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 CALEK 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 × 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 CO₂ 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 Ia-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°C or 41°C. Spleen
cell cultures were infected following the same method, except that \(5 \times 10^6\) 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 (\(\mu\) chain) (MAC1gm) (7), mouse monoclonal anti-chicken IA (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 (MAT3) (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 hemocytometer counter using trypan blue dye exclusion. Cells were washed once in PBS, resuspended in 0.1 ml of PBS and 10 \(\mu\)l 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 \(-70^\circ\)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 \(\mu\)l drop smears were air-dried, fixed in acetone and stained with the FITC-conjugated chicken antirotavirus. 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 104 TCD50 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 LMM 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

| Cells          | 4 × 10⁶ TCID₅₀ | 4 × 10⁵ TCID₅₀ |
|---------------|---------------|---------------|
|               | 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       | ...      | ...      |
| 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 × 10⁶ cells were infected with 4 × 10⁶ TCID₅₀ or 4 × 10⁵ TCID₅₀
c Not done

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

| Virus isolate  | 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 × 10⁶ lymphoblastoid cells were infected with 4 × 10⁶ TCID₅₀ 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<sup>c</sup> with Ch-2 at 24 and 48 hours in culture

| Inoculation in TCID<sub>50</sub><sup>a</sup> | Viable cells/ml | % pos.<sup>b</sup> | Viable cells/ml | % pos. |
|---------------------------------|-----------------|----------------|-----------------|--------|
| 4 × 10<sup>7</sup>              | 8.4 × 10<sup>5</sup> | 0.612          | 18 × 10<sup>5</sup> | 0.438  |
| 4 × 10<sup>6</sup>              | 10.0 × 10<sup>5</sup> | 0.071          | 24 × 10<sup>5</sup> | 0.057  |
| 4 × 10<sup>5</sup>              | 9.3 × 10<sup>5</sup> | 0.007          | 13 × 10<sup>5</sup> | 0.012  |
| None                            | 11.0 × 10<sup>5</sup> | ...            | 21 × 10<sup>5</sup> | ...    |

<sup>a</sup> Determined by titration on CKC cultures
<sup>b</sup> Each culture was centrifuged, resuspended in 100 μl and 3 smears were made using 10 μl/smear
<sup>c</sup> Cell cultures were initiated at 10 × 10<sup>5</sup> at the time of infection, cells were counted in a hematocytometer using trypan blue exclusion
<sup>d</sup> Not done

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

| Cell lines<sup>a</sup> | No. of viable cells × 10<sup>6</sup> (% virus-positive) at days in culture<sup>b</sup> |
|------------------------|------------------------------------------------------------------|
|                        | 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 (...)<sup>b</sup> | 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) |

<sup>a</sup> 1 × 10<sup>6</sup> lymphoblastoid cells were infected at day 0 with 4 × 10<sup>6</sup> TCID<sub>50</sub> of Ch-2 as determined by titration on CKC-cultures. Cells were subcultured at 48 hour intervals
<sup>b</sup> 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<sup>3</sup> and 10<sup>4</sup> TCID<sub>50</sub> 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: Ia, µ, and CLA. The actual numbers of cells positive for both rotavirus and surface markers were quite low. Lymphocytes positive for rotavirus and T3 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 serum dilution | Avg. number of positive cells |
|------|----------------------|------------------------------|
|      |                      | Neg. serum | Pos. serum |
| 1    | 80                   | 182 (36)   |     ...   |
|      | 200                  | 0          | 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) |

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*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 ($\lambda_\alpha$, $\mu$ 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: $4 \times 10^6$ 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, both 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.

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