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Cultivation of Avian Rotaviruses in Chicken Lymphocytes and Lymphoblastoid Cell Lines

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Summary

Avian rotavirus isolates were used to infect normal chicken spleen cells, lymphoblastoid T cell lines transformed by Marek's disease virus, an avian leukosis virus-transformed B cell line, and a reticuloendotheliosis virus-transformed line, which is a pre-B, pre-T cell line. All five isolates tested were able to infect spleen cells and the three types of lymphoblastoid cell lines, suggesting that avian rotaviruses can infect both B and T cells. Splenic lymphocytes were considerably less susceptible to infection than chick kidney cells. Lymphoblastoid cell lines remained virus-positive during a 10-day culture period. Virus was neutralized by the addition of low dilutions of normal chicken serum and high dilutions of chicken anti-rotavirus serum.

Introduction

The isolation and cultivation of rotaviruses were problematic until the recognition that treatment with pancreatic proteolytic enzymes enhances viral replication *in vitro* (reviewed in 10). Primary epithelial cells or epithelial cell lines are most often used for *in vitro* cultivation of mammalian rotaviruses (reviewed in 10). Similarly, avian rotaviruses are grown in either primary avian kidney (20) or embryo liver cell (12) cultures or in the epithelial cell line MA 104 (19).

In vivo studies on the pathogenesis of infection with rotavirus in mammals and birds confirmed that viral replication occurs mostly in the mature epithelial cells of the intestinal villi. Occasionally, macrophages with rotavirus particles have been observed in the lamina propria of rotavirus-infected calves (17) and virus antigens have been demonstrated in scattered

lymphocytes in the mesentric lymph nodes of infected piglets (18). Recently, YASON and SCHAT (21) described the presence of cells positive for rotavirus antigen in the lymphocytic areas of the cecal tonsils of experimentally infected chickens and turkeys. It was not clear if there was active virus replication or whether these cells were macrophages, antigen-presenting cells or lymphocytes.

There is a paucity of information on replication of rotaviruses in lymphocytes. Only NOZAWA and FONSECA (13) have reported that human rotavirus can replicate in mitogen-stimulated peripheral blood lymphocytes. In this paper we report that several avian rotaviruses can infect both nonstimulated avian splenic lymphocytes and lymphoblastoid cell lines transformed by avian tumor viruses.

Materials and Methods

Virus Strains

The following avian rotaviruses were used: chicken isolates Ch-1 and Ch-2, turkey isolates Tu-1 and Tu-2 and the pheasant isolate Ph-1 (20). Virus stocks were prepared from infected chick kidney cell cultures (CKC) between passage levels 5 and 10 and stored at −80° C.

Cell Cultures

The use of CKC cultures for rotavirus propagation has been described elsewhere (20). Short-term spleen cell cultures were prepared from spleens obtained from 5- to 8-week-old, specific-pathogen-free chickens as described by CALNEK *et al.* (4). Briefly: spleens were collected aseptically and gently forced through a 60 μm autoclavable screen (Tetco, Inc., Elmsford, NY). Cells were centrifuged over Ficoll-Paque (Pharmacia, Inc., Piscataway, NJ). The splenic lymphocytes collected from the interface, were washed twice in PBS and counted. These cells consist mainly of T and B cells, macrophages and natural killer cells. Five $\times 10^6$ spleen cells were cultured in 1 ml of LM-Hahn (LMH) medium (3) in plastic tubes (#2057, Falcon Plastics, Oxnard, CA) with caps loose at 41° C in a humidified atmosphere of 5 percent CO_2 in air. LMH-medium consists of equal parts of Leibovitz and McCoy medium (both from Gibco, Grand Island, NY) supplemented with 10 percent heat-inactivated chicken serum and 8 percent fetal bovine serum (FBS), 5 percent tryptose phosphate broth, 0.01 mM 2-mercaptoethanol, 2 mM glutamine, 1 mM sodium pyruvate and antibiotics. The use of chicken serum and FBS is essential for the cultivation of avian lymphoblastoid cells and for the short term (48 to 72 hours) cultivation of avian splenic lymphocytes. The following lymphoblastoid cell lines were used: i) Two Marek's disease virus-transformed cell lines: MDCC-CU 2 and CU 36 (2, 3), which are both I a-expressing T cells (15). ii) Two avian leukosis virus-transformed cell lines: LSCC-CU 10 (2) and RP-9 (14), which are B cells. iii) One cell line transformed by reticuloendotheliosis virus: RECC-CU 60, which is a pre-T, pre-B cell line (D. WEINSTOCK, personal communication).

Inoculation Procedures

Prior to infection of CKC, spleen cells or lymphoblastoid cell lines, the rotaviruses were treated with 5 μg/ml trypsin for 1 hour at 37° C (19). Lymphoblastoid cell lines were washed twice in PBS, and 1×10^6 cells/ml were resuspended in 0.4 ml PBS containing different concentrations of trypsin-treated rotavirus and incubated for 1 hour at 37° C. The cells were washed 1 to 6 times, placed in 1 ml of LMH and incubated at 38° or 41° C. Spleen

cell cultures were infected following the same method, except that 5×10^6 cells per culture were used. CKC cultures, cultivated on glass coverslips were infected as described elsewhere (20).

Antisera, Monoclonal Antibodies and Fluorescent Antibody Conjugates

Convalescent antisera against rotavirus were obtained from SPF-chickens infected with the Ch-2 strain of rotavirus. These sera were conjugated with fluorescein isothiocyanate (20) for immunofluorescence tests and also used in a virus-neutralization assay.

Mouse monoclonal IgG anti-chicken IgM (μ chain) (MACIgm) (7), mouse monoclonal anti-chicken I a (MAIa) (11), mouse monoclonal anti-chicken panlymphocyte antigen (MACLA), which detects immature B cells prior to hatching, some macrophages and circulating T cells (C.-L. CHEN, personal communication) and a mouse monoclonal antibody against circulating T cells (MAT_3) (A. BENEDICT, personal communication) were used as previously described (16).

Rabbit IgG antimouse Ig (heavy and light chain, Miles-Yeda Ltd, Rehovot, Israel) (RAM) and goat IgG antirabbit Ig/s conjugated with rhodamine (GARG-TRITC) (Cappel Laboratories, West Chester, PA) were used for indirect fluorescent antibody tests in conjunction with monoclonal antibodies.

Tests for Infected Cells in Culture

The numbers of infected cells in the lymphoblastoid and spleen cell cultures were determined as described by CALNEK *et al.* (4) for MDV-infected lymphocytes. Briefly: The number of live cells in each culture was estimated by counting cells in a hematocyte counter using trypan blue dye exclusion. Cells were washed once in PBS, resuspended in 0.1 ml of PBS and 10 μl drop smears were air-dried on glass slides and acetone-fixed. Smears were then stained with FITC-conjugated rotavirus antibodies or with the goat antiserum against human rotavirus followed by FITC-conjugated swine anti-sheep IgG. The number of positive cells was enumerated using a Leitz fluorescence microscope with epi-illumination. Supernatant fluids were harvested from lymphoblastoid cell line cultures, frozen at −70° C and assayed on CKC cultures for virus titers.

Dual Fluorescence Tests

Lymphocyte surface markers on rotavirus-infected spleen cells were detected following the technique described by CALNEK *et al.* (5) for MDV-infected lymphocytes. Briefly, spleen cells were washed in modified PBS (MPBS), containing 1 percent bovine serum albumin and 0.1 percent sodium azide, and then sequentially treated with the desired monoclonal antibody, RAM, and GARG-TRITC. Ten μl drop smears were air-dried, fixed in acetone and stained with the FITC-conjugated chicken antirotaserum. The Leitz microscope was equipped with filters suitable for exciting either conjugate, and by switching from one to the other it was possible to determine whether or not a given virus antigen-positive cell had a given surface marker.

Virus-neutralization Assays

Virus-neutralization assays were conducted using the Ch-2 rotavirus strain and heat-inactivated sera from rotavirus-infected birds, while sera from SPF birds were used as a negative control. Aliquots of 10^4 $TCID_{50}$ in 0.25 ml of trypsin-treated virus were mixed with 0.2 ml of appropriate serum dilutions and incubated for 1 hour at 37° C. The virus-serum mixture was added to 10^6 washed and drained MDCC-CU 36 cells, incubated for an additional hour at 37° C, washed again and resuspended in LMH medium. Cells were examined by immunofluorescence test for virus replication at 24 hours of incubation.

Results

Infection of Lymphoblastoid Cell Lines and Chicken Spleen Cells

In vitro exposure of different cell lines to the trypsin-treated Ch-2 strain of rotavirus resulted in the establishment of infection in a dose-dependent fashion (Table 1). Other avian rotavirus isolates were also able to infect lymphoblastoid cell lines (Table 2). Cells with fluorescent cytoplasmic staining were present in infected but not in control, mock-infected cultures after staining with the rotavirus-specific conjugate. Likewise, the use of the goat antirotavirus serum in an indirect FA assay showed positive cells in the infected, but not in the control cultures. Specific staining was absent when a normal goat serum, free of antibodies to rotavirus, was used.

Splenic lymphocytes exposed to the trypsin-treated Ch-2 strain showed similar specific cytoplasmic staining as the infected lymphoblastoid cell

Table 1. *Infection of spleen lymphocytes and lymphoblastoid cell lines with avian rotavirus strain Ch-2/p 5*

	No. positive cells/smear[a]			
	4×10^6 $TCID_{50}$[b]		4×10^5 $TCID_{50}$[b]	
Cells	24 hours	48 hours	24 hours	48 hours
LSCC-CU 10	100	231	9	20
LSCC-RP 9	120	210	10	13
RECC-CU 60	649	980	28	40
MDCC-CU 36	878	1420	112	142
MDCC-CU 2	147	664	12	68
Spleen bird 1	2	25	...[c]	...
Spleen bird 2	8	8	...	...
Spleen bird 3	2	10	...	...

[a] Each culture was centrifuged, resuspended in 100 µl and 10 µl was used to make a smear

[b] 1×10^6 cells were infected with 4×10^6 $TCID_{50}$ or 4×10^5 $TCID_{50}$

[c] Not done

Table 2. *Infection of lymphoblastoid cell lines by different isolates of avian rotavirus*

	No. positive cells / smear[b] at 48 hours			
Virus isolate[a]	LSCC-RP 9	LSCC-CU 10	MDCC-CU 2	RECC-CU 60
Tu-1	85	112	300	244
Tu-2	29	69	132	19
Ch-1	41	133	100	1140
Ph-1	20	660	26	31

[a] 1×10^6 lymphoblastoid cells were infected with 4×10^6 $TCID_{50}$ as determined by centrifugation on CKC-culture

[b] Each culture was centrifuged, resuspended in 100 µl and 2 smears were made using 10 µl/smear

Table 3. *Relationship between titer of the inoculum, number of virus-positive lymphoblastoid cells and cell numbers after infection of LSCC-CU 10[c] with Ch-2 at 24 and 48 hours in culture*

Inoculation in $TCID_{50}$[a]	24 hours		48 hours	
	Viable cells/ml	% pos.[b]	Viable cells/ml	% pos.
4×10^7	8.4×10^5	0.612	18×10^5	0.438
4×10^6	10.0×10^5	0.071	24×10^5	0.057
4×10^5	9.3×10^5	0.007	13×10^5	0.012
None	11.0×10^5	...[d]	21×10^5	...

[a] Determined by titration on CKC cultures

[b] Each culture was centrifuged, resuspended in 100 µl and 3 smears were made using 10 µl/smear

[c] Cell cultures were initiated at 10×10^5 at the time of infection, cells were counted in a hematocytometer using trypan blue exclusion

[d] Not done

Table 4. *Infection of lymphoblastoid cell lines with the Ch-2 strain of rotavirus*

Cell lines[a]	No. of viable cells $\times 10^6$ (% virus-positive) at days in culture[a]				
	2	4	6	8	10
LSCC-CU 10	1.46 (0.43)	2.88 (0.52)	8.64 (0.11)	18.0 (0.01)	36.0 (0.02)
MDCC-CU 2	1.06 (...)[b]	4.00 (1.11)	12.00 (0.47)	38.4 (0)	115.2 (0.01)
MDCC-CU 36	1.60 (0.28)	3.00 (0.52)	7.68 (0.25)	25.2 (0.01)	50.4 (0.01)

[a] 1×10^6 lymphoblastoid cells were infected at day 0 with 4×10^6 $TCID_{50}$ of Ch-2 as determined by titration on CKC-cultures. Cells were subcultured at 48 hour intervals

[b] Not done

lines. The number of infected cells was considerably lower in splenic lymphocytes than in the lymphoblastoid cell lines (Table 1).

An increase in the number of positive cells was noticed at 48 hours compared to 24 hours of incubation at 41° C. However, there was no increase when the number of positive cells was expressed as a percent of the total number of cells (Table 3). Cell lines remained positive for virus during a 10-day-culture period, but the percent of virus positive cells decreased markedly after 4 days (Table 4). Supernatant fluids collected from the infected lymphoblastoid cell lines at 8 and 10 days in culture were positive for cell-free virus. Titers were between 10^3 and 10^4 $TCID_{50}$ for each cell line. Spleen cell cultures seemed to be less susceptible to infection than the lymphoblastoid cell lines. Spleen cells do not survive very well under these conditions and the percentage of viable cells was only 44, 12 and 12 at 48 hours for the 3 spleen cell preparations in Table 1. In contrast, the lymphoblastoid cell lines were either stable or increasing in cell numbers at 48 hours (data not shown).

Culture Conditions

The importance of trypsin treatment prior to infection and temperature of incubation were examined. Absence of treatment of rotavirus with trypsin decreased the infection rate tenfold but infection could be established in lymphoblastoid cell lines with non-trypsin-treated virus. Due to the low level of infection, attempts were not made to infect splenic lymphocytes with non-trypsin-treated virus. Cultures were incubated at 38 and 41° C after infection with Ch-2 and smears were made at 24 and 48 hours. There were no differences detectable in infection rates between the two temperatures (data not shown).

Identification of Infected Spleen Cells by Surface Markers

Spleen cells infected with the Ch-2 strain of rotavirus were examined at 48 hours postinfection for the presence of rotavirus antigen and specific surface markers. The following surface antigens were present on some but not all of the rotavirus-infected spleen cells: I a, μ and CLA. The actual numbers of cells positive for both rotavirus and surface markers were quite low. Lymphocytes positive for rotavirus and T 3 were not detected. Uninfected spleen cells confirmed the presence of the surface markers.

Virus Neutralization Assays

The incubation of trypsin-treated rotavirus with dilutions of SPF serum, free of antibodies against rotavirus, resulted in considerable inhibition of infection of MDCC-CU 36 (Table 5). A dilution of 1 : 80 resulted in a reduc-

Table 5. *Serum neutralization of Ch-2 rotavirus assayed by infection of MDCC-CU 36*

Exp.	Final[a] serum dilution	Avg. number of positive cells[b, c]	
		Neg. serum	Pos. serum
1	80	182 (36)	...[d]
	200	...	0
2	2	0	0
	80	242 (31)	0
	160	443 (57)	0
	320	541 (70)	0
	640	714 (92)	0
	1280	671 (86)	50 (6)
	2560	771 (99)	216 (28)

[a] Equal volumes of serum and virus suspensions were mixed. The value is the reciprocal of the serum dilution in the mixture

[b] Average of two smears, the number of positive cells in the presence of PBS was 504 and 776 for experiment *1* and *2*, respectively

[c] The values between parenthesis are the percentage of positive cells based on the value for the PBS treatment

[d] Not done

tion of 69 percent compared to the PBS control. On the other hand, a 1 : 2560 dilution of known positive serum caused a similar level of inhibition.

Discussion

Thus far, only NOZAWA and FONSECA (13) have reported that rotaviruses can replicate in lymphocytes albeit after activation with mitogens. This paper extends their results and describes that avian rotaviruses can certainly infect and perhaps replicate in splenic lymphocytes in the absence of mitogens, but it can not be excluded that the low percentage of positive cells were activated cells. The surface markers present on virus-positive spleen cells are compatible with B and T cells (I a, μ and CLA). The finding that three different types of lymphoblastoid cell lines are susceptible to infection supports the concept that rotaviruses can infect both B and T lymphocytes. The question if cell-free infectious virus is indeed produced in lymphoblastoid cell lines is difficult to answer. The recovery of low levels of infectious virus at 8 and 10 days in culture (Table 4) suggest that it is possible. On the other hand it can not be excluded that some of the original inoculum survived outside the cells.

It is still unresolved how rotavirus enters a cell. Although the presence of receptors has been postulated, there is no conclusive proof for their existence (1). Infection of both CKC (10, 12) and lymphocytes requires activation of the virus by trypsin. However, the establishment of infection in lymphocytes is much less efficient than the infection of CKC: 4×10^6 $TCID_{50}$ in CKC infected between 200 and 1400 lymphoblastoid cells depending on the cell line. It is not clear if these differences are due to quantitative differences in expression of the putative receptors or that the lymphoblastoid cells lack certain enzymes important for viral replication. Clearly, the finding that lymphocytes and lymphoblastoid cells are susceptible to infection will be an important aspect in the search for a rotavirus receptor.

The neutralization of rotavirus by normal SPF serum was not expected. The negative serum was obtained from our SPF flock of chickens free of antibodies to rotavirus as determined by indirect immunofluorescence. Sera from the departmental SPF flocks were also tested elsewhere and found to be free of antibodies to rotavirus (Dr. Stewart McNulty, Veterinary Research Laboratories, Belfast, personal communication). Virus neutralizing substances (VNS) have been described in the albumin fraction of FBS interfering with the replication of duck hepatitis virus (6). LMH medium contains both chicken serum and FBS, buth the lymphoblastoid cells are washed prior to exposure with rotavirus. Apparently, the 1-hour incubation period with the chicken serum allows the VNS to interact with the trypsin-treated virus. It will be of interest to determine if the albumin fraction is responsible for the neutralization of the virus.

The relevance of the *in vitro* infection of rotavirus in lymphocytes and lymphoblastoid cells needs further investigation. The presence of viral antigens in the cecal tonsils (21) during virus replication in the intestinal tract raises the question of whether rotavirus may cause immunosuppression, especially in relation to intestinal immunity. We are currently investigating this aspect of the pathogenesis. The infection of lymphocytes may also be important for the establishment of a carrier state. Estes and Graham (9) and Chiarini *et al.* (8) described the establishment of carrier state cultures by cultivation of rotavirus in cell lines. The addition of FBS was essential for the establishment of their persistently infected cultures. The number of virus antigen positive cells remained low and only low levels of virus were released in the supernatant fluids. This system has remarkable similarities with the cultivation of avian rotavirus in lymphoblastoid cell lines: the use of FBS in addition to chicken serum in the medium, low level virus antigen expression decreasing over a 10- to 12-day period and only a low level release of virus into the supernatant fluids. It will be important to establish persistently infected cultures of lymphoblastoid cell lines and determine if these cells can play a role in the persistence and pathogenesis of rotavirus infection in avian species.

References

1. Babiuk LA, Sabara M, Hudson GR (1985) Rotavirus and coronavirus infections in animals. Prog Vet Microbiol Immun 1: 80–120
2. Calnek BW, Murthy KK, Schat KA (1978) Establishment of Marek's disease lymphoblastoid cell lines from transplantable versus primary lymphomas. Int J Cancer 21: 100–107
3. Calnek BW, Shek WR, Schat KA (1981) Spontaneous and induced herpesvirus genome expression in Marek's disease tumor cell lines. Infect Immun 34: 483–491
4. Calnek BW, Schat KA, Shek WR, Chen CLH (1982) *In vitro* infection of lymphocytes with Marek's disease virus. J Natl Cancer Inst 69: 709–713
5. Calnek BW, Schat KA, Ross LJN, Shek WR, Chen CLH (1984) Further characterization of Marek's disease virus-infected lymphocytes. I. *In vivo* infection. Int J Cancer Inst 33: 389–398
6. Chalmers WSK, Woolcock PR (1984) The effect of animal sera on duck hepatitis virus. Avian Pathol 13: 727–732
7. Chen CLH, Lehmeyer JE, Cooper MD (1982) Evidence for an IgD homologue on chicken lymphocytes. J Immunol 129: 2580–2585
8. Chiarini A, Arista S, Giammanco A, Sinatra A (1983) Rotavirus persistence in cell cultures–selection of resistant cells in the presence of foetal calf serum. J Gen Virol 64: 1101–1110
9. Estes MK, Graham DY (1980) Establishment of rotavirus persistent infection in cell culture. Arch Virol 65: 187–192
10. Estes MK, Palmer EL, Obijeski JF (1983) Rotaviruses: a review. Curr Top Microbiol Immunol 105: 123–184
11. Ewert DL, Munchus MS, Chen CLH, Cooper MD (1984) Analysis of structural properties and cellular distribution of avian I a antigen by using monoclonal antibody to monomorphic determinants. J Immunol 132: 2524–2530

12. McNulty MS, Allan GM, Todd D, McFerran JB (1979) Isolation and cell culture propagation of rotaviruses from turkeys and chickens. Arch Virol 61: 13–21
13. Nozawa CM, Fonseca MEF (1984) An attempt to cultivate human rotavirus in human leukocytes culture–preliminary report. Rev Inst Med Trop São Paulo 26: 228–229
14. Okazaki W, Witter RL, Romero C, Nazerian K, Sharma JM, Fadly A, Ewert D (1980) Induction of lymphoid leukosis transplantable tumours and the establishment of lymphoblastoid cell lines. Avian Pathol 9: 311–329
15. Schat KA, Chen CLH, Shek WR, Calnek BW (1982) Surface antigens on Marek's disease lymphoblastoid tumor cell lines. J Natl Cancer Inst 69: 715–720
16. Schat KA, Calnek BW, Weinstock D (1986) Cultivation and characterisation of avian lymphocytes with natural killer cell activity. Avian Pathol 15: 539–556
17. Stair EL, Mebus CA, Twiehaus MJ, Underdahl NR (1973) Neonatal calf diarrhea: electron microscopy of intestine infected with a reovirus-like agent. Vet Pathol 10: 155–170
18. Theil KW, Bohl EH, Cross RF, Kohler EM, Agnes AG (1978) Pathogenesis of porcine rotaviral infection in experimentally inoculated gnotobiotic pigs. Am J Vet Res 39: 213–220
19. Theil KW, Reynolds DL, Saif YM (1986) Isolation and serial propagation of turkey rotaviruses in a fetal Rhesis monkey kidney (MA 104) cell line. Avian Dis 30: 93–104
20. Yason CV, Schat KA (1985) Isolation and characterization of avian rotaviruses. Avian Dis 29: 499–508
21. Yason CV, Schat KA (1987) Pathogenesis of rotavirus infection in different age groups of chickens and turkeys. I. Clinical signs and virology. Am J Vet Res (in press)

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