Host Defense Mechanisms Against Infectious Bovine Rhinotracheitis Virus: In Vitro Stimulation of Sensitized Lymphocytes by Virus Antigen

BARRY T. ROUSE AND LORNE A. BABIUK

Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada

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Isolated peripheral blood lymphocytes (PBL) from cattle immunized or infected with infectious bovine rhinotracheitis (IBR) virus were cultured in vitro with ultraviolet light-inactivated IBR virus, and the degree of lymphocyte blastogenesis was quantitated by measurement of the uptake of [3H]thymidine into acid-insoluble material. Lymphocyte blastogenesis only occurred with PBL from immunized or infected animals. The optimal conditions for lymphocyte blastogenesis were defined. Blastogenesis was specific since cells from animals immunized against IBR failed to react with two other herpesvirus antigens tested, herpes simplex and equine rhinopneumonitis viruses. Blastogenesis could be prevented by reacting IBR antigen with IBR-specific antibody before adding to cultures, but incorporating IBR-specific antibody in the culture medium after adding free antigen failed to inhibit blastogenesis. With intranasally infected animals, lymphocyte blastogenesis was detectable after 5 days, reached peak levels between days 7 and 10, and then declined to low levels by day 19. In contrast, levels of neutralizing antibody were barely detectable on day 7 and reached maximal concentrations on day 19. The lymphocyte blastogenesis assay was emphasized as a convenient and useful in vitro correlate of cell-mediated immunity that should help define the role of cell-mediated immunity in immunity to herpesviruses.

The host response to a virus infection involves many components, including resistance of individual host cells to infection, local factors such as temperature or acidity, formation of interferon, resistance of macrophages and other leukocytes and specific humoral and cell-mediated immune responses (2). Much attention was focused previously on defining the part played by various immunoglobulins, both systemic and local, in resistance to and recovery from infection (18, 19, 22, 24). However, recently there has been a growing awareness that with many viruses, cell-mediated immunity (CMI) may be the major means of defense (3, 4, 9, 13, 25). For example, in the cases of herpesviruses, humoral antibody may play a minor role in the recovery process, since antibody levels are usually high during times of clinical illness (5), and the viruses persist in the host despite the presence of antibody (14). These viruses can spread between cells via intercellular bridges (6) and need not enter the extracellular environment. Consequently herpesviruses can avoid contact with extracellular antibody.

The factors that allow activation and those responsible for persistence of herpesviruses in a latent state are poorly understood, but it is thought that CMI may be involved in the overall process. A clear definition of the part played by CMI in recovery from and resistance to herpesvirus infections has been hampered by the lack of suitable quantitative in vitro assays. We describe here a lymphocyte stimulation assay that can be used to quantitate CMI against infectious bovine rhinotracheitis virus (IBR), a common herpes virus pathogen of cattle.

MATERIALS AND METHODS

Preparation of viruses. Strain P8-2 of IBR virus was isolated at Purdue University by J. R. Saunders. This virus received two passages through primary fetal bovine kidney monolayers before preparation of stock virus. Stock virus was prepared in Madin Darby bovine kidney (MDBK) cells. MDBK cells were grown to confluency in roller bottles (660 cm²), the medium was removed, and the cells were infected with 3 ml of IBR virus (approximately 0.1 to 0.5 plaque-forming units (PFU)/cell). Virus was allowed to adsorb at 37 C
for 90 min before the addition of fresh culture medium. The culture medium used was Eagle base minimal essential medium containing 10% heat-inactivated fetal calf serum (FCS). Each liter was supplemented with 10 ml of nonessential amino acids (Gibco, catalogue no. 114), 2 mmol of glutamine, and 100 mg of kanamycin. After 2 days of incubation at 37 °C, all cells showed virus-specific cytopathology, and approximately 98% were detached. The cells that remained attached were removed into the culture fluids by vigorous pipetting. All the cells and the culture fluids were subjected to two freeze-thaw cycles. Cellular debris was removed by centrifugation at 1,000 × g for 10 min. This constituted the stock virus.

Herpes simplex type 1 was obtained from the American Type Culture Collection by way of the University of Saskatchewan Department of Bacteriology. This virus was passed twice through MDBK cells, after which cytopathic effects were visible. The fourth MDBK passage was used as stock virus and was prepared essentially as described above. Equine rhinopneumonitis virus was isolated by J. R. Saunders, University of Saskatchewan. The virus received two passages through embryo swine kidney cells and then two passages through MDBK cells before the preparation of stock virus.

Preparation of viral antigens. Stock viruses were centrifuged at 40,000 × g at 4 C for 1 h and the virus pellet was resuspended in a minimal volume of Puck's solution G (PS). Samples (0.5 ml) of the virus pellet were layered onto a 12-mI gradient of 10 to 30% (wt/wt) sucrose in PS. The gradients were centrifuged at 35,000 × g at 4 C for 40 min, and 0.4-mI fractions were collected and assayed for infectivity. The fractions containing the most infectivity were pooled and repelleted at 40,000 × g for 60 min at 4 C. This constituted the purified virus. For use in the blast cell transformation studies, viruses were inactivated by exposure to ultraviolet irradiation. One milliliter of virus was placed in a 35-mm plastic petri dish and irradiated for 2 min by two General Electric G875 ultraviolet bulbs at a distance of 11 cm. The virus thus treated was rendered noninfectious.

Preparation of peripheral blood lymphocytes (PBL). Blood was collected by venipuncture into a syringe containing preservative-free heparin (5 IU per ml of blood collected). Theuffy coat was obtained after centrifugation at 800 × g for 20 min at 4 C, and these cells were diluted in PS. These leukocyte-rich cells were layered onto a 3-mI volume of Ficol-hypaque (density at 25 C, 1.077 g/cm3) (Ficoll-Pharmacia; Hyapaque-Winthrop) in a round-bottom centrifuge tube (13 by 120 mm). After centrifugation at 400 × g (at the interface) for 20 min at 25 C, the lymphocyte-enriched cells were collected from the interface of plasma and Ficol-hypaque. These cells were washed once in PS, and the red cells were lysed with 0.83% ammonium chloride (5 min at 37 C) and then washed two more times in PS before enumeration. Cells harvested by this technique were invariably more than 98% viable and consisted of greater than 99% mononuclear cells. Cells were enumerated and suspended at various concentrations in RPMI 1640 medium containing 10% heat-inactivated FCS and 100 μg of kanamycin per ml.

Blastogenesis of lymphocyte cultures. Samples (1 ml) of lymphocytes were cultured at 37 C in a humidified CO2 (5%) incubator in Falcon plastic tubes (no. 2064). Test cultures received 25 μl of stimulant (IBR, herpes simplex, and ERP viruses [approximately 2.5 × 109 PFU equivalents of each virus] or 6 μg of phytohemagglutinin [batch K 6644; Burroughs Wellcome, Beckenham, England]). Twenty-four hours before termination of cultures, they were pulsed with 1 μCi of [methyl-3H]thymidine (specific activity, 5 Ci/mmol; Amersham Searle, Oakville, Ontario). At the end of the experiment, the cells were pelleted, washed once in PS, and resuspended in 1 ml of PS containing 1% serum. The cells were precipitated at 4 C by adding 1 ml of ice-cold 10% trichloroacetic acid. The precipitate was collected onto glass fiber filter paper (Gelman) by using a Hoffer vacuum manifold (model no. FH100). The precipitate was washed with ice-cold 5% trichloroacetic acid and then with 100% methanol. Filter pads were collected, placed in scintillation vials, and dried at 56 C, and 10 ml of scintillation fluid (1,4-bis[5-phenyloxazolyl]benzene) [0.3 g/liter], 2,5-diphenyloxazole [5 g/liter] in scintanalyzed toluene; Fisher Scientific Co.) was added.

The samples were counted in a Nuclear Chicago Unilux II scintillation counter. All data presented are for triplicate cultures. Results are usually expressed as the mean counts per minute of [3H]thymidine incorporation plus and minus the standard error or are expressed as the stimulation index (the mean counts per minute of [3H]thymidine incorporation of test cultures divided by the mean counts per minute of [3H]thymidine incorporation of control cultures with no stimulant or culture fluids from noninfected MDBK cells as a stimulant). All experiments were repeated more than three times.

Immunization procedures. Young adult steers were immunized intramuscularly at monthly intervals with 104 PFU of IBR virus. The first injection was emulsified in Freund complete adjuvant. These animals were repeatedly bled after the second and third injections.

Determination of neutralization titer. Serial dilutions of plasma were prepared, and 0.5-ml samples were reacted with 0.5 ml of virus containing 102 PFU of IBR virus. Each dilution was added to four 7-mm wells containing MDBK cells and adsorbed for 1 h, after which the unadsorbed virus was removed and minimal essential medium with 5% FCS was added. The neutralization titer was computed by the Karber method (11).

RESULTS

Stimulation of sensitized lymphocytes by IBR antigen. To determine whether in vitro culture of lymphocytes with IBR antigen was a useful technique to demonstrate reactivity to this antigen, PBL were collected from several animals known not to have been exposed previ-
ously to IBR and from two cows immunized with IBR virus. Significant thymidine uptake (indicative of stimulation) was regularly recorded with immune PBL, but lymphocytes from nonimmunized animals failed to stimulate (Table 1). Such cells could, however, respond to other stimulants such as phytohemagglutinin, indicating that these cells were viable.

In a series of experiments to establish the optimal conditions for stimulation, it was found that maximal [3H]thymidine uptake and stimulation index occurred in cultures containing 2 × 10^6 PBL per ml (Fig. 1). Significant stimulation above background was first detectable at 2 days of culture, and both peak [3H]thymidine uptake and stimulation index (22.8) occurred on the 5th day of culture (Fig. 2). A low but significant degree of stimulation was still measurable on the 8th day of culture. The degree of lymphocyte stimulation was greatest with the highest dose of antigen used for stimulation (Fig. 3). Stock antigen diluted 1,000-fold (equivalent to 2.5 × 10^4 PFU of virus) gave significant stimulation. In the experiments reported below, the virus was used at a dilution of 100-fold (equivalent to 2.5 × 10^5 PFU of virus), cultures contained 10^4 lymphocytes per ml, and the

Table 1. In vitro stimulation of sensitized bovine peripheral blood lymphocytes by IBR virus antigen

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Lymphocytes from IBR-immunized animals</th>
<th>Lymphocytes from nonimmunized animals</th>
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<tbody>
<tr>
<td></td>
<td>Mean [3H]thymidine uptake (counts/min)</td>
<td>Mean [3H]thymidine uptake (counts/min)</td>
</tr>
<tr>
<td>IBR*</td>
<td>53,477a</td>
<td>3,425</td>
</tr>
<tr>
<td>BSS/</td>
<td>5,683b</td>
<td>3,201</td>
</tr>
<tr>
<td>PHA*</td>
<td>106,585</td>
<td>98,742</td>
</tr>
</tbody>
</table>

* Ten young calves were examined. These calves were from premises in which IBR had not been detected, and their dams were not vaccinated against the virus.
* SI: Stimulation index; = mean counts per minute of [3H]thymidine uptake in presence of antigen divided by mean counts per minute of [3H]thymidine uptake of cultures without antigen.
* Ultraviolet light-inactivated purified IBR virus antigen.
* Mean counts per minute of [3H]thymidine uptake of two immunized steers examined on five separate occasions.
* Range of stimulation indexes obtained in control animals.
* Balanced salt solution.
* 6 μg of phytohemagglutinin (PHA) (Burroughs-Wellcome).

![Fig. 1. Influence of cell numbers on magnitude of stimulation of immune PBL by IBR virus. PBL from an immunized cow were suspended, at the cell number shown, in RPMI 1640 medium plus 10% FCS, and test cultures were stimulated with the same concentration (2.5 × 10^4 PFU equivalents) of ultraviolet light-inactivated IBR virus antigen. Cultures were pulsed for 24 h on day 4 with 1 μCi of [3H]thymidine. The hatched areas represent the mean [3H]thymidine uptake by control cultures, the hatched area plus the open blocks represents the uptake by test cultures. The numbers at the top of each column indicate the stimulation index.](http://iai.asm.org/)

 Cultures were pulsed with thymidine on the 4th day.

**Specificity of lymphocyte stimulation by IBR virus.** To establish the immunological specificity of lymphocyte stimulation by IBR virus antigen, several approaches were adopted. First, as presented in Table 1, only lymphocytes from animals deliberately immunized against IBR antigen were stimulated by IBR viral antigen. PBL from both immunized and nonimmunized animals responded to phytohemagglutinin. Second, lymphocytes derived from young calves free of maternal antibody to IBR virus failed to be stimulated by IBR viral antigen. These young calves were then infected intranasally with 4 × 10^4 PFU of IBR virus. Virus replication probably occurred, since virus was recoverable on days 3 and 5 after infection and the animals showed mild clinical illness. Lymphocyte stimulation was detectable on day 5, peaked between days 7 and 10, and fell
Sera were to antigenicity of viral ERP demonstrating to stimulate. Also virus lymphocytes from MDBK antigens, virus lymphocytes from cultures. Error standard error reached a-° 120 a-O 140 150 130, dashed solid line was ultraviolet light-inactivated and the cultures were collected after day 19. Neutralizing antibody was first detectable on day 7 and reached peak levels on day 19 (Fig. 4).

In a third approach to show specificity, lymphocytes from immunized cows were cultured with IBR virus antigen and two other herpes-virus antigens, herpes simplex and ERP viruses. The lymphocytes were only stimulated by IBR virus antigen (Table 2). Culture fluids from control MDBK cell monolayers also failed to stimulate. Also shown in Table 2 are data demonstrating that the herpes simplex and ERP viral antigens used could stimulate lymphocytes from animals immunized with the homologous antigen.

The final approach used to investigate specificity of stimulation was to measure the effect of antiviral antibody on the ability of IBR virus antigen to stimulate immune PBL (Table 3). Sera were collected from cows immunized three times with IBR virus. The serum pool used had an antibody titer, as measured by viral neutralization, of 1:160. When the IBR antigen used for stimulation was reacted with 100 µl of undiluted antisera for 1 h at room temperature, the virus-antibody complex failed to cause lymphocyte stimulation. At lower antibody concentrations some stimulation did occur. Although premixing antisera with antigen before addition to cultures inhibited lymphocyte stimulation, incorporation of antisera (at a final antiserum concentration of 1:10) immediately after antigen addition had no inhibitory effect on lymphocyte stimulation.

**DISCUSSION**

Our studies demonstrate that PBL from animals immunized against IBR virus contain a population of cells that are stimulated by in vitro culture with IBR virus antigen. Lymphocytes from animals not previously exposed to antigen fail to stimulate, supporting the concept that blastogenesis represents a specific immunological response by PBL. Specificity was also shown by experiments indicating that the blast cell response only developed as a

![Fig. 2. Influence of culture time on stimulation of immune PBL by IBR virus antigen. PBL were adjusted to 10⁶/ml in RPMI 1640 + 10% FCS, and test cultures were stimulated with the same concentration of ultraviolet light-inactivated IBR virus antigen (2.5 x 10⁶ PFU equivalents). At the time intervals shown, triplicate cultures were pulsed with 1 µCi of [³H]thymidine, and the acid-insoluble [³H]thymidine uptake was determined after 24 h. The points shown on the solid line represent the mean counts per minute ± standard error of [³H]thymidine incorporated, the dashed line (x—x) indicates the stimulation index, and the lowest interrupted line (O—O) represents the [³H]thymidine uptake of unstimulated control cultures.](image)

![Fig. 3. Influence of concentration of IBR viral antigen on stimulation of immune PBL. Cultures containing 10⁶ PBL in RPMI 1640 + 10% FCS were stimulated with 10-fold dilutions of ultraviolet light-inactivated purified IBR virus preparation. The points represent the mean counts per minute ± standard error of [³H]thymidine incorporation by triplicate cultures. The dashed line represents the [³H]thymidine incorporation of control cultures. All cultures were pulsed for 24 h on day 4 with 1 µCi of [³H]thymidine. A 10⁻¹ dilution of viral antigen is equivalent to 2.5 x 10⁶ PFU.](image)
sequel to IBR virus infection or immunization and that PBL from animals immune to IBR antigens were not stimulated by other herpesvirus antigens. Lymphocyte stimulation by IBR virus antigen could be prevented by reacting the antigen with anti-IBR serum but not with normal serum before addition to cultures. This observation indicates that it was the IBR viral antigens, not some other component of culture fluids, that caused the stimulation. However, paradoxically, the addition of neutralizing quantities of antibody to cultures soon after addition of virus antigen failed to inhibit blastogenesis. An explanation for this paradox could be that the immune complexes formed before addition to cultures were stable and in antibody excess. Consequently, insufficient antigenic determinants were free to stimulate lymphocytes. Possibly antigen triggering of PBL occurred before the formation of stable complexes in the second circumstance. Previous data on the effects of antibody on antigen-induced blastogenesis have been conflicting. Thus, with herpes simplex, Rosenberg and Notkins (17) showed that antibody failed to inhibit stimulation by free antigen but could inhibit stimulation by cell-associated antigen. Working with Venezuelan equine encephalomyelitis virus, Adler and Rabinowitz (1) showed that antibody could block blastogenesis. A careful study with preformed complexes of varying ratios of antigen with immunoglobulins of different affinities could probably resolve these discrepancies.

Lymphocyte stimulation assays have been described for an expanding number of viruses since their introduction by Rosenberg et al. (1, 7, 8, 10, 16, 17, 20). Such assays appear to be in vitro correlates of cell-mediated immunity and consequently may represent useful assays to quantitate the importance of CMI in virus recovery. These assays may also provide an index of potential protective capacity of an animal to infection. Particularly in the case of herpesviruses, the part played by humoral antibody for protection has been questioned. Thus herpesviruses spread between cells by intercellular bridges and need not enter the extracellular environment of the cell (6). Accordingly they can avoid contact with extracellular antibody. Although cells infected with herpesviruses do express viral antigens on their cell surfaces (15) and are then susceptible to destruction by antibody and complement, this does not occur until after the virus has spread to contiguous cells (12). That antibody may have little protective importance in herpesvirus infections is

![Figure 4. Time of appearance of sensitized lymphocytes (x—x) and antibody (O—O) in the blood after intranasal infection of calves with IBR virus. At the intervals shown before and after infection, plasma was separated and PBL were isolated. 10^6 PBL were stimulated for 5 days with IBR antigen (2.5 x 10^4 PFU equivalents). Cultures were pulsed for 24 h on days 4 to 5 with 1 μCi of [3H]thymidine. The stimulation index was computed as described in the text. Serial dilutions of plasma were prepared to determine the neutralization titer. Also shown are the results of attempts to isolate virus from nasal secretions after infection. Virus was isolated on days 3 and 5. Symbols: +, Infectious virus isolated; −, infectious virus not isolated.

**TABLE 2. Specificity of stimulation of immune lymphocytes by IBR, herpes simplex, and ERP herpesviruses**

<table>
<thead>
<tr>
<th>Lymphocytes</th>
<th>Uptake of [3H]thymidine (counts/min ± standard error)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IBR</td>
</tr>
<tr>
<td>IBR immune*</td>
<td>19,086 ± 2,300</td>
</tr>
<tr>
<td>H. simplex immune*</td>
<td>4,150 ± 892</td>
</tr>
<tr>
<td>ERP immune*</td>
<td>3,022 ± 212</td>
</tr>
<tr>
<td>Normal bovine</td>
<td>1,479 ± 243</td>
</tr>
</tbody>
</table>

*Culture fluids from noninfected MDBK monolayers.
*Lymphocytes from a cow immunized three times with live IBR virus.
*Human lymphocytes collected from an individual recently recovered from labial herpetic lesions.
*ND, Not done.
*Lymphocytes collected from a rabbit 10 days after intradermal exposure to 2 x 10^4 PFU of ERP virus.
Lynn Franson, guide respect to immunity to reinfection and as a termine whether or can act as evidence of immunity. Unfortunately, quantitate that the recovery is cell mediated. In toto, these observations imply that recovery from herpesvirus infections has a cell-mediated mechanism and that spontaneous recurrences, characteristic of herpesviruses, including IBR (21, 23), may also involve cell-mediated immunity. To prove these notions, it is necessary to develop suitable in vitro assays to quantitate all aspects of the immune response. The blast cell assay is one such assay to quantitative CMI. Unfortunately this assay is a measure of antigen recognition and gives no direct information as to whether or not the cells being measured can act as effector cells. Evidence that the blast cell assay is a useful indicator of CMI could be obtained if it were shown that the same population of cells could prevent cytopathology by IBR virus in cell culture. Such an assay has recently been reported (B. T. Rouse and L. A. Babiuk, Cell Immunol., in press). Studies are in progress to determine whether the blast cell assay is useful to predict the immune status of an animal with respect to immunity to reinfection and as a guide to predict recurrence of infection.

ACKNOWLEDGMENTS

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LITERATURE CITED


### Table 3. Inhibitory effect of anti-IBR serum on the stimulation of sensitized lymphocytes by IBR virus antigen

<table>
<thead>
<tr>
<th>System</th>
<th>[H]thymidine uptake (counts/min ± standard error)</th>
<th>Stimulation index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes + 25 mliters of IBR</td>
<td>16,737 ± 1,254</td>
<td>4.1</td>
</tr>
<tr>
<td>Lymphocytes + 25 mliters of IBR + 100 mliters of anti-IBR</td>
<td>2,697 ± 921</td>
<td>0.7</td>
</tr>
<tr>
<td>Lymphocytes + 25 mliters of IBR + 25 mliters of anti-IBR</td>
<td>4,557 ± 764</td>
<td>1.1</td>
</tr>
<tr>
<td>Lymphocytes + 25 mliters of IBR + 5 mliters of anti-IBR</td>
<td>10,150 ± 1,073</td>
<td>2.5</td>
</tr>
<tr>
<td>Lymphocytes + 25 mliters of IBR + 100 mliters of NBS</td>
<td>15,823 ± 1,057</td>
<td>4.0</td>
</tr>
<tr>
<td>Lymphocytes + 25 mliters of IBR + 100 mliters of anti-IBR</td>
<td>15,343 ± 1,500</td>
<td>3.9</td>
</tr>
<tr>
<td>Lymphocytes + 25 mliters of IBR + 50 mliters of anti-IBR</td>
<td>16,855 ± 860</td>
<td>4.2</td>
</tr>
</tbody>
</table>

* 10⁶ PBL from an IBR-immune cow cultured with a stimulatory dose of ultraviolet light-inactivated IBR antigen (2.5 × 10⁶ PFU equivalents).
* IBR antigen was reacted with bovine IBR antisera for 1 h at 20°C, then the mixture was added to lymphocytes. Titer of anti-IBR was 1:160 against 100 PFU of virus.
* Protocol as in b above except that IBR antigen was reacted with normal bovine serum.
* 10⁶ lymphocytes cultured with a stimulatory dose of IBR antigen. Immediately after addition of antigen, anti-IBR antisera was added.