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Defense Mechanisms Against Bovine Herpesvirus: Relationship of Virus-Host Cell Events to Susceptibility to Antibody-Complement Cell Lysis

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The interaction of infectious bovine rhinotracheitis virus and susceptible host cells was examined to determine whether an infected cell could be destroyed by humoral immune mechanisms before or after the transmission of virus to susceptible adjacent cells. Viral antigens were detectable on cell membranes at 6 h postinfection, but cells were not susceptible to antibody-complement lysis until 10 h postinfection. Intracellular infectious virus was also detectable at 10 h postinfection, and transmission to adjacent cells by the intracellular route began at this time. Extracellular virus was not detectable until 12 to 13 h postinfection. By the continual addition of antibody and complement, virus dissemination could be reduced more than 50-fold. These results support the hypothesis that the humoral immune mechanism may be involved in the recovery from herpesvirus infections.

Most herpesvirus infections are characterized by recurrent infections, with the virus persisting in the body almost indefinitely. In infectious bovine rhinotracheitis (IBR) infection of cattle, usually there is only a single episode of symptomatic infection; nevertheless, the virus does persist and can be reactivated (4, 15, 16). One of the goals in the study of herpesvirus immunology is to delineate the mechanism of recovery from infection, and in this regard many components of both specific and nonspecific immunity have been investigated. The balance of evidence would seem to point to cell-mediated immunity as being of principal importance for the recovery from herpesviruses (1, 2, 8). However, it seems highly unlikely that cell-mediated immunity is of decisive importance, at least in herpes simplex virus, since in vitro parameters of cell-mediated immunity are usually detectable only in those individuals subject to recurrent herpesvirus infection and levels of cell-mediated immunity do not seem to change regularly prior to recurrent disease (9, 13, 14, 17). The levels of neutralizing antibody show a similar pattern (3). Such observations may call into question the role of any component of specific immunity in the recovery from herpesvirus infections.

In the present communication we have analyzed the interaction between IBR virus and susceptible host cells to determine whether an infected cell can be destroyed by humoral immune mechanisms before or after virus is disseminated to susceptible adjacent cells. Subsequent communications will examine this question with respect to other components of the immune response.

MATERIALS AND METHODS

Cells and virus. Madin Darby bovine kidney (MDBK) or Georgia bovine kidney cells were cultured in Eagle minimal essential medium (MEM) containing 10% fetal calf serum. Each liter was supplemented with 2 mmol of glutamine (Gibco, no. 503), 50 mg of gentamycin (Schering Diagnostics), and 2.5 g of sodium bicarbonate. During assays, the medium used contained 4% fetal calf serum.

Strain P8-2 of IBR virus was prepared in MDBK cells as previously described (10).

Time course of virus production. MDBK cells were grown to confluency in 60-mm plastic petri dishes (Falcon Plastics), the medium was removed, and the cells were infected with 0.5 ml of IBR virus at a multiplicity of infection of 1. After absorption at 37 C for 60 min, the monolayers were washed twice in Puck solution G and fresh medium was added. At intervals postinfection (PI), the culture fluids were removed and the amount of extracellular virus was quantitated by plaquing in microtiter plates with an antibody overlay (11). To measure the quantity of intracellular virus, cells were removed from the washed monolayers with a rubber policeman and then were subjected to three freeze-thaw cycles prior to titration.

Antisera and complement. Young adult steers were immunized intramuscularly at monthly intervals with 10^9 plaque-forming units of IBR virus. The first injection was emulsified in Freund complete adjuvant (Difco Laboratories). These animals were

repeatedly bled after the second and third injection. The neutralization titers were determined by preparing serial dilutions of the serum and reacting 0.5 ml with 0.5 ml of virus (10^2 plaque-forming units). Serum-virus mixtures were added to four 7-mm wells in a microtiter plate (Falcon Plastics, no. 3040) containing MDBK cells. After adsorption for 1 h, the unadsorbed virus was removed and fresh medium was added. The serum neutralization index was computed by the Karber method (6). In all assays described subsequently in which antiserum was used, four neutralizing units of anti-IBR serum was employed.

Rabbit complement (Nutritional Biochemicals Corp., lot no. 6282) was used at a dilution of 1:20 in cytotoxicity and plaque inhibition assays.

Plaque inhibition by antibody and complement. Quadruplicate monolayers of confluent MDBK cells (Falcon Plastics, no. 3040) were infected with varying concentrations (10 to 15,000 plaque-forming units) of IBR virus. At 6 h PI, the culture fluids were removed and replaced with MEM containing antibody alone, antibody plus complement, or complement alone. In experiments in which fresh complement was added every 2 h, the above procedure was repeated for 20 h at 2-h intervals. After a further 48 h of incubation, the monolayers were fixed, stained, and examined for viral plaques (11).

Infectious center assay. MDBK cells grown in 35-mm plastic petri dishes were infected with IBR at a multiplicity of infection of 0.001 to 0.005. After a 1-h adsorption period, monolayers were washed twice with Puck solution G and overlaid with MEM plus 4% fetal calf serum. Then 2 h later, the culture fluids were removed and replaced with MEM containing anti-IBR serum. At various times thereafter, the monolayers were treated with trypsin and washed, and the cells were resuspended in 5 ml of MEM containing anti-IBR serum. Fourfold dilutions of these cells were made in MEM–anti-IBR, and 1 ml of each dilution was added to confluent MDBK cells in 16-mm wells of a microtiter plate (Linbro Plastics, no. 16-24-TC). The cells were allowed to settle without disturbance for 3 days after which the monolayers were fixed and stained, and the viral plaques were enumerated (12).

In experiments designed to test the inhibition of spread by antibody and complement, fresh MEM containing antisera and complement was added every 2 h, prior to the infectious center assay.

Antibody-complement lysis of infected cells. MDBK or Georgia bovine kidney cells were grown to confluency in microtiter plates (Falcon Plastics, no. 3040). Each well was labeled with 2 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear Corp., Dorval, P.Q.) and simultaneously was infected with 1 plaque-forming unit of IBR virus per virus (0.1 ml). After 90 min of labeling and infection, the monolayers were washed three times before fresh MEM was added. At appropriate times PI, the medium was removed and the monolayers were incubated for 1 h at 37 C with anti-IBR antibody, antibody and complement, or complement alone. The amount of radioactivity released into the medium was compared to that released by controls. Total releasable chromium was determined by adding five successive portions of distilled water to microtiter wells containing labeled cells. These portions were then pooled and centrifuged, and the amount of radioactivity in the supernatant was taken as the total releasable ^{51}Cr. Specific release (SR) was calculated by the following formula:

$$SR = \frac{\text{release by Ab + C} - \text{release by control}}{\text{total releasable } ^{51}Cr - \text{release by control}} \times 100$$

where Ab represents antibody and C represents complement.

Immunofluorescence. MDBK cells (10^7 cells/ml) were infected in suspension at a multiplicity of infection of 5 in a volume of 2 ml. After 90 min, the cells were washed and suspended at a concentration of 5×10^5 cells /ml in Spinner culture MEM plus 10% fetal calf serum. At various times, a sample of the suspension culture was removed, washed, and suspended in 250 μl of anti-IBR serum. After reaction at 4 C for 30 min, the cells were washed three times and suspended in 200 μl of a 1:20 dilution of fluorescein-labeled goat anti-bovine immunoglobulin G antisera (Cappel). After reaction at 4 C for 30 min, the cells were washed three times and suspended in 10% glycerol-saline. Fluorescent microscopy was performed with a Carl Zeiss Ultraphot III microscope using incident light from an Osram HBO 200 lamp with a BG 3 exciter filter in combination with a permanent BG 38 filter and a 50/44 barrier filter. These filters gave a peak excitation intensity of 320 to 400 nm.

RESULTS

Appearance of virus-induced membrane antigens, production of infectious virus, and spread to adjacent cells. To determine how soon after infection new virus-specific membrane antigens appeared, cells were infected with a multiplicity of infection of 5 and, at appropriate times thereafter, cells were assayed to determine the percentage of cells with membrane fluorescence. A few fluorescent cells were observed as early as 4 h PI, and the percentage increased rapidly to include 100% of the cells by 7 h PI (Fig. 1).

Infectious intracellular virus was not detectable until approximately 9 h PI, but such virus was not released extracellularly until 2 to 3 h later (11 to 13 h PI, Fig. 2). Thus, in the experimental systems employed, virus-specific membrane antigens were detectable at least 3 h before the production of infectious intracellular virus and 5 h prior to the release of extracellular infectious virus.

Having established the time at which extracellular virus first appeared, we designed a subsequent experiment to determine the time when virus could spread to adjacent uninfected

FIG. 1. *Time of appearance of IBR virus-specific membrane antigens. MDBK cells were infected and cultured in suspension. At various times PI portions were removed, and the cells were assayed for the presence of virus-specific antigens by the indirect immunofluorescence technique. At least 250 cells were enumerated at each time.*

cells. Cell monolayers were infected at a multiplicity of infection of 0.005 and then were overlaid with MEM containing anti-IBR serum to prevent secondary infection by extracellular virus. This procedure, however, would not prevent secondary infection by virus spreading directly between adjoined cells. At various times PI, the cells were treated with trypsin and the number of infected cells was determined by the infectious center assay. These experiments showed that virus dissemination began at approximately 10 h (i.e., 1 to 3 h before the appearance of extracellular virus) and gradually increased thereafter (Fig. 3).

Cytotoxicity of antibody and complement against IBR-infected cells. The data in Fig. 4 indicate that specific ^{51}Cr release from IBR-infected cells began at approximately 10 h PI and increased until a plateau was reached at 14 h PI. The release of ^{51}Cr from infected monolayers overlaid with a medium containing either antibody or complement was not elevated over that released by the medium alone. The percentage of specific ^{51}Cr released by two different

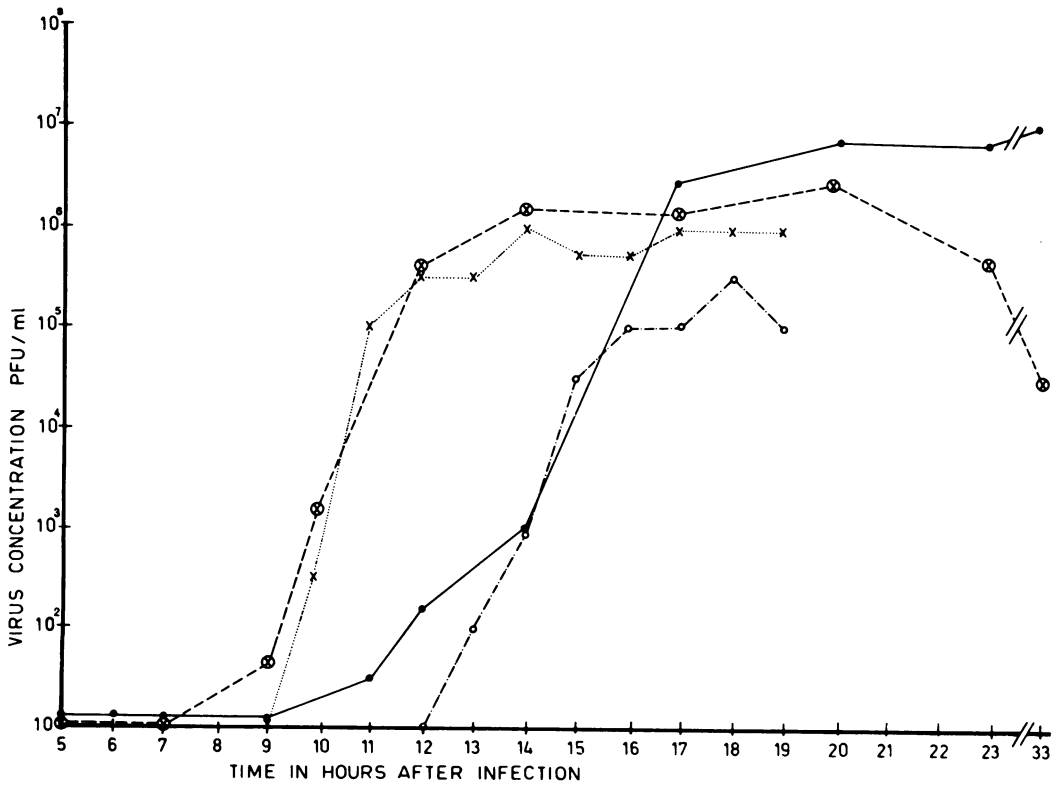

FIG. 2. *Time course of intracellular and extracellular IBR virus production. MDBK cells were infected at a multiplicity of infection of 1. At various times PI, the culture fluids were removed and titrated for extracellular virus (●, ○) and intracellular virus (⊗, ×) after subjecting the cells to three freeze-thaw cycles. The results of two separate experiments are illustrated. PFU, Plaque-forming units.*

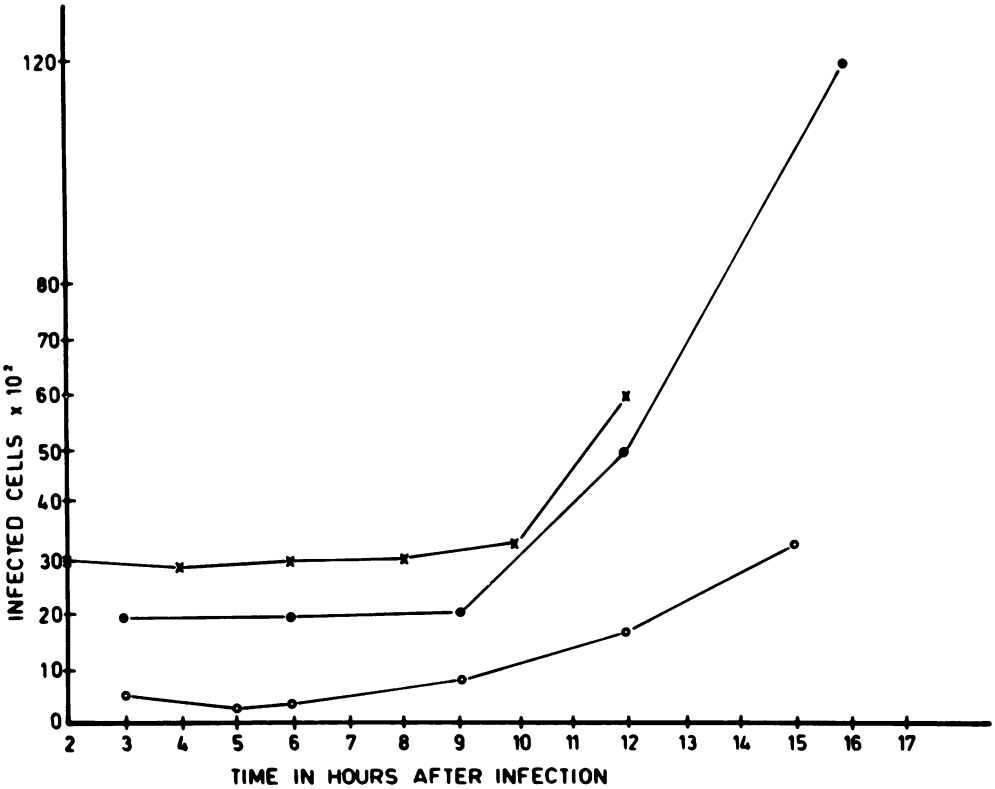

FIG. 3. *Spread of IBR virus to adjacent cells. Confluent cultures of MDBK cells were infected at a multiplicity of infection of 0.001 to 0.005 and overlaid with four neutralizing units of anti-IBR serum. At various times the cells were removed, diluted in MEM containing anti-IBR serum, and replated onto fresh MDBK cell monolayers to determine the number of infected cells. The three curves represent three separate experiments.*

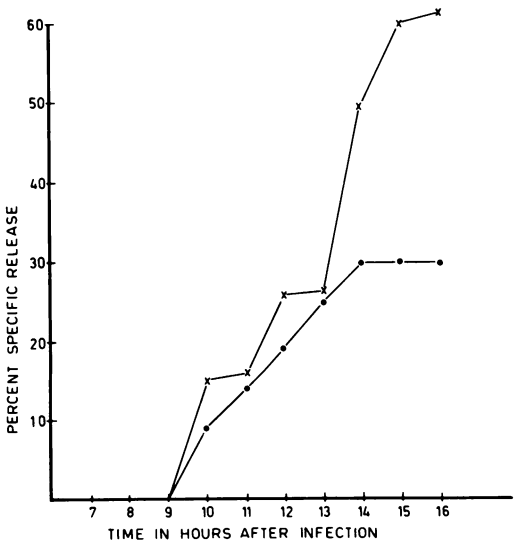

FIG. 4. *Immune lysis of virus-infected cells. Virus-infected and ^{51}Cr-labeled cells were incubated for 1 h in the presence of antibody and complement, and the amount of specific ^{51}Cr released was determined for MDBK cells (●) and Georgia bovine kidney cells (×).*

IBR-susceptible cell lines was compared, and it was found that, although Georgia bovine kidney cells were much more susceptible to antibody-complement lysis (60% specific lysis at 14 h) as compared with MDBK cells (30% specific lysis), the time when both cell lines became susceptible to antibody-complement lysis was essentially identical (10 h) (Fig. 4).

Prevention of virus spread by antibody and complement. The previous experiments had indicated that cells become susceptible to antibody-complement-mediated lysis at about the same time infectious virus appeared intracellularly and was capable of spreading to adjacent cells. These observations suggested that antibody and complement may serve to reduce or prevent virus dissemination by killing infected cells before viral spread. To evaluate this concept two separate types of experiments were performed. The first was to infect confluent

MDBK monolayers with known concentrations of IBR virus (15 to 15,000 plaque-forming units) and to measure the ability of each infectious unit to produce visible plaques in the continual presence of antibody alone, antibody plus complement, or complement alone. The addition of fresh antibody and complement every 2 h greatly reduced the number of visible plaques in comparison with cultures incubated in the presence of antibody or complement alone (Table 1). Whereas the continual addition of antibody and complement every 2 h greatly reduced virus cytopathology, the addition of antibody and complement only once at the start of the experiment had little or no effect on plaque numbers (Table 1).

In the second series of experiments, infectious center assays were used to determine whether antibody and complement could prevent the spread of IBR virus to adjacent cells. In cultures with antibody alone, 8 and 28 times more cells had become infected by 16 and 22 h PI, respectively, as compared with the number of cells infected originally (Table 2). In contrast, there was a decrease in the number of infected cells both at 16 and 22 h PI in those cultures in which fresh antibody and complement were added every 2 h. However, if antibody and complement were added only once, at 7 h PI, infected-cell numbers increased compared with the number originally infected although this increase was slightly less than was seen in control cultures with antibody alone. In cultures receiving only complement, the increase in the number of infected cells at 22 h PI was most marked, presumably as a result of secondary infections caused by extracellular virus released from the originally infected cells.

TABLE 1. *Effect of antibody (Ab) and complement (C) on plaque-forming ability of IBR virus in MDBK cell monolayers*

Input virus	Effect of treatment			
	C[a]	Ab	Ab + C, ×1[b]	Ab + C, 2 h[c]
15	~10% Lysis	2.5[d]	6.5	1.2
150	~50% Lysis	TNC[e]	TNC	3.7
1,500	CL[f]	CL	CL	16.2
15,000	CL	CL	CL	TNC

[a] C (25 μl) was added every 2 h beginning 6 h PI.
[b] C (25 μl) was added ×1 at 6 h PI.
[c] C (25 μl) was added every 2 h in fresh medium.
[d] Plaque numbers given are the average of quadruplicate cultures.
[e] TNC, Too numerous to count.
[f] CL, Complete lysis.

TABLE 2. *Effect of antibody (Ab) and complement (C) on spread of IBR virus in MDBK cells*

Time after infection (h)	Infected cells (no.)			
	C[a]	Ab	Ab + C, ×1[b]	Ab + C, 2 h[c]
7	980	1,040	ND	1,029
16	ND[d]	8,320	ND	600
22	140,800	28,160	11,520	200

[a] C (25 μl) was added at 6 h PI.
[b] C (25 μl) was added once at 6 h PI.
[c] C (25 μl) was added every 2 h in fresh medium.
[d] ND, Not done.

DISCUSSION

The principal aim of the present communication was to examine the kinetics of IBR virus-host cell interactions to determine whether humoral immune mechanisms could destroy virus-infected cells prior to virus dissemination, thereby being of importance in the virus recovery process. It is well known that antibody can neutralize extracellular virus but, because such neutralizing antibody cannot enter viable cells, antibody alone has been assumed to play little or no role in the recovery from viruses that can be transmitted directly between contiguous cells (2, 7, 8, 11, 14). Although virus-specific antigens were detectable on the cell surface by indirect immunofluorescence as early as 6 h PI, these cells did not become susceptible to immune lysis by antibody and complement until approximately 4 h later. The reason for this discrepancy is not known, but it could be that virus determinants were sparsely distributed so that the cytotoxic immunoglobulin G molecules used (the sera were from hyperimmunized animals) were unable to form doublets and, consequently, fix complement. Alternative explanations could be either that there were qualitative differences between early and late membrane antigens or that the indirect immunofluorescence technique was more sensitive than was the antibody-complement lysis. The importance of density and type of antigenic determinants in relation to destruction by antibody and complement or cell-mediated immunity has been poorly defined with viral antigens and is certainly worthy of further investigation.

Although antibody- and complement-mediated cell destruction did not occur until 10 h PI, such a mechanism may, nevertheless, be of some importance in the recovery process. Thus, although intracellular infectious virus was present at this time, such virus was not released extracellularly until 2 h later; transmission to contiguous cells via the intracellular route be-

gan at around 10 h. These observations supported the concept that, if both antibody and complement were present from 6 to 7 h onwards, then virus dissemination would be limited or curtailed. By means of an infectious center assay, this was shown to the the case. Thus, if antibody and complement were continually added, virus dissemination was reduced more than 50-fold. It was necessary, however, to continually add fresh complement because if antibody and complement were added only once, such as had been done by Lodmell et al. (7), then the reduction of transmission was barely detectable. Presumably one reason previous workers failed to demonstrate a reduction of viral spread by antibody and complement was because the latter reagent had become inactivated by the time it could have been involved in infected-cell destruction. Because the continual addition of complement more closely mimics the in vivo situation, we suggest that antibody-complement lysis may be an important mechanism in limiting the spread by herpesviruses.

Although our studies demonstrate that humoral immunity is important in the virus recovery process, this does not mean that it is the only process involved. Indeed, studies by others as well as by ourselves have also inferred an important role for cell-mediated immunity mechanisms (5, 7, 9, 11, 12, 14). Presumably, in vivo many components could interact to cause recovery from acute disease. Studies are needed to define how the various components interact and to decide which components can destroy virus-infected cells prior to the transmission of virus to contiguous cells.

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LITERATURE CITED

1. Allison, A. C. 1972. Immunity against viruses, p. 51–73. *In* I. Gilliland and J. Francis (ed.), Scientific basis of medicine annual review. Athlone Press, London.
2. Allison, A. C. 1974. Interactions of antibodies, complement components and various cell types in immunity against viruses and pyogenic bacteria. Transplant. Rev. 19:3–55.
3. Cesario, T. C., J. D. Poland, H. Wulff, T. Chin, and H. Wenner. 1969. Six years study with Herpes simplex in a children's home. Am. J. Epidemiol. 90:416–422.
4. Davies, D. H., and J. F. Duncan. 1974. The pathogenesis of recurrent infections with infectious bovine rhinotracheitis virus induced in calves by treatment with corticosteroids. Cornell Vet. 64:340–366.
5. Ennis, F. A. 1973. Host defense mechanisms against Herpes simplex virus. II. Protection conferred by sensitivity. Infect. Immunol. 7:898.
6. Karber, G. 1931. Beitrag. zur Killektiren Behandung pharmakologischer Reihenversuche. Arch. Exp. Pathol. Pharmakol. 162:480–483.
7. Lodmell, D. L., A. Niwa, K. Hayashi, and A. L. Notkins. 1973. Prevention of cell-to-cell spread of Herpes simplex virus by leucocytes. J. Exp. Med. 137:706–720.
8. Notkins, A. L. 1974. Immune mechanisms by which the spread of viral infections is stopped. Cell. Immunol. 11:478–483.
9. Rasmussen, L. E., G. W. Jordan, D. A. Stevens, and T. C. Merigan. 1974. Lymphocyte interferon production and transformation after Herpes simplex infections in humans. J. Immunol. 112:728–736.
10. Rouse, B. T., and L. A. Babiuk. 1974. Host defense mechanisms against infectious bovine rhinotracheitis virus. In vitro stimulation of sensitized lymphocytes by virus antigen. Infect. Immun. 10:681–687.
11. Rouse, B. T., and L. A. Babiuk. 1974. Host responses to infectious bovine rhinotracheitis virus. III. Isolation and immunologic activities of bovine T lymphocytes. J. Immunol. 113:1391–1398.
12. Rouse, B. T., and L. A. Babiuk. 1975. Host defense mechanisms against infectious bovine rhinotracheitis virus. II. Inhibition of plaque formation by immune peripheral blood lymphocytes. Cell. Immunol. 17:43–56.
13. Russell, A. S. 1973. Cell-mediated immunity to Herpes simplex virus in man. Am. J. Clin. Pathol. 60:826–830.
14. Russell, A. S. 1974. Cell-mediated immunity to Herpes simplex virus in man. J. Infect. Dis. 129:142–146.
15. Sheffy, B. E., and D. H. Davies. 1972. Reactivation of a bovine Herpes virus after corticosteroid treatment. Proc. Soc. Exp. Biol. Med. 140:974–976.
16. Snowdon, L. A. 1965. The IBR-IPV virus: reaction to infection and intermittent recovery of virus from experimentally infected cattle. Aust. Vet. J. 41:135–142.
17. Wilton, J. M. A., L. Ivanyi, and T. Lehner. 1972. Cell-mediated immunity in herpes virus hominis infection. Br. Med. J. 1:723–726.