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In Vitro Replication of Infectious Bursal Disease Virus in Established Lymphoid Cell Lines and Chicken B Lymphocytes

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The in vitro susceptibility of chicken lymphocytes to a wild strain of infectious bursal disease virus was investigated by using immunofluorescence and virus assays as infection criteria. A variety of Marek's disease lymphoblastoid cell lines, all of thymus (T-cell) origin, were refractory to virus exposure. However, a bursa (B-cell)-derived lymphoblastoid cell line from an avian leukosis virus-induced tumor was highly susceptible. Viral antigen appeared in the cytoplasm of 20 to 30% of the cells, and large amounts of cell-free virus were released, with maximum yields occurring by 3 days postinfection. The virus also replicated in a small percentage of normal lymphocytes prepared from lymphoid tissues and peripheral blood of chickens. Pretreatment of the lymphocytes, with heat-inactivated anti-B-cell serum or with antiserum against fowl immunoglobulin M before inoculating them with the virus blocked the virus infection; no blocking occurred with anti-T-cell serum or with specific antiserum against fowl immunoglobulin G or immunoglobulin A. This suggests that surface immunoglobulin M-bearing B-lymphocytes were the target cells for infection.

Infectious bursal disease is a disease of young chickens for which the causative virus (infectious bursal disease virus [IBDV]) is tentatively classified as a member of Reoviridae (3, 10, 12, 13). The virus induces atrophy of the bursa of Fabricius as a result of necrosis of lymphocytes and also causes a general lymphocidal effect in other lymphoid organs, including the thymus and spleen. It has been suggested that IBDV may replicate in lymphocytes (6) since virus antigen could be localized by immunofluorescence (IF) in cells thought to be of that type.

This disease is of interest immunologically, since the function of the bursa-dependent lymphoid system is affected in young chickens (8, 15). The mechanism of the immunosuppression is not fully understood, but presumably it results from the loss of immunocompetent lymphocytes. Hitchner (17) reviewed studies on laboratory host systems for IBDV and noted that the virus replicates in chicken embryos and that embryo-adapted virus could be cultivated in cell cultures of chicken embryo origin, with consequent cytopathic effects. No published information is available on replication of virulent, nonadapted strains of the virus in cultured lymphocytes. Since a specific lymphocyte type might serve as the target for IBDV infection in vivo, it was of interest to study virus replication in vitro.

The present experiments were performed to determine the susceptibility to virulent IBDV of normal chicken lymphocytes and established lymphoblastoid tumor cell lines of both thymic and bursal origin (T- and B-cells, respectively).

MATERIALS AND METHODS

Cell culture medium. RPMI 1640 medium was supplemented with 5% tryptose phosphate broth, 8% bovine fetal serum, 10% chicken serum, 2 mM glutamine, 1 mM sodium pyruvate, 10^{-5} M 2-mercaptoethanol, and antibiotics. The medium was described by Hahn et al. (9) and is referred to here as Hahn medium.

Virus. Virulent strain 73688 of IBDV, isolated in this laboratory and kindly supplied by B. Lucio, was prepared from passage 4 of the virus in susceptible chickens. Stock virus consisted of a 10% suspension of bursal tissue harvested from 6-week-old chickens 4 days after infection as described previously (14). The virus was stored at −70°C until use.

Established cell lines. Six lymphoblastoid cell lines initiated from Marek's disease tumors were studied. The MSB-1 (1) and RPL-1 (19) lines were originally obtained from K. Nazerian, U.S. Department of Agriculture Regional Poultry Laboratory, E. Lansing, Mich.; the other lines (GACL-1, JMCL-1, CUCL-1, and GBCL-1) were derived in this laboratory (5). All have T-cell markers. A single B-cell lymphoblastoid line was studied; TLT-1 (5) originated from Olson's transmissible lymphoid tumor (20) induced by avian leukosis virus, a retrovirus.

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All cells were grown in Hahn medium as suspension cultures seeded at 0.5×10^6 to 1.0×10^6 cells per ml. The Marek's disease lines were maintained at 41°C, and TLT-1 was held at 37°C.

Chickens and cell preparation. The 4-week-old White Leghorn chickens used as donors of cultured cells were from the departmental PDRC flock maintained free of most avian viral pathogens, including IBDV. Minced tissues from bursa, thymus, and spleen were processed by gently forcing them through a 60-μm mesh autoclavable screen (Tetko, Inc., Elmsford, N.Y.). The lymphocytes from these suspensions and from heparinized whole blood were separated by centrifugation over Ficoll-Hypaque and washed in phosphate-buffered saline (PBS). The centrifugation on Ficoll-Hypaque and washing procedures were repeated three times, and the cells were finally suspended in Hahn medium. Total and viable cells were counted in a hemacytometer with trypan blue dye exclusion as the criterion for viability.

Antisera. Rabbit anti-B-cell and anti-T-cell sera used for lymphocyte treatment were prepared as previously described (5). The anti-T-cell and anti-B-cell sera were repeatedly absorbed with normal bursal and thymic cells, respectively, until no cross-reactions were detected in IF tests. These sera were shown to be cytotoxic for more than 90% of the homologous cell type when used with complement (C'), but they did not affect the heterologous cell type (Calnek, unpublished data). The antisera alone clumped but did not kill cells, and C' alone was nontoxic.

Rabbit anti-chicken immunoglobulin G (IgG), IgM, and IgA sera used for lymphocyte treatment were from stocks prepared by Higgins and Calnek (11). These stocks were not rendered monospecific for use in this study.

Anti-B-cell and anti-T-cell serum treatment. One-milliliter suspensions of lymphocytes (6×10^6 cells per ml) were treated by adding 0.2 ml of either anti-T-cell serum or anti-B-cell serum. After incubation at 37°C for 1 h, the cells were washed in PBS and resuspended in 0.1 ml of a 1:5 dilution of guinea pig serum as a source of C'. After incubation at 37°C for an additional 45 min, the cells were again washed with PBS. Thereafter, the cells were centrifuged twice on Ficoll-Hypaque, washed two times with PBS, resuspended in Hahn medium, and examined with trypan blue for cell viability.

Cytotoxicity tests. Cytotoxicity of the rabbit anti-chicken IgM and IgG for TLT-1 and MSB-1 cells was tested by mixing 0.05 ml of cell suspension (containing 5×10^5 cells), 0.05 ml of antiserum dilution, and 0.05 ml of C'. For controls, C' or antiserum or both were deleted. After incubation for 90 min at 37°C, the percent viable cells was determined by counting with trypan blue dye. The cytotoxic antibody titer was expressed as the reciprocal of the highest dilution which reduced viability by 50% or more compared with the controls.

Infection. Infection of cells with virus was performed as follows. To 0.5 ml of cell suspension 0.5 ml of the virus suspension, representing a multiplicity of infection of 1.0, was added. The cell cultures were incubated at 37°C for 1 h for virus adsorption and then washed two times with PBS to remove unadsorbed virus, resuspended in Hahn medium, and incubated at 37 or 41°C in plastic flasks (Falcon Plastics, Los Angeles, Calif.). Periodically, samples of cells were collected from the flasks and examined by IF tests for the presence of IBDV antigen-positive cells and by virus assays for infectivity.

IF tests. Antibodies from chickens immunized against IBDV were precipitated from pooled sera and conjugated with fluorescein isothiocyanate by previously described procedures (4). The conjugate was specific for IBDV, based on its failure to stain uninfected cells or cells infected with viruses other than IBDV and its consistent staining of cells or tissues known to be infected with IBDV.

For IF assays, cell suspensions were washed once with PBS. A small drop of concentrated cell suspension was smeared on a cover slip and air-dried at 37°C for 2 min. The cells were fixed with acetone for 10 min and were used for IF staining within 2 h after preparation or were held at 4°C for use within 2 days. The cover slip preparations were stained and examined by previously described procedures (4). The percentage of cells containing viral antigen was determined by examining from 500 to 1,000 cells.

Virus titrations. Virus titrations were done in 10-day-old embryonating eggs from the departmental PDRC strain by inoculating serial 10-fold dilutions of cell culture supernatant fluids into the allantoic cavity. Three to five embryos per dilution each received 0.2 ml. All embryos were incubated for an additional 9 days at 37°C. The 50% embryo infective dose was calculated by the method of Reed and Muench (23), based on embryo mortality or the appearance of characteristic lesions in surviving embryos or both (16).

Electron microscopy. Cells were pelleted by low-speed centrifugation and fixed with 2% glutaraldehyde and 1% osmium tetroxide. After dehydration in graded concentrations of ethyl alcohol and substitution with propylenoxide, the cells were embedded in an Epon-Araldite mixture and thin sectioned with glass knives on a Porter-Blum microtome. The sections were stained with 2% uranyl acetate and lead citrate and examined with a Hitachi HU-11A electron microscope.

Morphological identification of virus particles was based on previous observations (6, 13).

RESULTS

Susceptibility of established lymphoblastoid cell lines to IBDV. The ability of a virulent strain of IBDV to replicate in cultures of several lymphoblastoid cell lines transformed by avian tumor viruses was first studied by the direct IF technique (Table 1). Virus-specific antigen detectable in the TLT-1 cell line was distributed throughout the cytoplasm or appeared as a focus of fluorescence (Fig. 1a and b). The percentage of IF-positive cells ranged from 18 to 28% at 72 h post-inoculation. No virus-specific antigen was identified in uninoculated TLT-1 control cultures or in any of the lymphoblastoid T-cell lines induced by Marek's disease virus. This difference in susceptibility between TLT-1 and Marek's disease lines was not due to the

TABLE 1. *Susceptibility of chicken lymphoblastoid cell lines to infection by virulent IBDV*[a]

Cell type	Cell Line	Passage	% of cells positive for IBDV antigen in IF tests at 72 h post-inoculation
B-cells transformed by avian leukosis virus	TLT-1	7-20	22
T-cells transformed by Marek's disease virus	GACL-1	5	0
	JMCL-1	13	0
	CUCL-1	10	0
	GBCL-1	37	0
	MSB-1	200	0
	RPL-1	206	0

[a] Multiplicity of infection, approximately 1.0. The results represent the average of five experiments.

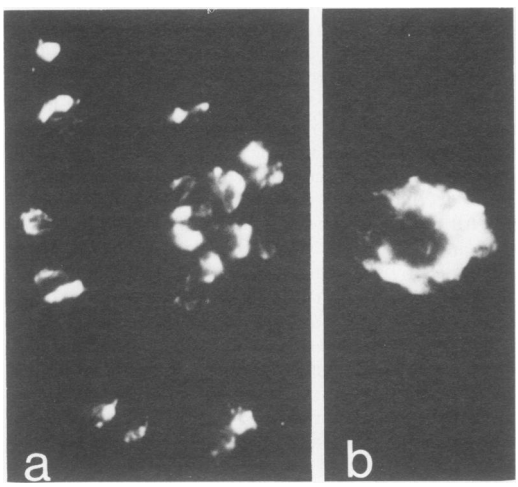

FIG. 1. *Virus-specific IF in TLT-1 cells. The cells were cultured at 37°C for 3 days. (a) ×200. (b) ×300.*

difference in incubation temperature. Subsequent tests, in which TLT-1 cells were exposed to virus and then divided into replicate cultures incubated at 41 and 37°C, showed both temperatures to be equally permissive for virus replication.

IBDV replication in TLT-1 cells. The correlation between production of infectious viral progeny and IF-positive cells was examined. Cell-free virus was found in the supernatant fluids sampled at various intervals (Fig. 2), with the infectivity titer gradually increasing from 1 to 3 days post-inoculation. A plateau of $10^{7.0}$ to $10^{7.5}$ 50% embryo infective doses per 0.2 ml was maintained between 3 and 7 days. The percentage of IF-positive cells paralleled the infectivity titers. Cell viability remained constant throughout the time of incubation.

In thin-sectioned preparations, numerous virus particles 53 to 58 nm in size, with features characteristic of IBDV, were seen in crystalline arrays in the cytoplasm of the TLT-1 cells (Fig. 3a). In uninoculated TLT-1 cells, a large number of C-type virus particles were observed on, or budding from, the cell membrane (Fig. 3b). These were presumed to be the avian leukosis retrovirus known to be associated with TLT-1 cells. Very interestingly, cells in the IBDV-inoculated culture which contained infectious bursal disease virions in the cytoplasm did not have C-type particles on the membrane. This observation was consistent in extensive examinations of several preparations of IBDV-infected TLT-1 cells.

Susceptibility of normal lymphocytes to IBDV. Chicken lymphocytes prepared from lymphoid organs and peripheral blood were tested for susceptibility to IBDV (Table 2). The percentage of IF-positive cells was much higher with bursal lymphocytes than with those from thymus, spleen, and peripheral blood. The percentage of positive cells increased only slightly during the experimental period, reaching 3.81% in the bursal lymphocytes at 72 h after infection. IF-positive cells in the cultures of peripheral blood lymphocytes were rarely detected throughout the observation periods, although a slight increase in the percentage of such cells was found at 72 h after infection.

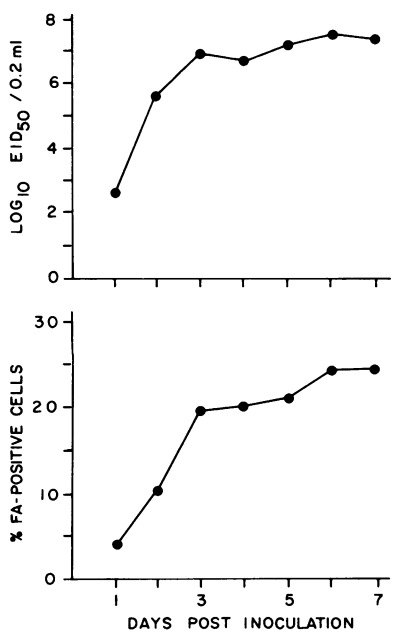

FIG. 2. *Replication of IBDV in TLT-1 cells. Uninoculated control cells tested in parallel were negative in all cases. FA, Fluorescent antibody; EID_{50}, 50% embryo infective dose.*

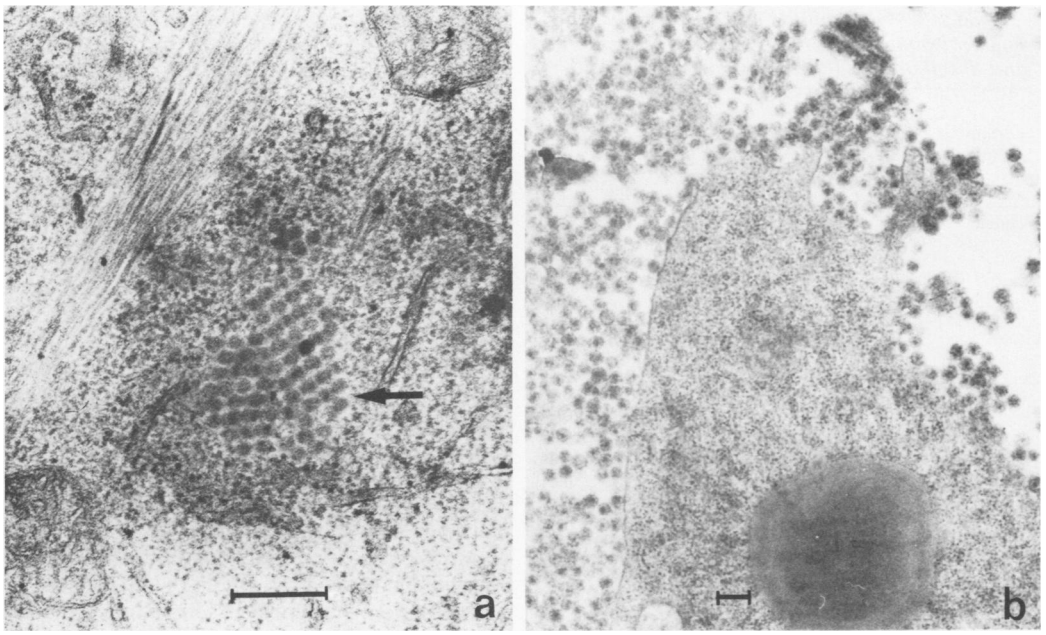

FIG. 3. *(a) Electron micrograph of TLT-1 cell section containing an intracytoplasmic crystalline array of IBDV (arrow). ×30,000. Bar = 100 μm. (b) Normal TLT-1 cell. Avian leukosis virus particles are seen on the cytoplasmic membrane. ×10,000. Bar = 100 μm.*

TABLE 2. *Susceptibility to IBDV of cultured lymphocytes prepared from the bursa of Fabricius, spleen, thymus, and peripheral blood of 4-week-old chickens*[a]

Origin of lymphocytes	% of cells positive in IF tests for IBDV antigen in cultures sampled at:		
	24 h p.i.[b]	48 h p.i.	72 h p.i.
Bursa	2.22	3.07	3.81
Spleen	0.05	0.35	0.75
Thymus	0.40	0.48	0.49
Peripheral blood	0.07	0.11	0.16

[a] Multiplicity of infection, approximately 1.0. The results represent the average of two experiments.
[b] p.i., Post-inoculation.

These data from normal and transformed cell cultures of different origins suggested that the cells susceptible to infection by IBDV may be B-cells but not T-cells. This was further investigated by treating normal lymphocytes with anti-T-cell or anti-B-cell serum before infection of the virus (Table 3). Pretreatment of bursal lymphocytes with anti-T-cell serum with or without C′ had no effect on susceptibility to infection with IBDV. However, pretreatment of thymic lymphocytes with anti-B-cell serum completely blocked infection. Similarly, in suspensions of spleen lymphocytes, IF-positive cells were observed after anti-T-cell serum treatment but not after anti-B-cell serum treatment. The effect of anti-B-cell serum was seen whether or not C′ was added to the system, suggesting that there was a blocking of infection rather than a simple elimination of target cells. In either case, the cumulative data of these experiments clearly suggest that the cells susceptible to IBDV infection are B-cells.

Relationship between IBDV susceptibility and the presence of surface immunoglobulin. Since the susceptible cells for IBDV infection were found to be present in the B-cell populations, a possible relationship between susceptibility and surface immunoglobulin-carrying B-cells in the bursal lymphocyte preparations was examined in three experiments (Table 4). Bursal lymphocytes were first treated with anti-T-cell serum and C′ and then treated with anti-immunoglobulin sera before infection with IBDV. Based on the percentage of IF-positive cells at 24 h post-inoculation, neither anti-IgG nor anti-IgA affected susceptibility to virus infection. On the other hand, the percentage of IF-positive cells in the suspensions pretreated with anti-IgM serum or with combinations of sera containing anti-IgM was markedly reduced.

In cytotoxicity tests, anti-IgM and anti-IgG sera alone were nontoxic for TLT-1 or MSB-1 cells, and these cells were unaffected by C′ alone. In the presence of C′, both antisera were highly cytotoxic for TLT-1 cells (anti-IgM titer, 20;

TABLE 3. *Susceptibility of bursa, thymus, and spleen lymphocytes to IBDV after treatment with anti-T-cell or anti-B-cell serum with or without C'*[a]

Cell source	Pre-inoculation treatment	% of IBDV-positive cells in IF tests at:		
		24 h p.i.	48 h p.i.	72 h p.i.
Bursa	None	1.86	3.34	3.53
	C'	1.43	2.94	3.86
	Anti-T-cell serum	2.34	4.56	6.37
	Anti-T-cell serum + C'	2.52	3.29	7.55
Thymus	None	0.22	0.37	0.42
	C'	0.21	0.25	0.55
	Anti-B-cell serum	0	0	0
	Anti-B-cell serum + C'	0	0	0
Spleen	None	0.52	0.53	0.58
	C'	0.50	0.80	0.78
	Anti-T-cell serum	0.60	0.76	0.82
	Anti-T-cell serum + C'	0.65	0.62	0.58
	Anti-B-cell serum	0.01	0.01	0.01
	Anti-B-cell serum + C'	0.01	0	0

[a] The results represent the average of two experiments.
[b] p.i., Post-inoculation.

TABLE 4. *Effect of pretreatment of bursal lymphocytes with antisera against specific fowl immunoglobulin classes (IgG, IgA, IgM) on their susceptibility to IBDV*[a]

Treatment antisera against:	% of IF-positive cells at 24 h post-inoculation		
	Expt 1	Expt 2	Expt 3
Anti-IgG	2.25	1.4	3.24
Anti-IgM	0.18	0.05	0.43
Anti-IgA	2.83	1.85	2.56
Anti-IgG + anti-IgM	ND[b]	0.08	0.37
Anti-IgA + anti-IgM	ND	0.05	0.17
Anti-IgA + anti-IgG	ND	1.65	3.32
Anti-IgG + anti-IgM + anti-IgA	ND	0.01	0.16
Untreated control	2.74	2.82	4.51

[a] Bursal cells were treated with anti-T-cell serum and C' before treatment with anti-immunoglobulin sera.
[b] ND, Not done.

anti-IgG titer, 10) but not for MSB-1 cells. At the lowest dilution tested (1:2.5), there was virtually no loss of viability with MSB-1 (controls, average of 87.7% viable; anti-IgM and anti-IgG treated, 93.0 and 90.4% viable, respectively). In contrast, TLT-1 cells (controls, average of 94.3% viable) dropped to 0.2 and 1.6% viable with the same respective treatments.

DISCUSSION

These experiments provided significant new information about IBDV. First, they clearly indicated that the susceptible target for infection is B-cells and that T-cells are either totally refractory or have very low susceptibility to the virus. This was borne out by the data from trials with lymphoblastoid T- and B-cell lines, in which no cells in the former, but many cells in the latter became infected, and also by the inability of anti-T-cell serum plus C' to decrease the susceptibility of various cell suspensions, whereas pretreatment with anti-B-cell serum markedly decreased susceptibility.

It is generally accepted that B-lymphocytes in young chickens can be found in several organs and peripheral blood, with the percentage of the total cell population decreasing in the following order: bursa of Fabricius, spleen, peripheral blood, and thymus. In this study the percentage of IF-positive cells was highest in bursal lymphocytes and lowest in peripheral blood lymphocytes, again lending support to the hypothesis that B-cells are the major or only target. However, even in the bursa cell suspensions, the total number of positive cells (generally less than 5%) was much lower than would be anticipated if all B-cells were susceptible. This implies that only a proportion of the B-cell population constitutes the target for IBDV infection.

The anti-B-cell serum treatment of susceptible cell populations prevented the appearance of infected cells after IBDV exposure whether or not C' was added to the system. Since this serum, by itself, was not cytotoxic, it can be concluded that it blocked infection rather than eliminated susceptible cells. This observation, coupled with the above conclusion that perhaps not all B-cells are targets, led to the hypothesis that surface antigens found on some but not all B-lymphocytes might be associated with susceptibility. Surface immunoglobulins were considered probable candidates, and, indeed, it was found that anti-IgM but not anti-IgG or anti-IgA serum specifically blocked susceptibility. Although our anti-immunoglobulin sera were not rendered monospecific, the major activity of each was directed against a specific immunoglobulin class, and if there was minor cross-reactivity, it was not strong enough to cause confusion.

It could be that only B-cells bearing IgM are susceptible to infection and that the blocking activity of the anti-IgM reagent could have resulted from blocking or destroying receptors for IBDV on these cells. This would account for the observation that only a proportion of the total population of B-cells presumed to be present in bursal or spleen cell suspensions were susceptible to the virus. Up to 30 to 50% of bursal lymphocytes may fail to express either B- or T-cell markers. Some investigators suggest that these may be null cells (2, 18, 21). The lack of an

appropriate technique for the separation of subpopulations of chicken B-lymphocytes prevented us from pursuing this point and determining whether specific surface immunoglobulin-bearing cells are, in fact, the targets for IBDV. Along these lines, it is interesting that in a previous study, it was observed that levels of IgM in the sera of IBDV-infected chickens from 1 to 8 weeks old were significantly lower than those in uninfected controls (Hirai et al., unpublished data).

The studies with TLT-1 were of considerable interest from two standpoints. First, this lymphoblastoid cell line was established from a transplantable lymphoid tumor of chickens induced by a retrovirus, avian leukosis virus (20). Cooper et al. (7) found that avian leukosis virus-induced tumor cells invariably had detectable surface IgM but not IgG or IgA, and they interpreted their observations to suggest that avian leukosis virus-induced transformation could be related to an interruption in the normal switch from IgM to IgG gene expression in certain bursa cell clones. If, indeed, the presence of surface IgM relates to susceptibility to IBDV, then it would be expected that TLT-1 cells would be highly susceptible to infection, as our data indicated. Precise definition of the immunoglobulin class(es) present on the membrane of TLT-1 cells must await studies with monospecific reagents. However, it is clear from cytotoxicity studies that immunoglobulins are present. Anti-IgM serum was highly cytotoxic for TLT-1; the reactivity of the anti-IgG serum was lower and could have represented cross-reactivity with this agent.

A second intriguing observation with TLT-1 cells was the absence of retrovirus particles associated with those cells containing IBDV particles, as seen by electron microscopy. It would be of interest to know whether only cells not producing C-type particles are susceptible to IBDV, or if IBDV infection inhibits replication of the retrovirus. Purchase and Cheville (22) found that IBDV infection of chicks previously infected with avian leukosis virus prevented the subsequent appearance of lymphoid neoplasms normally caused by the latter. They postulated that IBDV infection eliminated bursal cells which would serve as targets for transformation. If the hypothesis of Cooper et al. (7), i.e. that a switch from IgM or IgG gene expression is blocked by transformation, is followed, then the high susceptibility of avian leukosis virus tumor cells gives additional support for postulating that IgM-bearing cells are the specific target for IBDV infection.

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