Alteration of Alveolar Macrophage Functions after Aerosol Infection with Bovine Herpesvirus Type 1

H. BIELEFELDT OHMANN†* AND L. A. BABIUK

Department of Veterinary Microbiology, University of Saskatchewan, and the Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan, Canada S7N 0W0

Calves were aerosol challenged with bovine herpesvirus type I, and bronchoalveolar cells were subsequently retrieved by lavage from days 1 to 8 postinfection. Alveolar macrophages (AM), which were depleted of contaminating cells, were characterized with respect to phenotypic markers and functional activities. In most aspects, the changes suggested a stimulation of the AM. With variations in kinetics the percentage of AM expressing an MHC II antigen and Fc (immunoglobulin G)-mediated phagocytosis increased, as did the activity level of two ectoenzymes and the lysosomal hydrolase β-glucoronidase. The generation of prostaglandin E_2 by the AM also rose significantly. However, selective suppression of cellular cytotoxicity and interleukin-1 generation was observed. These findings may have important implications for understanding the events involved in the virus-bacterial interaction in respiratory diseases.

Pulmonary virus infections predispose animals and humans to secondary bacterial pneumonia (16, 17, 21); it is assumed that this is at least in part due to virus-induced impairment of alveolar macrophage (AM) functions such as bacterial phagocytosis and killing (for a review, see reference 18) and production of neutrophil chemotactic factors (22). However, there often appear to be discrepancies between in vitro and in vivo observations of the AM-virus interaction (11, 12, 24, 29). Bovine AMs are susceptible to infection with bovine herpesvirus-1 (BHV-1) in vitro, resulting in impairment of immune receptor functions and antibody-dependent cell-mediated cytotoxicity (ADCC) (11). In contrast, less than 0.1% of bronchoalveolar cells retrieved from experimentally infected calves are productively infected with BHV-1 and neither ADCC nor receptor functions are altered (12). However, the finding that the generation of neutrophil chemotactic factors is impaired (22) could indicate that selective alterations of AM functions might occur. The present investigation was conducted to explore this possibility. To avoid any unintentional activation of the AMs or loss of the more slowly adhering subpopulation (7), or both, the AMs were purified by Percoll gradient centrifugation and immediately used in various assays (H. Bielefeldt Ohmann and L. A. Babiuk, J. Leukocyte Biol., in press).

Hereford calves (7 to 9 months old) that were seronegative for BHV-1 (12) were initially used for serial lavages to establish base-line activities of the AMs. After an appropriate resting period all animals were aerosol challenged on the same day with BHV-1 strain 108 as previously described (8). Body temperature and clinical signs were recorded daily. Bronchoalveolar cells were retrieved by lavage from three calves at 24, 48, and 96 h, 6 and 8 days after challenge. To eliminate the effects of lavage on AM functions, 7 days were allowed to pass before lavage was repeated. To deplete polymorphonuclear leukocytes (PMN), lymphocytes, and epithelial cells, the alveolar cells were centrifuged on a discontinous Percoll gradient as described elsewhere

(Bielefeldt Ohmann and Babiuk, in press). Briefly, up to 1 × 10^8 cells were suspended in 10% Percoll in RPMI 1640 plus 10% fetal bovin serum and layered over a 5-step gradient of from 65 to 25% Percoll. Centrifugation was for 45 min at 350 × *g* at 4°C. Cells at the 10 to 25% interphase were discarded, and the remaining bands were collected separately. Siliconized glassware was used throughout the procedure. After three washings in cold Hanks balanced salt solution cell counts were performed, and cytosmears were made and stained with Wright's stain for differential counts of each cell sample. Samples contaminated with PMN or lymphocytes were discarded (fraction 5, containing <4% of the total AMs), the remaining fractions were pooled for each animal, and the cells were suspended in Hanks balanced salt solution to 1 × 10^7 CFU/ml. In a normal uninfected animal this protocol usually produces an AM population that is >98% pure, consisting of cells from interphases 2, 3, and 4. However, in BHV-1-infected animals some cell stickiness occurred on days 2 and 4 postinfection (p.i.), resulting in some retention of PMN and lymphocytes at low densities. Thus the final AM pool could be contaminated with up to 10 to 15% of these cells. Cell purification was followed immediately by functional assays and phenotypic characterization.

Surface antigens present on bovine macrophages (10) and defined by monoclonal antibodies H34A and B18A were assayed for by antibody plus complement killing. The H34A monoclonal antibody detects an Ia-like (MHC II) antigen, whereas the B18A-defined antigen has not yet been related to any specific function (10). Complement (C3b) and Fc immunoglobulin G (IgG) receptor-mediated phagocytosis was examined as previously described (9) by incubating AMs that had been pelleted with IgM-C3b- or IgG-sensitized erythrocytes for 30 min at 37°C. After carefully suspending the cells, samples were examined under a light microscope, and a minimum of 300 AMs were counted to determine the percentage of phagocytizing cells (lymphocytes and PMN were excluded from these counts). Superoxide anion (O_2^-) generation by nonstimulated and opsonized zymosan-stimulated cells was measured as described previously (7). Cells in suspension were incubated for 1 h. For generation of prostaglandin E_2 (PGE2), interleukin-1 (IL-1), and interferon

* Corresponding author.
† Present address: Department of Veterinary Virology and Immunology, The Royal Veterinary and Agricultural University, Copenhagen, Denmark.

(IFN), 1×10^6 cells in 1 ml of modified Iscove's medium (4) without serum were plated in 24-mm-diameter wells (Corning Glass Works, Corning, N.Y.). Quadruplicate cultures were set up for each animal, two of which received 0.1 mg of opsonized zymosan.

Cultivation was terminated after 24 h by collecting the medium, which was subsequently cleared for cell debris by centrifugation, filtered (0.45-μm membrane filter; Millipore Corp., Bedford, Mass.), and stored at −70°C until assayed. PGE_2 was quantified by radioimmunoassay with commercially available test kits (NEK 020A; New England Nuclear Corp., Boston, Mass.) according to the manufacturer's instructions. IFN levels were determined in a virus inhibition assay with vesicular stomatitis virus and Georgia bovine kidney cells as previously described (3). IL-1 was assayed in a thymocyte proliferation assay as previously described (25) by using bovine fetal thymocytes. The results are reported as counts above control (counts per minute [cpm]) for triplicate determinations or as a stimulation index. Spontaneous cellular cytotoxicity and ADCC toward BHV-1-infected fibroblasts were assayed in a 6-h ^{51}Cr-release assay at an effector-to-target cell ratio of 50:1 as previously described (9). Cytosmears were stained for nonspecific butyryl esterase according to Koski et al. (20) and evaluated on an arbitrary scale from 0 to +++ by the same person throughout the investigation. The ectoenzymes leucine amino-peptidase, alkaline phosphodiesterase-I, and 5'-nucleotidase, as well as the lysosomal hydrolase β-glucuronidase were assayed on cell lysates in 0.05% Triton X-100 according to previously published procedures (2, 16). To test for viral infection of the AMs, infectious center assays were carried out as described by Forman et al. (12) with BHV-1-infected fibroblasts as target cells. All data were analyzed for statistical significance by one-way analysis of variance with multiple comparisons.

Within 24 to 48 h of aerosol challenge with BHV-1, all calves developed pyrexia, and the rectal temperature remained elevated until days 7 to 9 p.i. Anorexia, depression, oculonasal discharge, light coughing, and mild to moderate lung involvement was observed in all calves, but symptoms resolved withing 9 to 15 days p.i. The total yield of live bronchoalveolar cells remained fairly constant throughout the experimental period; however, the composition changed, i.e., the proportion of AMs descreased concomitant with an increase in PMN by day 2 p.i. (Fig. 1A). Changes in the AM population were also observed. These changes included alterations in the nonspecific butyryl esterase score and the number of cells positive for the B18A-defined antigen (Fig. 1). Within 1 day p.i. the nonspecific butyryl esterase score rose dramatically ($P < 0.05$) and remained elevated for the entire observation period. In the case of the B18A-defined antigen there was a rapid decrease in the number of cells expressing this antigen at day 1 p.i., but by day 8 p.i. the B18A-defined antigen returned to normal (Fig. 1C). In contrast, the proportion of cells positive for an Ia-like antigen defined by H34A remained constant until day 8 p.i., at which time a significant increase was observed ($P < 0.05$; Fig. 1C). No major changes were observed in the level of leucine amino-peptidase (Fig. 1D), whereas alkaline phosphodiesterase-I increased by day 6 p.i. to become significantly elevated on day 8 p.i. ($P < 0.05$), and 5'-nucleotidase increased during the first 4 days p.i. ($P < 0.05$ on days 2 and 4 p.i.), after which activity returned to the preinfection level (Fig. 1D). Fc(IgG)-mediated phagocytosis increased briefly on day 1 p.i., but C3b-mediated phagocytosis remained constant (Fig. 2A). In contrast, the nonstimulated and
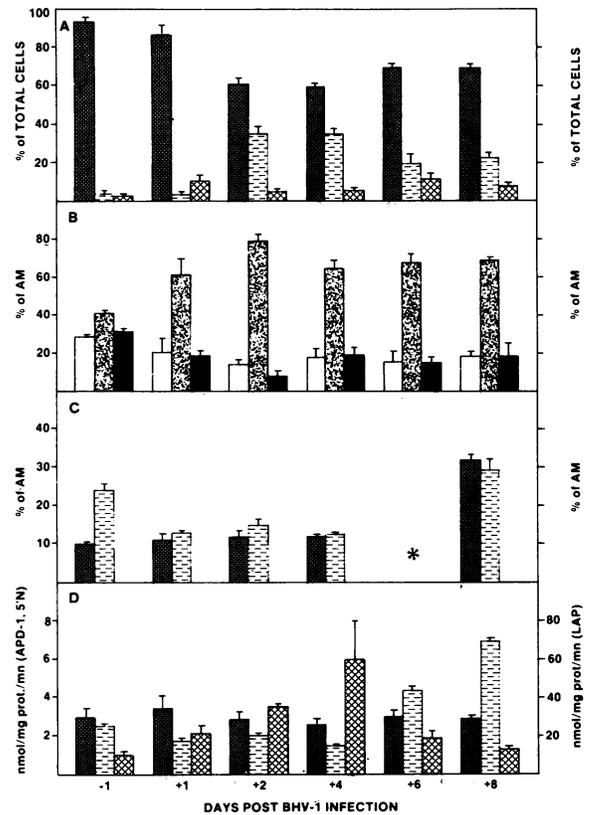

FIG. 1. Changes in proportion of AMs in bronchoalveolar lavage cells and their phenotypic characteristics after BHV-1 infection of calves. (A) Proportions of AMs (■), PMN (▤), and lymphocytes (▨) in retrieved cells. (B) Nonspecific butyryl esterase score of AMs: □, −/+; ▨, ++; ■, +++. (C) Proportion of AMs with H34A- (■) and B18A- (▤) defined surface antigens; *, no data. (D) Activity level of the ectoenzymes leucine amino-peptidase (■), alkaline phosphodiesterase-I (▤), and 5'-nucleotidase (▨). Each column represents the average of three animals, bars indicate standard error of the mean. Calves lavaged on day 8 are the same ones lavaged on day 1.

zymosan-stimulated O_2^- generation had decreased significantly ($P < 0.05$) by 1 to 2 days p.i. (Fig. 2B), and some initial decrease was also observed in ADCC activity (Fig. 2C). However, by day 6 p.i. ADCC activity rose significantly ($P < 0.05$) over the preinfection level. Notably, a low level of spontaneous cellular cytotoxicity was observed from day 1 p.i. and throughout the observation period ($P < 0.05$; Fig. 2C). Both spontaneous and in vitro-stimulated PGE_2 generation increased after infection, the former significantly ($P < 0.05$) on day 6 p.i. Spontaneous in vitro IL-1 generation by the AMs was characterized by minor, inconsistent variations, whereas the in vitro zymosan-stimulated release of IL-1 decreased progressively from day 1 to 6 p.i. (Table 1). The small increase of IL-1 from days 6 to 8 p.i. might indicate the beginning of recovery. The AMs produced low levels of IFN (8 to 32 μm/ml) from days 1 through 6 p.i., when cultured for 24 h; however, by day 8 p.i. this activity had ceased (data not shown). The amount of the lysosomal hydrolase β-glucuronidase rose significantly ($P < 0.05$) from day 4 p.i. and did not appear to return to normal levels within the observation period (data not shown). As found in a previous study (12), at all times p.i. less than 0.05% of the
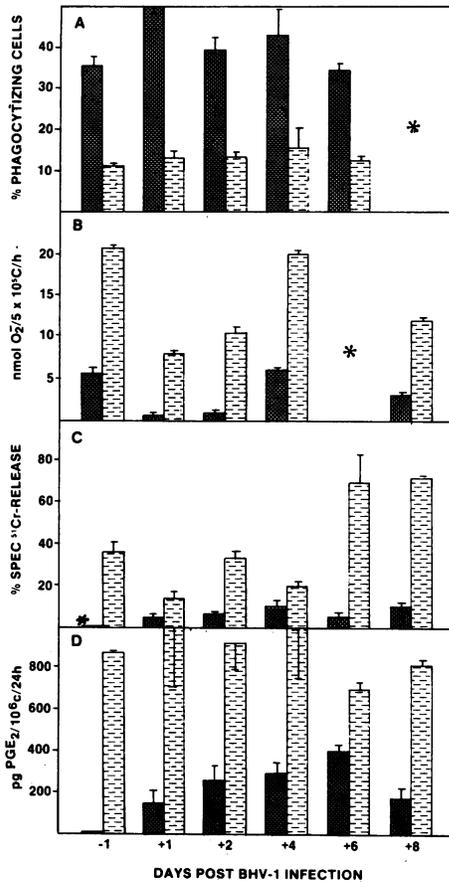
FIG 2. Changes in functional activities of bovine AMs after lung infection with BHV-1. (A) Fc(IgG)- (■) and C3b- (▨) mediated phagocytosis. (B) Superoxide anion generation by nonstimulated, i.e., in vitro (■) and opsonized zymosan-stimulated (▨) AMs; *, no data. (C) Spontaneous cytotoxicity (■) and ADCC (▨) against BHV-1-infected fibroblasts. Incubation was for 6 h with an antibody concentration of 1:500. The effector-to-target cell ratio was 50:1 (*– 1.76 ± 2.26). (D) Release of PGE₂ by AMs not stimulated further in vitro (■) and by zymosan-stimulated (▨) cells during a 24-h incubation in serum-free medium. Average of three animals ± standard error of the mean.

TABLE 1. Production and release of thymocyte-activating factor (IL-1) by AMs from BHV-1-challenged calves

Day p.i.	Phytohemagglutinin response of thymocytes[a]			
	Spontaneous release of IL-1[b]		Zymosan-stimulated IL-1[c]	
	cpm[d]	Stimulation index	cpm	Stimulation index
−1	2,697 ± 551	4.6 ± 0.7	10,608 ± 1,157	15.2 ± 1.5
+1	2,369 ± 127	4.1 ± 0.1	6,407 ± 202	9.5 ± 0.3
+2	2,507 ± 222	4.4 ± 0.3	5,897 ± 1,282	8.9 ± 1.7
+4	2,369 ± 95	4.1 ± 0.1	4,665 ± 422	7.2 ± 0.5
+6	2,786 ± 200	4.7 ± 0.3	4,364 ± 730	6.8 ± 1.0
+8	2,593 ± 702	4.4 ± 0.9	5,292 ± 357	8.1 ± 0.4

[a] 5 × 10⁵ thymocytes per well plus phytohemagglutinin (5 μg/ml) plus AM supernatant.
[b] Supernatant from AM cultures without in vitro stimulant, at 1:8 dilution.
[c] Supernatant from AM cultures was stimulated with opsonized zymosan at 1:8 dilution.
[d] Counts per minute were determined by experimental (phytohemagglutinin added) minus base-line values (without phytohemagglutinin), mean cpm for three calves plus over minus standard error of the mean at all time points. (Base line cpm for thymocyte cultures without phytohemagglutinin was less than 750 cpm).

AMs were productively infected as evidenced by infectious center assay.

The present investigation suggests that a stimulated AM population occurs after BHV-1 infection rather than a population of compromised functional activity (1, 19), although selective inhibition of some functions such as ADCC and IL-1 generation was noted. The latter finding suggests that despite stimulation the AMs may be impaired in some important antimicrobial and immunoregulatory functions. The latter aspect may be further intensified by increased PGE₂ release (Fig. 2D), which could contribute to the apparent immunosuppression in BHV-1 infection (8), including the antibacterial activities of the PMN (14). The present investigation does not reveal whether the observed alterations in functional activities, i.e., both increases and decreases, are caused by such changes in the AM subpopulation as a whole or whether different AM subpopulations (Bielefeldt Ohmann and Babiuk, in press) account for the disparate effects. An increased influx of monocytes (28) may contribute to some of the changes in activity, as for example

the emergence of spontaneous cellular cytotoxicity (6; H. Bielefeldt Ohmann, W. C. Davies, and L. A. Babiuk, Immunobiology 165:503–519, 1985) and the increase in number of cells expressing MHC-II antigen (H34A⁺). However, this cannot explain the increase in 5′-nucleotidase activity, which is undetectable on circulating bovine blood monocytes (Bielefeldt Ohmann and Babiuk, in press). Stimulation of the resident AMs is likely to take place as a result of local IFN release by virus-infected epithelial cells, the AMs themselves, and lymphocytes (9) and perhaps after release of lymphokines (including gamma IFN) by infiltrating antigen-specific lymphocytes in the later stages of the infection (Fig. 1A) (13, 26). The latter may account for the increased expression of Ia-like (MHC II) antigen, as detected with the monoclonal antibody H34A, an event which may be of decisive importance for an amplification of the virus-specific immune response (29).

Rather than playing a beneficial role, the functionally altered AM population may actually contribute to lung injury via its proinflammatory activities (26). Thus, future studies of BHV-1-induced respiratory disease should pay more attention to the AM and its capacity to produce and secrete complement components, procoagulant (of which bovine AMs are avid producers after in vitro stimulation [H. Bielefeldt Ohmann, unpublished data]), other arichidonic acid derivatives, and neutral proteases. These factors may contribute to increased vascular permeability, coagulation, fibrinolysis, and tissue damage (for a review, see reference 26), thereby creating an environment which promotes secondary bacterial invasion and growth and at the same time interferes with normal clearance mechanisms (23). In this respect BHV-1 infection of cattle could also serve as a natural model to determine events in the pathogenesis of virus-induced respiratory disease (6–8).

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