidence of antibody against chick HTV in adult chickens and virus has been isolated in high incidence, the incidence of the disease is generally not very high. This discrepancy might be explained by the widespread existence of nonpathogenic chick HTV and of an optimal age for susceptibility to infection with chick HTV for development of the disease. Rispens et al. (1969) reported that 12 sera of adult chickens originating from SPF stock raised in isolators all gave a negative reaction in the indirect FA test. The present report also showed that sera of 11 chickens raised in vinyl isolators were all negative for up to 165 days after hatching. This supports the idea that the increase in the incidence of sera with antibody in chickens raised under ordinary conditions is due to horizontal infection with the virus.

It is unknown why chickens with MD and chickens with viremia of chick HTV showed high serum titers of antibody. However, this phenomenon might be related with the immunological pathogenesis of Marek's disease which involves the nervous tissues.

Unexpectedly it was demonstrated that most human sera examined showed antibody activity against chick HTV antigen, and also that most adult chicken sera tested also showed antibody activity against EB virus antigen. The infectivities of these viruses to cultured cells of heterologous hosts including human cells for chick HTV and chick cells for EB virus, has not been demonstrated (Kato et al., 1970; Onoda et al., 1970). The possibility that these viruses can infect heterologous hosts cannot be entirely excluded, but the cross reaction due to antigenicity common to both viruses, as demonstrated in the agar gel diffusion test by Ono et al. (1970), must be one reason for the cross immunofluorescence. On the other hand we also noticed some discrepancy between the antibody titers against the two virus antigens, as shown in Table 6. The reason for this discrepancy is unknown, but these sera may contain various amounts of antibodies against other B group herpes viruses which share common antigenicity.

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