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Inhibition of T-Lymphocyte Mitogenic Responses and Effects on Cell Functions by Bovine Herpesvirus 1

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The mitogenic response of bovine peripheral blood mononuclear cells stimulated by concanavalin A (ConA) was suppressed by infectious bovine herpesvirus 1 (BHV-1). Proliferation in response to interleukin-2 (IL-2) by IL-2-dependent lymphocyte cultures was also inhibited by BHV-1. Although inhibition of mitogenesis approached 100%, less than 1 cell in 1,000 was productively infected by BHV-1 in ConA-stimulated cultures. Neither conditioned medium from mitogen-stimulated peripheral blood mononuclear cell cultures nor human recombinant IL-2 reversed suppression by the virus. Infection by BHV-1 did not influence the expression of IL-2 or IL-2 receptor mRNA in ConA-stimulated cultures, nor did it affect the cytolytic capabilities of lymphocytes. The data suggest that the inhibition of T-lymphocyte proliferation is the result of a nonproductive BHV-1 infection.

Pulmonary infection with bovine herpesvirus 1 (BHV-1) often leads to immune suppression and increased host susceptibility to secondary bacterial infections (18). The syndrome known as infectious bovine rhinotracheitis is the result of a synergistic interaction between viruses and bacteria (37). Similar viral-bacterial interactions have been observed in humans and other animals. In humans, for example, serious bacterial infections can follow influenza (17) and measles (3) virus infections.

During BHV-1 infection, antibody responses in the host usually remain normal (12). The cell-mediated response, however, appears to be depressed. Peripheral blood mononuclear cells (PBMC) from infected animals produce less interleukin-2 (IL-2) (2) and have reduced mitogenic responses (6, 13) and lower natural cytotoxic activities (2, 6) than cells from uninfected animals. Additionally, the migratory response of polymorphonuclear neutrophils is decreased (6, 13) during BHV-1 infection.

It is generally believed that BHV-1 primarily infects monocytes and not lymphocytes and that immune suppression is due to infection of the nonlymphoid cells. However, we present data here which suggest that BHV-1 has a direct effect on lymphocyte proliferation. In the presence of BHV-1, DNA synthesis in lymphocytes was almost totally inhibited, although T-cell activation by mitogens occurred normally.

MATERIALS AND METHODS

Viruses and cell cultures. All cell lines and viruses were obtained from the American Type Culture Collection, Rockville, Md. All cell cultures were maintained in Dulbecco modified Eagle medium (Flow Laboratories, Inc., McLean, Va.) supplemented with 5% fetal bovine serum (Flow), 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and antibiotics. Viruses were propagated as described by Rouse and Babiuk (32) with Madin-Darby bovine kidney cells (MDBK) or bovine turbinate cells at a multiplicity of infection (MOI) of 0.01. Virus concentrations were determined by a plaque assay with MDBK cells and a methylcellulose overlay. To inactivate BHV-1 with UV light, we irradiated a virus preparation with a low-pressure mercury lamp at 67 ergs/mm^2.

Bovine PBMC were prepared from blood obtained by venipuncture in the presence of citrate as an anticoagulant. Gradient centrifugation was then used to isolate PBMC as described previously (32). Lymphocytes from bovine lymph nodes were obtained as described by Namen and Magnuson (25). Conditioned medium was prepared from 24-h concanavalin A (ConA)-stimulated bovine PBMC or lymph node cultures as described previously (25). IL-2-dependent lymphocyte cultures were initiated and maintained as described by Carter and co-workers (9). Briefly, PBMC were stimulated either with an allogeneic mixed-lymphocyte culture or with ConA (2.5 μg/ml). All cultures were maintained in medium containing IL-2 (human recombinant IL-2; Chiron Corp., Emeryville, Calif.) at a concentration of 50 U/ml. Cells used in IL-2 bioassays had been maintained in continuous cultures for at least 14 days.

Assay for suppression of mitogenesis by BHV-1. BHV-1 was incubated with freshly isolated PBMC (2×10^7/ml) in serum-free medium for 1 h at 37°C. Additions were then made to bring the final medium concentrations to 5% calf serum, 2.6×10^6 cells per ml, and 2.5 μg of ConA per ml or 10 nM phorbol 12,13-dibutyrate (PDB; Sigma Chemical Co., St. Louis, Mo.) and 0.5 μM ionomycin (Calbiochem-Behring, San Diego, Calif.) (21). On various days, 1 μCi of [^{3}H]thymidine ([^{3}H]TdR) (New England Nuclear Corp., Boston, Mass.) was added to 200 μl of each sample in a 96-well plate. The contents of the plate were collected 4 h later with a semiautomatic cell collector (Flow). All experiments were performed in triplicate.

Infectious-center assay. Freshly isolated PBMC or nylon wool-nonadherent cells (24) were infected with BHV-1 at an MOI of 10. The suspension was incubated on ice for 1 h and at 37°C for 1 h. The infected cells were diluted to 2×10^6/ml, and ConA (2.5 μg/ml) was added. After incubation for 72 h at 37°C in 5% CO_2, the PBMC were washed three times and counted. Various numbers of infected PBMC were added to monolayers of MDBK cells in six-well plates (Flow). Uninfected, ConA-stimulated PBMC were added to maintain a

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constant number of cells per well (1×10^6 to 2×10^6). Medium was added to a total volume of 1 ml per well. The medium was covered with a 2-ml overlay of 1% methylcellulose. After 3 days, the wells were washed with saline, stained with crystal violet, and inspected for plaques.

Suppression of IL-2-dependent cell growth. (i) Time course. Cultures were established in microdilution plates containing 50 U of IL-2 per ml in the presence or absence of BHV-1 at an MOI of 1. Each day following infection, triplicate samples of infected and uninfected cultures were pulsed and harvested as described above.

(ii) Dose response. BHV-1 was added to IL-2-dependent bovine lymphocytes at various concentrations in a microdilution plate in triplicate (10^5 cells per ml). All of the cultures received IL-2 (50 U/ml) unless indicated otherwise. After 48 h, cultures were pulsed and harvested as described above.

(iii) Assay for inhibition of IL-2. Human recombinant IL-2 (Chiron) was serially diluted across a microdilution plate in 50 μl of medium in triplicate. Virus was added (MOI, 1) to the IL-2-dependent cell cultures (depleted of IL-2) just before distribution of the cells to the microdilution plates (10^5 cells per well). The plates were incubated for 48 h and then pulsed for 4 h with [^{3}H]TdR.

Cytotoxicity assay. Nonspecific cytotoxicity was determined by a lectin-dependent cytotoxic assay with YAC-1 cells as targets. A modification of the method of Berke and co-workers (4) was used as previously reported (9). In brief, cytotoxic effector cells which had been generated as described above were infected with BHV-1 (MOI, 1) for various lengths of time. The effector cells were serially diluted (1:2) across a microdilution plate. YAC-1 cells were incubated with $Na_2{}^{51}CrO_4$ (New England Nuclear) for 1 h and washed. The YAC-1 cells were suspended in medium containing ConA (2.5 μg/ml) and added to the microdilution plates at 10^4 cells per well. All experiments were performed in triplicate.

Isolation of RNA and Northern (RNA) blot analysis. Bovine lymphocytes (10^9) were stimulated with ConA and 4-phorbol-12-myristate-13-acetate (10 ng/ml). After incubation at 37°C in 5% CO_2 for 24 h, the cells were collected by low-speed centrifugation. RNA was isolated from the cell pellet with guanidine isothiocyanate (11). Typical yields of total RNA from 10^9 cells were 0.5 to 1.0 mg. Oligo(dT)-cellulose chromatography was used to enrich for mRNA (1). The mRNA was isolated from BHV-1-infected (MOI, 1) or uninfected bovine lymphocytes at various times after stimulation. After electrophoresis in a 1.25% agarose-formaldehyde gel (23), the RNA was transferred to nitrocellulose and hybridized to a nick-translated bovine IL-2 cDNA probe (30) or a nick-translated IL-2 receptor cDNA probe (analogous to the human 50-kilodalton subunit) (36). The hybridization conditions and washes were as described previously (30).

RESULTS

Suppression of mitogenic responses by BHV-1. Bovine PBMC cultures were infected with BHV-1 at MOIs of 0.0005 to 5. Following infection the cultures were stimulated with ConA. The cultures were assayed each day for 6 days for the uptake of [^{3}H]TdR. Figure 1 shows the result of one such experiment. After 3 days there was greater than 50% inhibition at an MOI of 5. After 4 days, in all cultures having an MOI of 0.05 or greater, thymidine uptake was reduced by greater than 50%, as compared with that in uninfected controls.

To determine if BHV-1 could alter proliferation when lymphocytes were stimulated in an accessory cell-independent manner (20), we treated the cultures with PDB and ionomycin. In these experiments one culture had BHV-1-permissive cells depleted by nylon wool treatment prior to stimulation by PDB and ionomycin. BHV-1 inhibited PDB- and ionomycin-mediated stimulation to the same extent as ConA-mediated stimulation (Fig. 2). Depletion of BHV-1-permissive cells did not alter the suppressive effects of the virus.

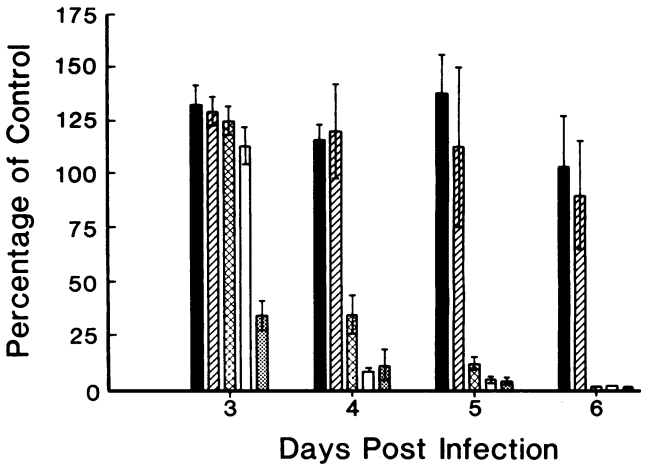

FIG. 1. Time course and dose response of BHV-1-induced suppression. PBMC were stimulated with ConA and infected with BHV-1 at an MOI of 0.0005 (■), 0.005 (▨), 0.05 (▩), 0.5 (□), or 5 (▦). Aliquots of each culture were removed on the indicated days and pulsed with [^{3}H]TdR for 4 h. Bars represent the percent counts per minute relative to those in uninfected controls plus or minus the standard error.

Suppression of lymphocyte mitogenic responses was not a result of cell death, as lymphocytes were viable for 4 days after exposure to virus, as determined by trypan blue dye exclusion and staining with ethidium bromide and acridine orange. Suppression was due to viable virus, as heat-killed and UV-inactivated viruses were not suppressive (data not shown).

Determination of the number of PBMC productively infected by BHV-1. Infectious-center assays were conducted to

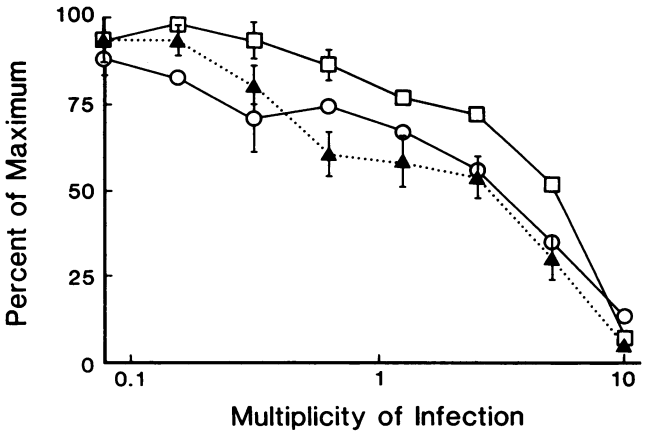

FIG. 2. Suppression of ionomycin- and PDB-stimulated cultures. PBMC were infected at the indicated MOIs following ionomycin-PDB stimulation (○), nylon wool treatment and ionomycin-PDB stimulation (▲), or ConA stimulation (□). Cultures were pulsed with [^{3}H]TdR on day 3 for 4 h. Data represent the percent maximum counts per minute for each culture plus or minus the standard error.

TABLE 1. Determination of the number of PBMC infected by BHV-1 at an MOI of 10

Expt	Animal no.	Nylon wool treatment[a]	No. of uninfected cells/infected cell[b]	% Infected
1	16	−	2,100 ± 200	0.048
		+	$>5 \times 10^5$	<0.0002
2	64	−	4,200 ± 700	0.024
		+	$>9 \times 10^5$	<0.0001
3	64	−	4,900 ± 300	0.020
		+	$>1 \times 10^5$	<0.0006

[a] Nylon wool-adherent cells were removed prior to infection with BHV-1.
[b] The data are presented as means plus or minus standard deviations when at least three wells could be counted. The data without standard deviations represent the wells which had the maximum number of plaques.

determine the maximum number of PBMC which could be productively infected. The data indicate that the total number of PBMC which could be infected was less than 0.05% (Table 1). Table 1 also shows that greater than 90% of the cells which were susceptible to infection by BHV-1 could be removed by nylon wool. In control experiments it was found that the number of infectious centers did not increase significantly when MOIs greater than 10 were tested (data not shown).

Effect of BHV-1 on IL-2-dependent cell proliferation. The effect of BHV-1 on IL-2-dependent cell growth was characterized in three assays systems. In time course studies, IL-2-dependent lymphocytes were infected at an MOI of 1, and the uptake of [^{3}H]TdR was monitored each day following infection. At 1 day after infection there was no significant difference between infected and uninfected cultures (Fig. 3a). At 2 and 3 days after infection the uptake of [^{3}H]TdR in infected cultures was less than 5% of that in uninfected cultures. In parallel experiments it was found by trypan blue dye exclusion that after 3 days of infection (MOI, 1) greater than 90% of the infected cells were viable. A dose-response experiment was conducted with the same cells as those used above. After 2 days in culture there was significant inhibition of [^{3}H]TdR uptake at all concentrations of virus tested (an MOI as low as 0.01) (Fig. 3b). The ability of additional IL-2 to overcome the virus-induced inhibition of IL-2-dependent mitogenic responses was evaluated with IL-2-dependent cultures which were either uninfected or infected with BHV-1. The addition of BHV-1 (MOI, 1) inhibited the uptake of [^{3}H]TdR by greater than 90%, and additional IL-2 could not reverse the effect (Fig. 3c).

Effect of BHV-1 on cytotoxic activity. To determine if the T-cell effector function was altered by viral infection, we generated IL-2-dependent cytotoxic cells in a mixed-lymphocyte culture and propagated them in medium containing IL-2. Virus (MOI, 1) was added to these cultures, which were incubated for 1 to 4 days before determination of cytotoxic activity. Because BHV-1 inhibited lymphocyte proliferation, the infected cultures contained approximately one-half the number of cells as the uninfected cultures after 4 days of incubation. Although inhibition of lymphocyte proliferation occurred in these cultures as soon as 2 days postinfection (Fig. 3), there was no detectable difference in the cytolytic capabilities of infected and uninfected cells when similar effector cell/target cell ratios were used. Figure 4 shows the results of a typical cytotoxic assay with cultures which had been incubated for 4 days after infection.

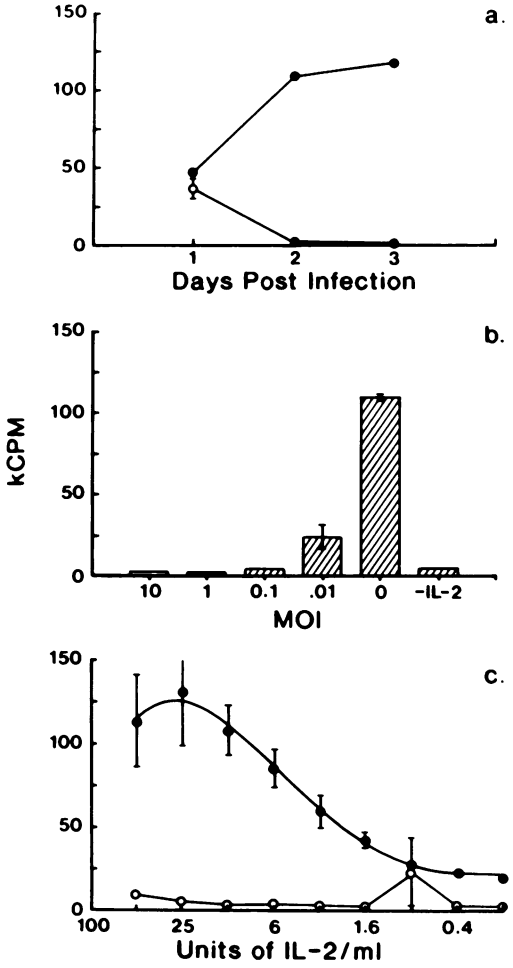

FIG. 3. Effect of BHV-1 on IL-2-dependent cell proliferation. (a) IL-2-dependent cultures were infected (MOI, 1) (○) or not infected (●) with BHV-1. The uptake of [^{3}H]TdR was measured on each day following infection. (b) IL-2-dependent cultures were infected with BHV-1 at the indicated MOIs, and [^{3}H]TdR uptake was measured at 48 h postinfection. (c) IL-2-dependent cultures were infected with BHV-1 at an MOI of 1 (○) or mock infected (●) and added to a microdilution plate containing IL-2. The uptake of [^{3}H]TdR was measured at 48 h postinfection. In all cases the data represent the average of three replicates plus or minus the standard error.

Effect of BHV-1 on the expression of IL-2 and the IL-2 receptor. To determine whether lymphocytes were activated by ConA in the presence of virus, we monitored the expression of IL-2 and IL-2 receptor mRNAs. At various times (15 to 92 h) after ConA stimulation, in the presence or absence of virus, cells were collected and the mRNA was isolated and fractionated by electrophoresis. Northern blotting was performed with ^{32}P-labeled IL-2 (Fig. 5A) or IL-2 receptor (Fig. 5B) cDNA probes. The sizes of the mRNAs for IL-2 (800 nucleotides) and the IL-2 receptor (2,600 nucleotides) had been established in previous studies (30, 36). There was no difference between infected and uninfected cells in the expression of either IL-2 or IL-2 receptor mRNAs at any time tested. As a control, the uptake of [^{3}H]TdR was measured on each day following infection. Incorporation in infected cultures was greater than 25% 3 days postinfection and greater than 10% 4 days postinfection relative to that in

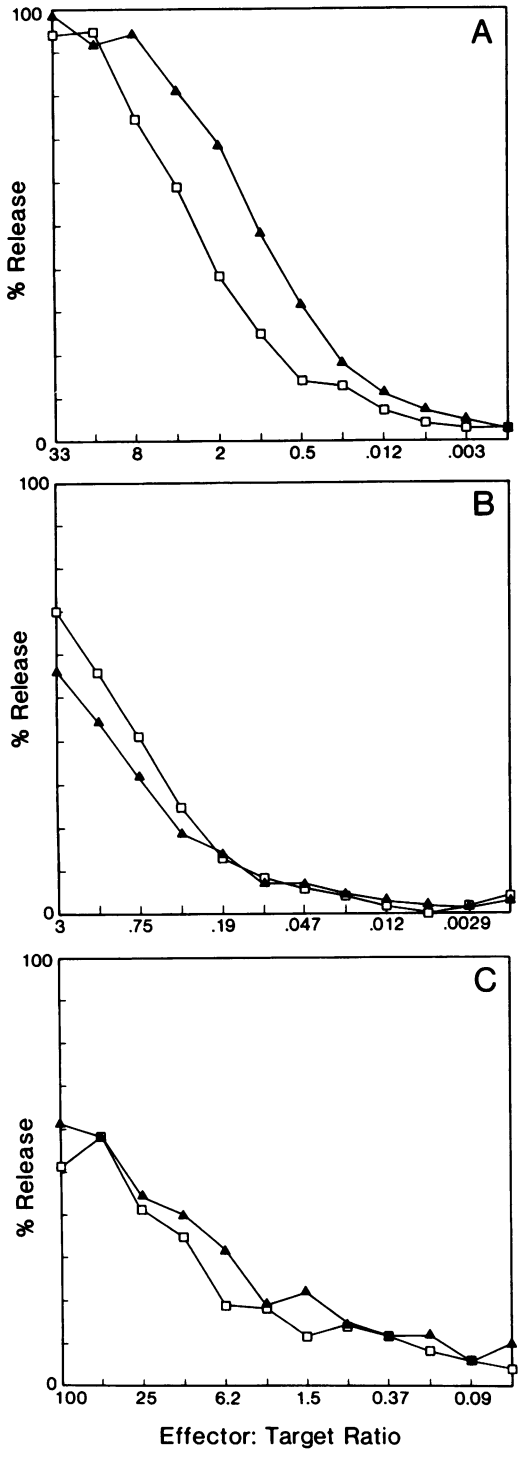

FIG. 4. Effect of BHV-1 on cytotoxic IL-2-dependent cell cultures. Controls (▲) and cultures infected with BHV-1 (MOI, 1) (□) were incubated for 4 days prior to testing in a lectin-dependent ^{51}Cr release assay. The cultures had been maintained for 6 weeks (A), 4 weeks (B), and 3 weeks (C) prior to the assay. The highest value on the abscissa represents the starting effector cell/target cell ratio. Tick marks on the abscissa represent the series of subsequent 1:2 dilutions of the effector cells.

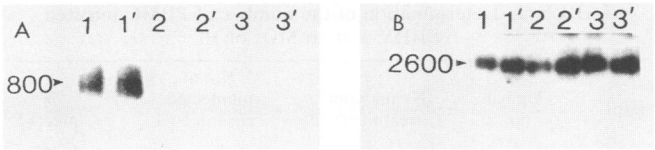

FIG. 5. Northern blot analysis of IL-2 and IL-2 receptor mRNA expression in BHV-1-infected and uninfected cultures. Bovine lymph node cells were stimulated by ConA in the presence or absence (numbers with primes) of BHV-1. At various times after stimulation cells were collected, and the poly(A)$^+$ RNAs were isolated and separated by electrophoresis. Northern blot analysis was conducted with labeled IL-2 cDNA (A) or IL-2 receptor cDNA (B). The bands in panel A represent 800-base polynucleotides, and those in panel B represent 2,600-base polynucleotides. Numbers at the top represent times of harvest: A, 1 is 15 h, 2 is 24 h, and 3 is 48 h; B, 1 is 24 h, 2 is 28 h, and 3 is 48 h.

uninfected cultures. Results from two different animals were identical.

To determine whether the expression of IL-2 protein was altered by the presence of virus, we tested the supernatants from the infected and uninfected cultures for IL-2 activity by a bioassay. When the 24-h uninfected-culture supernatant was titrated on IL-2-dependent bovine cells, a maximum [^{3}H]TdR incorporation of 46,000 cpm was recorded after 24 h of incubation. When the infected-culture supernatant was titrated, a maximum of 47,000 cpm was incorporated.

DISCUSSION

Infection of ConA-stimulated or IL-2-dependent cells with BHV-1 inhibited lymphocyte proliferation in vitro. We showed here that this inhibition occurred in the absence of significant proliferation of BHV-1 in lymphocytes. This finding is similar to those reported for the inhibition of lymphocyte mitogenic responses by a variety of viruses (14–16, 22, 28, 29). Inhibition of replication is not simply due to the lysis of lymphocytes because effector functions, such as cytotoxicity, remain intact. BHV-1-induced inhibition of replication occurred to the same extent when BHV-1-permissive cells were removed by nylon wool treatment. This result suggests that suppression may result either directly from a nonpermissive infection of lymphocytes or indirectly from a nonproductive BHV-1 infection of a subpopulation of cells. We suspect the first possibility because we have been unable to detect suppressive activity in the supernatant of infected cultures; however, until the mechanism of suppression is determined, this remains speculative.

The kinetics of BHV-1-induced suppression of IL-2-dependent cell proliferation were found to be quite different from those of the suppression of lectin-stimulated PBMC. This result is not surprising, as the kinetics of proliferation are quite different in each case. In lectin-stimulated cultures, cells enter the DNA synthesis phase of the cell cycle (S phase) at 2 to 3 days following stimulation, whereas IL-2-dependent cells enter the S phase after 24 h of incubation with IL-2 (8). Suppression occurred 2 days sooner in the IL-2-dependent cultures than in the ConA-stimulated cultures with similar virus concentrations. In both cases we suspect that suppression resulted from the production of new virus in a subpopulation of cells. Indeed, infectious-center assays confirmed that a very small percentage of cells (>0.05%) were infected in ConA-stimulated cultures. Therefore, the difference in the rate of suppression between IL-2-dependent cultures and ConA-stimulated cultures may have reflected a difference in the rate of production of new

virus in these cultures, although this possibility has not been fully investigated.

The suppression of mitogenic responses was not due to a reduction in available IL-2, as exogenously added IL-2 did not eliminate the suppression. Bielefeldt Ohmann and Babiuk (6) showed that PBMC removed from cattle infected with BHV-1 were not as responsive to mitogen stimulation as were PBMC removed from uninfected animals and that additional IL-2 did not eliminate the suppressive effects of the virus. It appears, therefore, that whether lymphocytes are infected in vivo or in vitro, their proliferative capabilities are greatly reduced even in the presence of exogenously added IL-2.

It has been shown previously that PBMC from infected animals have a slight reduction in the amount of IL-2 produced when stimulated in vitro (2) and have reduced natural killer cell activity (5). Our results with lymphocytes infected in vitro indicated little or no change in the ability to produce IL-2 or to kill in cytotoxic assays. Therefore, the observations made with PBMC from infected animals (2, 5) may have been the result of a virus-induced change in the population of circulating lymphoid cells rather than a virus-caused alteration of lymphocyte cell function. This possibility was postulated previously by Bielefeldt Ohmann and Babiuk (6). Changes have been observed in bovine lymphoid cell populations following BHV-1 infection (6). Furthermore, changes in the ratios of lymphocyte subpopulations have been shown to occur in human herpesvirus infections (34).

PDB and the ionophore ionomycin, in combination, stimulate both B and T cells (21). Therefore, our results showing the suppression of PDB- and ionomycin-induced stimulation demonstrate that suppression is not limited to T-cell mitogens. Whether suppression of B-cell mitogenesis occurs independently of suppression of T-cell mitogenesis or is a result of T-cell suppression is not known at this time.

Nyaga and McKercher (26) demonstrated that BHV-1 could multiply in the adherent PBMC population. It has been suggested, therefore, that BHV-1 may cause immune suppression by affecting macrophage functions, including the production of interleukin-1 and prostaglandin E2 (7). The results from our infectious-center assays also show that the majority of cells productively infected with BHV-1 are adherent cells (Table 1).

Although cells of the monocyte-macrophage linage appear to be one site of viral replication, our data suggest that infection of these cells is not the sole cause of immune suppression. There are three reasons for this contention. Firstly, suppression occurred in the IL-2-dependent cultures, which did not contain monocytes or macrophages, as determined by nonspecific esterase staining. Secondly, cells of the monocyte-macrophage linage are known to play a key role in the activation of T cells (19). We showed that T cells are activated normally in the presence of BHV-1, as evidenced by the expression of IL-2 and IL-2 receptor mRNAs. Thirdly, it was found that when PBMC were stimulated with PDB and ionomycin, suppression occurred to the same degree regardless of whether the adherent cells were removed.

We suggest that a nonproductive infection of lymphocytes by BHV-1 may be occurring. Such an infection may be similar to the type of infection observed to cause suppression with human cytomegalovirus (31, 33), measles virus (10), and rabbit fibroma virus (35). In these examples, viral genes are expressed in the absence of viral cytolysis (27), and although the infected cells survive, their activity is altered.

BHV-1 can infect many different types of cells, and immune suppression in cattle probably occurs by more than one mechanism. We believe that infection of lymphocytes by BHV-1 may be a major contributing factor to immune suppression. Experiments are now under way to determine the extent of viral gene expression in bovine lymphocytes.

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