INFECTION AND IMMUNITY, Mar. 1982, p. 1041-1047
0019-9567/82/031041-07$02.00/0

Vol. 35, No. 3

Effect of Infectious Bovine Rhinotracheitis Virus Infection on Bovine Alveolar Macrophage Function†

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Received 10 July 1981/Accepted 27 October 1981

Bovine alveolar macrophages isolated in culture were assessed for immunological activity in assays for Fc and complement receptors, for phagocytosis, and for effector cell function in antibody-dependent cell cytotoxicity. In the case of uninfected alveolar macrophages, Fc receptors were detected on approximately 94% of macrophages and complement receptors were detected on 39%. Phagocytosis of immunoglobulin G-coated sheep erythrocytes occurred in 58% of macrophages, and phagocytosis of opsonized *Candida parapsilosis*, mediated by the complement receptor, was observed in 68% of cells. Alveolar macrophages were efficient effector cells in antibody-dependent cell cytotoxicity. Infection of macrophages with infectious bovine rhinotracheitis (IBR) virus resulted in reductions in Fc-mediated receptor activity and phagocytosis after approximately 12 and 6 h, respectively. Complement receptor activity was initially elevated and then markedly reduced. Macrophages retrieved from IBR-immune and -susceptible donors were affected to a similar extent. The ability of macrophages to participate in antibody-dependent cell cytotoxicity was reduced dramatically from 2 h after IBR virus infection, suggesting that IBR virus-infected alveolar macrophages undergo alterations in immunological activity long before morphological changes in the cells become apparent.

Viral infection of the lung often increases susceptibility to secondary bacterial infection (12, 24). Antibacterial activity of the alveolar macrophage (AM) is considered to be the major component of pulmonary bacterial clearance (8), and there is considerable evidence that viruses exert their effect by suppressing phagocytic activity in the lung (9, 26, 33).

Whereas macrophages are susceptible to infection in vitro with a number of viruses that predispose individuals to bacterial pneumonia (19, 25, 28), this does not necessarily imply that the observed reduced competence in vivo is directly due to infection of the AM rather than being an indirect effect of virus replication in the lung. Thus, whereas Warshauer et al. (33) showed that infection of mice with influenza virus impaired macrophage antibacterial activity, infection of mouse AM in vitro with influenza virus was abortive and there was no demonstrable effect on macrophage phagocytic or bactericidal functions (17).

Although infectious bovine rhinotracheitis (IBR) virus more commonly causes disease in the upper respiratory tract of cattle, it also replicates in the lung and produces pathological lesions there (10). In many cases, both the virus and *Pasteurella* spp. can be isolated from cattle dying of pneumonia (13). Furthermore, cattle challenged experimentally with IBR virus have an increased susceptibility to *Pasteurella haemolytica* (11). These observations suggest that viral infection alters the defense mechanisms of the lung in such a way as to make the animals more susceptible to infection of the lung with a bacterium which is a common resident of the bovine upper respiratory tract (27). In view of the importance of AM in antibacterial defense, we have initiated studies to determine whether IBR virus can infect bovine AM as well as whether such infection can alter their functional capacity. This report demonstrates that IBR virus infection of bovine AM results in a rapid alteration of their function.

MATERIALS AND METHODS

Calves. Holstein calves were obtained at birth, artificially reared in isolation, and used between 4 and 12 months of age. IBR-immune calves had been challenged by intranasal instillation of IBR virus (Colorado-1 strain; ATCC VR-864). They developed typical clinical signs of disease and showed a serological response. IBR-susceptible calves were periodically monitored serologically to ensure that their status was unchanged.

† Journal article no. 13 of the Veterinary Infectious Disease Organization.

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Macrophage cultivation and virus infection. AM were obtained by fiberoptic bronchoscopy, as described by Wilkie and Markham (35). Calves were sedated with 100 to 140 mg of xylazine (Rompun; Cutter Laboratories, Mississauga, Ont., Canada) intravenously and placed in left lateral recumbency. The endoscope was passed via the trachea into the right diaphragmatic lobe. Normal saline (0.15 M NaCl) containing 5 mM glucose, 2 mM EDTA, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 50 μg of gentamicin per ml, and 5 μg of amphotericin B (pH 7.2) per ml was used for lung lavage. An initial wash was made by passing 50 ml of fluid into the lung followed by retrieving approximately 25 ml by mild vacuum. The initial wash was discarded. A second wash involved injecting a further 250 ml of fluid into the lung and removal of the fluid as above. The cells present in the second lavage fluid were filtered through four layers of sterile gauze to remove particulate debris and mucus, centrifuged at 1,000 × *g* for 10 min, washed once with Eagle minimal essential medium (MEM; GIBCO Laboratories, Grand Island, N.Y.), and suspended in MEM with 5% fetal bovine serum. The cells were plated at 5×10^4 cells per cm^2 in two-well chamber slides (no. 4802; Lab-Tek Products, Westmont, Ill.) for detection of Fc or complement receptors and for phagocytic assays. They were also placed in 100-cm^2 petri dishes (no. 25020; Corning Glass Works, Corning, N.Y.) at 1.3×10^5 cells per cm^2 for later use in antibody-dependent cell cytotoxicity (ADCC) experiments. Cultures were incubated for 3 h at 37°C in a humidified 5% CO_2 atmosphere, washed twice with MEM to remove nonadherent cells, and reincubated in MEM with 20% fetal bovine serum until used for the assays. These cells were >99% macrophages, as determined by morphology, nonspecific esterase activity, and phagocytosis of latex particles. Mammary gland macrophages were obtained as described previously (30) by eliciting with bacterial lipopolysaccharide (O128, B12; Difco Laboratories, Detroit, Mich.). They were plated in chamber slides as described above.

For infection, monolayers were again washed twice with MEM to remove serum and inoculated with IBR virus at a multiplicity of infection of 5 to 10. After 1 h of adsorption at 37°C, the monolayers were washed again to remove unadsorbed virus and reincubated in MEM with 20% fetal bovine serum until used for assays.

ADCC. Details of the ADCC assay have been described previously (29). Briefly, Georgia bovine kidney cell monolayers were infected at a multiplicity of infection of 1 and labeled with $Na_2{}^{51}CrO_4$ (New England Nuclear Corp., Lachine, P.Q., Canada). Sixteen hours later, the labeled infected cells were removed by mild trypsinization, enumerated, and used as target cells in the assays. Effector cells were cultured uninfected AM, infected AM at various times postinfection (p.i.), or mammary gland macrophages. All assays were performed in microtiter plates at an effector/target cell ratio of 25:1 and a final concentration of anti-IBR antibody of 1/50. The assays were carried out for 6 to 10 h at 37°C in a humidified 5% CO_2 incubator. At the end of an assay 50% of the supernatant fluids were collected for radioactivity measurements (counts per minute), and the percent specific release was computed by the formula: specific release = [(counts per minute, test − counts per minute, control)/(total releasable counts per minute − counts per minute, control)] × 100. Total releasable counts per minute was taken as the amount released from targets in the presence of 3% Triton X-100. The controls contained either antibody-sensitized target cells in MEM or nonsensitized target cells plus AM. In all cases the controls released nonspecifically approximately 10% of the radioactivity.

Detection of Fc receptor activity. Uninfected or IBR-infected AM (at different times p.i.) were assayed for the presence of Fc receptors and erythrocyte phagocytosis by the method of Hearst et al. (6). Sheep erythrocytes (SRBC) were washed three times in complement fixation buffer (Oxoid Ltd., London, England) containing 0.1% (wt/vol) gelatin. A 5% suspension of SRBC was incubated with a 1/200 dilution of 7S (immunoglobulin G [IgG]) rabbit anti-SRBC serum (lot no. 80759; Cordis Laboratories, Miami, Fla.) for 30 min at 37°C and 18 h at 4°C. This was the maximum subhemagglutinating concentration of the serum. Treated erythrocytes were washed twice in complement fixation buffer and suspended to a 0.5% suspension in MEM. These cells (EA) were then used to detect Fc receptors or phagocytosis by AM. Duplicate chambers (Lab-Tek) containing either uninfected or infected AM were washed twice with MEM to remove serum and incubated with 0.8 ml of the 0.5% EA suspension for 1 h at 22°C for detection of Fc receptors or 37°C for measurement of phagocytosis. To differentiate between phagocytosis and Fc receptor adherence, nonphagocytized EA were lysed by incubation with a 0.83% solution of NH_4Cl for 5 min at 37°C. Monolayers were washed three times in 0.15 M NaCl with 0.1% gelatin, pH 7.2, rapidly air dried, fixed in methanol, and stained with Wright-Giemsa. A minimum of 200 AM was examined in each assay. AM were considered EA positive if three or more erythrocytes were attached. For positive phagocytosis, a minimum of one erythrocyte had to be internalized.

Detection of complement receptor activity. Complement (C3b) receptors were detected by the formation of rosettes with antibody–complement-sensitized SRBC (EAC) (6). For this purpose SRBC prepared as above were incubated for 30 min at 37°C with a 1/40 dilution of 19S (IgM) rabbit anti-SRBC serum (lot no. 14030; Cordis Laboratories) and then for 15 min at 37°C with a 1/10 dilution of C5-deficient mouse serum.

TABLE 1. Effect of pretreatment with trypsin on EA adherence, EAC adherence, and *Candida* phagocytosis by AM

Assay	Treatment	% AM positive[a]
EA adherence	None	98.8
	Trypsin[b]	89.1
EAC adherence	None	34.2
	Trypsin	0
Candida phagocytosis	None	36.4
	Trypsin	5.9

[a] A minimum of 200 cells was examined to compute percentages.

[b] Macrophages were pretreated for 20 min at 37°C with 0.5 mg of trypsin per ml immediately before the assay.

TABLE 2. Effect of IBR virus infection on immune adherence and phagocytosis by AM

Time after infection (h)	% Positive by given assay			
	EA adherence	EAC adherence	EA phagocytosis	*Candida* phagocytosis
Uninfected	99.0	19.4	62.4	77.9
3	99.5	46.0	51.6	83.8
6	98.5	51.2	32.2	82.5
12	89.7	30.6	31.7	60.7
24	40.4	15.2	10.0	36.6

After treatment, cells were washed twice with complement fixation buffer and made up to a 0.33% suspension in MEM. The presence of C3b on the EAC preparations was confirmed by the method of Ross and Polley (20) by demonstrating rosetting with human erythrocytes. Complement receptors were determined by incubating EAC for 30 min at 37°C with AM cultures. Unadsorbed EAC were removed by washing, and EAC-positive cells were enumerated as described above for Fc receptor activity.

Microbial phagocytosis by AM. Microbial phagocytosis was measured with *Candida* cells opsonized as described by Roth et al. (21). *Candida parapsilosis* was grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) for 96 h at 33°C. The organisms were washed three times with Dulbecco phosphate-buffered saline, pH 7.2, suspended to a concentration of 10^8/ml, and incubated with 10% bovine serum for 30 min at 37°C. They were washed twice with 10 mM EDTA in 0.15 M NaCl and resuspended in phosphate-buffered saline. Monolayers were incubated with 6.4×10^5 organisms in 0.8 ml of MEM with 25% fetal bovine serum. After incubation for 30 min at 37°C, phagocytosis was determined as described above. Macrophages which had phagocytosed one or more organisms were scored as positive. Trypan blue staining of monolayers inoculated with heat-killed opsonized *Candida* indicated that, under the assay conditions, all organisms associated with the macrophages were internalized, since they remained unstained.

To determine the receptors involved in mediating *Candida* adherence and phagocytosis, AM were preincubated with 0.5 mg of trypsin (no. T1055; Sigma Chemical Co., St. Louis, Mo.) per ml for 20 min at 37°C and washed twice with 1 mg of ovomucoid inhibitor (no. T2011; Sigma) per ml to terminate trypsin activity. Monolayers were then assayed for phagocytosis of opsonized *Candida* and for adherence with EA and EAC to monitor the effect of trypsin treatment on Fc and C3b receptors.

RESULTS

Receptor activity in AM. Immune adherence and phagocytic assays were carried out in 10 separate experiments with uninfected AM from normal animals. The percentage of AM positive for Fc receptor activity as measured by EA adherence was 94.4 ± 5.7 (mean ± standard deviation). In contrast, only 39.0 ± 22.2 were positive for C3b receptors using EAC, 57.6 ± 10.7 for EA phagocytosis, and 68.1 ± 15.8 for *Candida* phagocytosis. In assays carried out with untreated SRBC, IgM-coated SRBC, and unopsonized *Candida*, there was no adherence or phagocytosis observed. Pretreatment of macrophage monolayers with trypsin reduced EA adherence slightly and eliminated C3b receptor activity, as detected by EAC adherence. *Candida* phagocytosis was almost completely abolished (Table 1), suggesting that the phagocytosis of opsonized *Candida* was mediated mainly through C3b receptor activity.

In an attempt to determine what effect IBR infection had on AM function, we infected macrophages and at various times p.i. assayed them for alterations in activity. Table 2 illustrates that Fc receptor binding did not change for the first 6 h p.i., gradually declined by 12 h p.i., and was dramatically reduced by 24 h p.i., a time when macrophages were showing cytopathic effect and detaching from the monolayers and virus was being released (3a). In contrast, infection of macrophages resulted in a substantial increase in the number of C3b receptor-positive cells. Thus, there was a greater than twofold increase in the number of C3b receptor-positive cells 6 h p.i. when compared with uninfected cells. This was repeatable in numerous experiments, and the extremes are illustrated in Table 3, which demonstrates that regardless of the percentage of C3b-positive cells before infection these values increased by 6 h p.i. Since there was no adherence of uncoated SRBC or IgM-coated erythrocytes, we assumed that EAC adherence was specific and mediated through the C3b receptor.

Analysis of the ability of IBR virus-infected cells to phagocytose either erythrocytes or

TABLE 3. Complement receptor activity in different animals after infection with IBR virus

Animal no.	% EAC-positive cells at time p.i.:	
	0 h	6 h
1	19.4	51.2
2	10.2	49.8
3	3.6	17.5
4	52.4	75.0

TABLE 4. Effect of IBR virus infection on functional capacity of macrophages from IBR-immune and nonimmune calves

Macrophage source	Treatment	% Positive by given assay			
		EA adherence	EAC adherence	EA phagocytosis	*Candida* phagocytosis
Immune	Control	98.5	6.6	32.2	41.7
	IBR[a]	66.8	12.5	17.9	26.7
Nonimmune	Control	89.9	11.6	42.6	37.0
	IBR	71.6	33.6	18.0	33.1

[a] Infected 12 h before assay with IBR virus at a multiplicity of infection of 10.

microorganisms indicated that, even though Fc receptor activity was not decreased significantly 6 h p.i., phagocytosis of EA was reduced by approximately 50%, whereas *Candida* could still be phagocytosed at the same efficiency as uninfected cells. However, by 24 h p.i. all activity was dramatically decreased.

In an attempt to determine whether the donor animals' immune status influences the ability of virus to alter macrophage function, macrophages were obtained from IBR-immune and nonimmune animals. Cultured macrophages from both groups were equally susceptible to infection (3a). At 12 h after IBR infection, they responded functionally in an identical manner (Table 4).

Activity of AM in ADCC. AM may potentially be involved in elimination of virus infections by a variety of mechanisms, including ADCC. It was of interest to first determine whether AM could participate in ADCC and, second, whether infection of AM with IBR virus could reduce their effectiveness in such activities. We had previously shown that bovine mammary macrophages were extremely effective in ADCC (23). Thus, we felt that a comparison of the activity of AM with mammary gland macrophages might reflect the relative effectiveness of AM in antiviral defense. Figure 1 illustrates that AM are capable of killing virus-infected cells as efficiently as are mammary gland macrophages with respect to kinetics of killing (Fig. 1A) and antibody levels required for sensitization (Fig. 1B). However, IBR virus infection of macrophages resulted in a total elimination of ADCC by 4 to 6 h p.i. (Fig. 2), a time when >99% of the macrophages were still viable as determined by adhesion to glass and trypan blue exclusion. Even

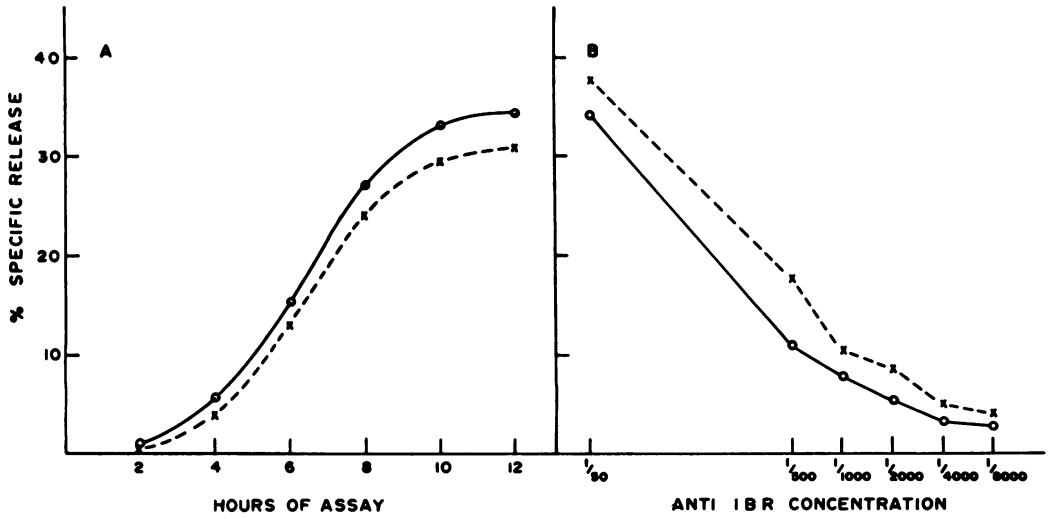

FIG. 1. ADCC of IBR virus-infected cells by bovine AM (○) and mammary macrophages (×). (A) IBR virus-infected cells were incubated for various times with a 1/50 dilution of anti-IBR serum at an effector/target cell ratio of 25:1. (B) IBR virus-infected cultures were incubated for 10 h with various concentrations of anti-IBR serum at an effector/target cell ratio of 25:1. Background release values were 9.8 and 10.2% for macrophages and targets without serum and 9.5 and 9.9% for targets and serum without macrophages in (A) and (B), respectively. Nonspecific release by targets without serum or macrophages was 9.7% in (A) and 10.2% in (B). These values were subtracted before data were plotted.

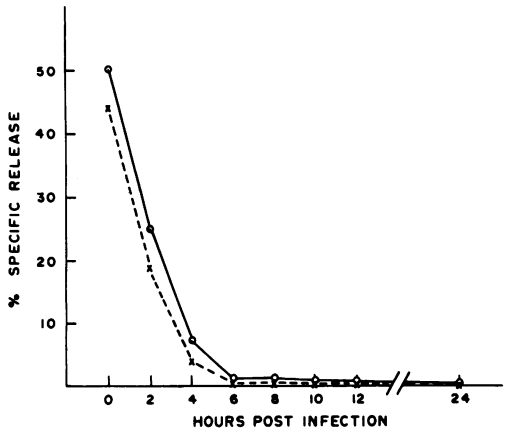

FIG. 2. Effect of IBR virus infection of macrophages on their ability to participate in ADCC. AM (○) or mammary macrophages (×) were infected at various times and then tested for ability to kill IBR virus-infected, ^{51}Cr-labeled target cells in a 10-h assay. The effector/target cell ratio was 25:1. Background release was 8.7% for targets in the absence of macrophages or serum. Release values in the presence of macrophages or serum individually were 8.5 and 8.6%, respectively.

though a large proportion of infected macrophages still possessed Fc receptors and could form rosettes with EA as late as 24 h p.i. (Fig. 3), they could not perform the subsequent events required for target cell lysis after 4 to 6 h p.i., indicating that a substantial alteration in macrophage function occurred even in the early stages of IBR virus infection before actual death of the macrophages occurred. Thus, even at 12 h p.i., in excess of 60% of the macrophages were considered viable by adhesion to glass and trypan blue exclusion but were unable to participate in ADCC even at 6 h p.i.

DISCUSSION

The characterization of surface markers on various leukocyte populations has received extensive investigation, since this information has been useful in aiding the separation of different cell populations as well as in helping to understand the functional capacity of each individual cell population. The present study was directed at determining the surface markers present on bovine AM obtained by lung lavage and the effect of IBR virus infection on receptor activity. In our studies, the adherent cells obtained by lung lavage were almost 100% AM, as determined by morphology, nonspecific esterase activity, and phagocytosis of latex particles (unpublished data). Over 90% of these cells also possessed Fc receptors (Tables 1, 2, and 4). There was no non-immunological binding of erythrocytes and no binding of IgM-coated erythrocytes, consistent with findings of others (3, 7, 18). However, Warr and Jakab (31) and Hearst et al. (6) found that a high proportion (45 to 80%) of mouse AM form rosettes with nonopsonized *C. krusei*. The explanation for this discrepancy requires further investigation. It could, however, be related to nonspecific opsonizing factors in the mouse lavage fluids, since only a very low percentage of mouse peritoneal macrophages formed non-immunological rosettes.

In contrast to Fc receptors, only a small proportion of AM also possessed C3b receptors, the proportion being highly variable between samples, although somewhat less variable between samples from individual animals on different days (data not shown). Initially, we were concerned that our C3b coating of erythrocytes might be inefficient or variable, resulting in variable and inefficient detection of C3b receptors. However, in all assays a large proportion of complement receptor-positive cells had more than 10 erythrocytes bound, suggesting that cells with C3b receptors avidly bound sensitized erythrocytes. Thus, the percentage of C3b receptor-positive cells determined in the assays was probably an accurate estimate of the proportion of AM with C3b receptors at the specific time tested. Therefore, we feel that the resident AM population is in a dynamic state, varying from day to day and animal to animal, possibly as a result of external stimuli which may alter the degree of activation or differentiation at any one time and be reflected in changes in complement receptor activity (6).

It is of interest to note that there appears to be some species variation in the presence of complement receptors of AM. Thus, approximately 80% of resident human AM and 70% of rabbit AM have complement receptors (18), whereas mouse AM are almost devoid of C3b receptors.

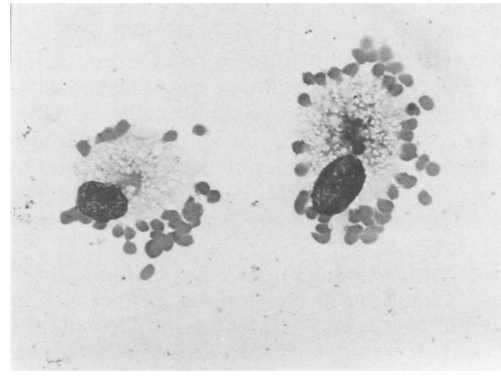

FIG. 3. AM 24 h after infection with IBR virus. Although cytopathology is apparent (vacuolation of cytoplasm and clumping of chromatin), IgG-coated SRBC are adhered, demonstrating that Fc receptor activity is still present.

However, the proportion of C3b receptor-positive murine AM can increase to 50% after viral infection of the lung (32). Since viral infection of the lung can induce a recruitment of macrophages into the alveolar spaces (19), this may again support the proposal that variation in C3b receptor activity reflects the dynamic state of the AM population.

Support for the suggestion that the C3b receptor may play a major role in phagocytosis by bovine AM was obtained from experiments wherein AM were treated with trypsin before assays were performed. As has been shown by Bianco et al. (1), such treatment eliminated C3b receptor activity and almost abolished opsonized *Candida* phagocytosis. Our conclusion that *Candida* phagocytosis was mediated by complement receptor activity is consistent with the findings of other workers (15, 16).

Since our ultimate goal is to determine the mechanism whereby infection with IBR virus increases the susceptibility of the bovine to subsequent bacterial pneumonia (11), we assessed the effect of IBR virus infection of AM on Fc and complement receptor activity, on phagocytosis, and on ADCC activity. Although morphological alteration of AM monolayers was not evident until 8 h p.i., there was a dramatic decrease in the ability of infected AM to participate in ADCC of virus-infected cells even at 2 h p.i. (Fig. 2). In contrast to ADCC, there was no significant decrease in Fc receptor binding of EA or attachment of macrophages to sensitized targets for approximately 12 h p.i. These results suggest that for ADCC to occur there are requirements other than just Fc receptor binding. This has been demonstrated earlier by the observation that, even though bovine peripheral blood lymphocytes do possess Fc receptors, they cannot kill virus-infected cells but can kill chicken erythrocytes (23).

Most herpesviruses induce Fc receptors on the surface of infected cells (34). We have not been able to demonstrate Fc receptor induction by IBR virus on any cell type (unpublished data) and our present results confirm this, since infection of AM resulted in an abrogation of these receptors. We are presently using this model to study turnover of AM Fc receptors and the molecular events which lead to Fc receptor removal.

In contrast to reduction in Fc receptors, there was a significant increase in the number of C3b receptors between 3 and 12 h p.i., followed by a marked decrease by 24 h p.i. Whether these results indicate an actual synthesis of complement receptors or unmasking of receptors after infection with IBR virus requires further investigation. We are intrigued by this observation, since we have previously shown that complement does enhance ADCC (22). Furthermore, it has been shown that herpesvirus and infected cells may directly activate complement (14) and that such infected cells may be killed by complement alone or by a complement-dependent cell cytotoxic mechanism (4, 5). For this to occur it is proposed that some viral antigen is expressed on the surface of herpesvirus-infected cells which can bind complement. Thus, the increase in C3b receptors on AM after infection may reflect actual synthesis of complement receptors rather than unmasking of preexisting receptor sites. Experiments are presently in progress to isolate this complement receptor and determine whether it is viral or cellular.

The present observation that IBR virus can infect, and thereby alter, the functional capacity of bovine AM suggests that infection of AM may be one of the reasons for increased susceptibility of IBR virus-infected animals to secondary bacterial infections (2, 13). Thus, if the AM could not phagocytize and clear the bacteria from the lung, they could get established and cause pneumonia (31). However, for viral infection of AM to be a direct cause of reduced bacterial defense in the lung, a substantial proportion of macrophages would have to be compromised at any one time. The possibility and extent of macrophage infection in vivo is currently being investigated.

ACKNOWLEDGMENTS

We thank J. M. Heise and M. L. Buckley for valuable technical assistance.

This work was supported in part by grants from the Saskatchewan Horned Cattle Trust Fund, Farming for the Future, and the Medical Research Council of Canada.

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