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Alteration of Pulmonary Macrophage Function by Respiratory Syncytial Virus Infection In Vitro

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Alveolar macrophages (AL) are the first line of defense against inhaled pathogens and are exposed to virus during the course of a respiratory syncytial virus (RSV) infection. Interference of virus with alveolar macrophage functions may contribute to the risk of aquiring secondary bacterial infections during or after respiratory tract infections with RSV or other viral agents. We studied whether murine AL get infected with RSV and whether they support viral replication in vitro. In addition, the effects of RSV on microbicidal and on immunoregulatory functions were examined. Only a subpopulation of AL expressed viral F proteins after exposure of these cells to RSV. Infected AL released only small amounts of infectious virus into the supernatant. The extent of virus replication in AL seemed to be dependent in part on the amount of IFN induced by the virus, as has been demonstrated by infection of lung tissue macrophages and AL in vitro. In general, RSV infection of pulmonary macrophages appeared to be abortive. Nevertheless, release of reactive oxygen intermediates, phagocytosis, and killing of protozoa were reduced in RSV-infected AL in comparison to noninfected AL. In contrast, RSV stimulated secretion of TNF-α, IL-1, and IL-6 in an infectious-dose dependent manner. Along with the increased cytokine release, accessory functions of AL were increased after RSV exposure. Thus, exposure of AL to RSV appeared to stimulate their immunoregulatory functions, whereas the microbicidal activity of these cells seemed to be severely diminished. *The Journal of Immunology,* 1995, 154: 268–280.

Respiratory syncytial virus (RSV)[2] is the major cause of acute lower respiratory tract illness in infants and can cause severe, even fatal, infections in the elderly (1–3). RSV demonstrates peculiar tropism for the lower respiratory tract, where it induces inflammation of the airway epithelium accompanied by peribronchiolar infiltrations of leukocytes. In contrast to the proteins of influenza virus, RSV proteins are highly conserved (4). Nevertheless, reinfection with RSV is quite common (5), but the mechanisms leading to repeated RSV infection are poorly understood. Attempts to correlate protective immunity with levels of RSV Abs in serum or lung lavage fluid have yielded conflicting results (6–8). Some investigations showed clear evidence for the importance of a specific T cell response in resolution of RSV infections (9, 10). However, it has also been suggested that the specific response itself is responsible for the pathogenicity of severe RSV infections in the lung (11).

The development of animal models, especially the murine model, for RSV infection facilitated more detailed investigations of the mechanisms involved in the pathogenesis of this infection, including with respect to reinfections (11–13). Most studies dealt with specific immune responses against RSV; little is known about the influence of RSV infections on mononuclear phagocytes. Based on in vitro experiments with monocytes, it has been postulated that reinfections would be in part caused by inhibitory effects of RSV on Ag-presenting cell function (14, 15). Because during the natural course of RSV infection alveolar macrophages (AL), rather than monocytes, are exposed to RSV, we raised the question of whether these inhibitory effects are also induced in AL infected with RSV in vitro. In

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[2] Abbreviations used in this paper: RSV, respiratory syncytial virus; AL, alveolar macrophages; INT, interstitial lung macrophages; MOI, multiplicity of infection; PFU, plaque forming units; p.i., postinfection; ROI, reactive oxygen intermediates; NTE, NaCl-Tris-EDTA-buffer.

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the human system it has been shown that RSV is able to infect AL either in vitro (16) or in vivo (17).

RSV infections are often accompanied by secondary bacterial infections leading to a more severe course of the disease (18, 19), which might be the result of different mechanisms: on the one hand, injury of lung tissue during RSV infection could facilitate the adherence and expansion of bacteria within the lung, on the other hand, there might be a viral-dependent inhibition of the nonspecific defense function. Because the alveolar macrophage is thought to play an important role in defending the lower respiratory tract against pathogens, it is of interest to know whether RSV infection influences the microbicidal functions of these phagocytes. There are a few studies on AL infected with bovine RSV that indicate an inhibitory effect of RSV on phagocytic function (20). Therefore we investigated the influence of RSV exposure on the microbicidal function of AL. Furthermore we examined the course of virus-infection of interstitial lung macrophages in vitro. This macrophage population is located within the lung tissue and might also get in contact with RSV during the course of RSV infection.

Materials and Methods

Mice

Inbred female BALB/c mice at 6 to 8 wk of age were obtained from Charles River Laboratories (Würzburg, Sülzfeld, Germany).

Cytokines and LPS

Human rIL-1, human rIL-6, and murine rTNF-α were obtained from Genzyme Corp. (Cambridge, MA). Murine IFN-γ was kindly provided by Böhringer Ingelheim, Ingelheim, Germany. LPS from *Escherichia coli* strain III:B4 was purchased from Sigma Chemical Co. (Munich, Germany).

Cell lines and microbes

7TD1 hybridoma cells were kindly provided by Dr. Edgar Schmidt (University of Mainz, Germany) and were maintained in tissue culture with RPMI 1640 supplemented with 10% supernatant from Con A-stimulated spleen cells. D10.G4.1, a T helper cell line, was obtained from American Type Culture Collection (Rockville, MD) and was cultivated in Iscove medium supplemented with 5% FCS, 1% L-glutamine (2 mM), 2% HEPES (5 mM), 2% 2-ME (0.5 mM) and 10% Con A-stimulated rat spleen cell supernatant. *Leishmania donovani*, strain LRCLD 51 was kindly provided by Dr. Ebert (Hamburg, Germany). HEp-2 cells and L929 cells were obtained from American Type Culture Collection. RSV (a subgroup of the long strain) has been cultivated in vitro in HEp-2 cells since it was isolated from patient specimens in 1988 at the Medizinische Hochschule in Hannover and has not been mixed with other virus isolates. All media and supplements were obtained from Life Technologies, Europe (Eggenstein-Leopoldhafen, Germany).

Abs

Neutralizing polyclonal Abs against TNF-α and IL-1 were obtained from Genzyme, against IL-6 were purchased from Herbert Biermann (Bad Nauheim, Germany), and against IFNα/β from Lee Biomolecular (San Diego, CA).

Anti-I-A^d FITC-conjugated mAb was obtained from Paesel und Lorei (Frankfurt, Germany).

Isotype-matched control Abs and FITC conjugated anti-rat $F(ab')_2$ fragments were obtained from Dianova (Hamburg, Germany). Nonspecific mouse serum and nonspecific goat serum were obtained from Vector Laboratories (Burlingame, CA).

Unconjugated (30–555L, c65062 M) and FITC-conjugated (RSV Accu-Clone) mouse anti-RSV IgG mAbs were purchased from Whittaker (Walkersville, MD) and from Biodesign (Kennebunkport, ME). Bovine anti-RSV serum was obtained from Wellcome Diagnostic (Dartford, Great Britain) and neutralizing antiserum against RSV (1/100 dilution of goat anti-RSV serum) was from Biodesign.

Isolation of alveolar macrophages

AL were harvested by a modification of a method previously described by Kobzik et al. (21). Briefly, the lungs were flushed with 1 ml of ice-cold Mg_2- and Ca_2-free PBS containing 0.6 mM EDTA (PBS-EDTA). The lavage procedure was repeated ten times. After washing the lavage cells, differential cell counts were done. Lavage fluid from untreated mice contained 98% AL. AL were >96% viable as measured by trypan blue dye exclusion.

Isolation of lung interstitial cells

INT were harvested by a modification of a method previously described by Kobzik et al. (21). Briefly, after lung lavage and perfusion of the vascular bed, the lung tissue was sliced into 0.4 mm pieces and digested in RPMI containing DNase Type I (66 U/ml; Sigma Chemical Co.) and Collagenase (50 U/ml; Worthington Type IV, Copper Biomedical, Malvern, PA) for 60 min at 37°C in a gently shaking water bath. Cell debris was removed by passing through thin layer of premoistened nylon wool. The digested lung cells were washed twice with RPMI and layered on top of a 20% to 50% percoll gradient. After centrifugation at 300 × *g* at 4°C the interphase between the 20% and 50% percoll layer was harvested, washed again, and resuspended in RPMI with 10% FCS. To further enrich the isolated cells for macrophages, cells were seeded in microtiter plates and washed carefully after an incubation period. The remaining cells were >95% macrophages as determined by nonspecific esterase staining and phagocytic activity.

Exposure of cells to RSV

Five × 10^4 AL or HEp-2 cells were placed in 0.1 ml serum-free medium in polypropylene tubes or flat-bottom microtiter plates. For virus infection, cells were exposed for 3 h at 37°C to virus at a multiplicity of infection (MOI) of 0.1 to 5.0 with gentle shaking of the cells during this time period. For all experiments cells were also exposed to UV-inactivated RSV. The cells were washed three times either by centrifugation and resuspension with RPMI + 10% FCS or by removing medium from microtiter plates and replaceing with fresh serum-containing medium. Cells were further incubated in culture medium at 37°C, 5% CO_2 for an additional time period as indicated.

Anti-RSV Ab staining

Cytospots of AL were air-dried, fixed with cold acetone for 10 min at 4°C, and incubated with 5% nonspecific mouse serum for 20 min to block nonspecific binding to Fc receptors. Slides were washed with PBS (containing 2% BSA and 0.2% sodium azide) and incubated either with 40 μl specific Ab solution (FITC-linked anti-RSV Ab) or FITC-linked isotype-matched control Ab at the same dilution for 30 min at room temperature. After washing three times with PBS, staining was evaluated by fluorescence microscopy. AL in fluid culture were stained after preincubation of 2 × 10^5 cells with nonspecific mouse serum as described above and analyzed by flow cytometry (FACScan, Becton Dickinson, Heidelberg, Germany).

If an unlabeled anti-RSV Ab was used, cells were washed three times with buffer after the first Ab-staining step and incubated with 40 μl FITC-conjugated goat anti-rat $F(ab')_2$ in a final dilution of 1/40 for indirect immunofluorescence. After 45 min, cells were washed three times and analyzed on a FACScan (Becton Dickinson, Heidelberg, Germany) for increase in green fluorescence. All staining and washing steps were done at 4°C.

Virus stock preparation with semipurification

The long strain of RSV was grown in HEp-2 cells in tissue culture flasks. Monolayers of HEp-2 cells were infected with RSV. When the cytopathic

effect reached 80%, flasks were shaken with sterilized glass beads. The cell suspension was sonicated, centrifuged at 2000 × *g* and pellets were mixed with polyethylene glycol (50% w/v in NTE buffer (0.15 M NaCl, 0.05 M Tris, and 1.00 mM EDTA, pH. 7.5), Sigma Chemical Co.) to a final dilution of 10% and stirred for 60 min at 4°C. The virus-containing solution was centrifuged at 10,000 × *g* (J-21C Beckman centrifuge; Beckman Instruments, Fullerton, CA) for 15 min; the pellets were resuspended in 0.1 to 0.2 ml 20% sucrose-NTE buffer and were carefully placed on top of 30% sucrose-NTE buffer in a one to one ratio. After centrifugation at 51,000 × *g* for 60 min at 4°C in a J-21C Beckman centrifuge, the pellets were resuspended in a small amount of 20% sucrose-NTE buffer. A (60%–45%–35%) discontinuous sucrose gradient was prepared by using the same volume for each layer, and virus solution was placed on top of it. After centrifugation at 165,000 × *g* in an ultracentrifuge (Beckman centrifuge L5.65) for 60 min, the interphase between 35% and 45% was collected, mixed with 1 ml 20% sucrose-NTE buffer and aliquotted. The virus stock aliquots were quick frozen (on dry ice and alcohol) and stored at −70°C. Virus titer was approximatly 1×10^7 PFU/ml when assayed on HEp-2 cells. Each step was done at 4°C.

Methylcellulose plaque assay

Infected cells were disrupted by sonification at 4°C before virus determination. Virus titer was measured by incubation of serial dilutions of virus stock or sonicated AL on HEp-2 cell monolayers. After a 3-h incubation at 37°C, 5% CO_2, the supernatant was removed. The monolayer was overlayed with 2% methylcellulose (w/v in MEM + 2% FCS) and incubated for 5 days at 37°C, 5% CO_2. After fixation with 10% formalin and crystal violet staining, PFU were counted using an inverse microscope.

Infection center assay

One × 10^4 test cells were seeded in small petri dishes (ϕ = 35 mm). For infection, medium containing RSV was added in a total volume of 0.5 ml and washed several times after 3 h of incubation. After the indicated time period p.i., cells were overlaid with 1×10^6 HEp-2 cells and incubated for 5 h. Supernatants were removed and 3 ml of 2% methylcellulose (w/v in MEM + 2% FCS) was added to each petri dish. After incubation for 4 to 6 days, the cells were fixed with 4% formalin and plaques were counted using light microscopy.

Phagocytosis assay

Saccharomyces cerevisiae (a kind gift of Dr. Auling, University of Hannover, Germany) were heat killed and labeled with FITC (Sigma Chemical Co.). Macrophages were seeded in polypropylene tubes, mixed at a 1:10 ratio with labeled *S. cerevisiae*, and incubated at 37°C, 5% CO_2 for the indicated time period. An increase in green fluorescence resulting from phagocytosis was measured by flow cytometry. As a control for nonspecific binding, tubes containing macrophages and *S. cerevisiae* were incubated on ice. Furthermore, nonphagocytic P815 cells together with labeled yeast particles were incubated at 37°C, 5% CO_2.

IL-1 assay

IL-1 activity was determined as the ability to stimulate proliferation of IL-1-dependent D10.G.4.1 cells (22). One unit of IL-1 is defined as the amount of IL-1 required to stimulate 50% maximal proliferation. As a positive control, human rIL-1 was used. Preincubation of macrophage supernatants with neutralizing antiserum against murine IL-1 completely abolished IL-1 activity in macrophage supernatants.

IL-6 assay

IL-6 levels in supernatants of macrophage cultures were determined by a 3-(4,5-dimethylthiazol-2 yl)-2,5 diphenyltetrazolium bromide thiazolylblan reduction assay using the IL-6-dependent B cell hybridoma 7TD1 (23). One unit of IL-6 is defined as the amount of IL-6 required to stimulate 50% maximal proliferation. Human rIL-6 served as a positive control. Preincubation of macrophage supernatants with neutralizing antiserum against murine IL-6 completely inhibited their IL-6 activity.

TNF-α assay

TNF-α activities of macrophage supernatants were measured in a biologic assay on actinomycin D-treated (Sigma Chemical Co.) L929 cells as described by Ruff and Gifford (24). The units given are the reciprocal values of the supernatant dilution that would cause lysis of 50% of an L929 cell monolayer. As a positive control for the cytopathic effect, rTNF-α was used. No activity was detectable after preincubation of supernatants with neutralizing Abs against murine TNF-α.

Interferon assay

IFN content of macrophage supernatants was measured using a cytopathic assay as described by Lüttig et al. (25). In brief, serial dilutions of supernatants were added to L929 cell monolayers; after a 24-h incubation, the plates were washed, and fresh media containing appropriate concentrations of vesicular stomatitis virus were added; following a 24-h incubation, L929 cells were evaluated microscopically for cytopathic effects. IFN units are defined as the reciprocal values of the supernatant dilution that would prevent virus induced lysis in 50% of the L929 cell monolayer. Murine rIFN-γ was used as a positive control. Protection was completely inhibited by preincubation of supernatant with neutralizing Abs against IFN-α/β.

PGE_2-ELISA

PGE_2 levels in supernatants of macrophage cultures were determined using a commercially available ELISA (IBL, München, Germany). The competitive ELISA was performed according to instructions.

Measurement of lucigenine-dependent chemiluminescence

The chemiluminescence assay, previously described by Lüttig (25), is outlines here: 1×10^5 freshly isolated macrophages per vial were cultured in 10 mM HEPES-buffered RPMI 1640 supplemented with 10% FCS and incubated for 24 h. The adherent macrophages were washed gently and fresh medium was placed into the vials. Shortly before running the assay, 0.01 ml of lucigenine (Sigma Chemical Co.) at a concentration of 12 μM was added. When background activity as monitored in a six-channel Berthold Biolumat (Berthold, Wildbad, Germany) reached constant values, 10 μl of zymosan (Sigma Chemical Co.) was added. Generation of reactive oxygen intermediates resulting in chemiluminescence was measured for a period of 30 min. Software for computerized calculation of peak activities and intergrals was supplied by Berthold.

Leishmanicidal assay for cytotoxicity against microorganisms

This method has been described previously by Hockertz et al. (26). [^{3}H]thymidine-labeled (70 μCi, 25°C, 24 h) *Leishmania donovani* promastigotes were added to macrophages with an effector to target ratio of 1:5. After an incubation period of 18 h at 37°C, 5% CO_2, the plates were centrifuged and half of the supernatants were collected for reading the radioactivity in a β-scintillation counter (Beckman LS 7500, Beckmann Instruments). The percentage of specific lysis was calculated according to the formula:

$$\%\ \text{spec. lysis} = \frac{\text{cpm}_{\text{experimental}} - \text{cpm}_{\text{spontaneous}}}{\text{cpm}_{\text{total}}} \times 100$$

The total release of [^{3}H]thymidine was determined by lysis of labeled *L. donovani* promastigotes using 0.01% SDS. The spontaneous release is the amount of radioactivity released by labeled *Leishmania* during the incubation period without addition of macrophages.

Assessment of accessory cell function

Determination of accessory function of macrophages was performed according to a modification of the method described by Kaltreider et al. (27). In brief, nonadherent spleen cells were prepared by nylon wool

column as described by Baccarini et al. (28). Recovery averaged 40% of applied cells. Remaining mononuclear phagocytes were <1% as determined by phagocytosis assay and nonspecific esterase staining. These fractionated spleen cells were seeded at 2×10^5 cells per well in microtiter plates, macrophages were added in different proportions ($0-8 \times 10^3$ cells/well), and lymphocyte proliferation was stimulated by 2 μg/ml Con A. In previous experiments using 0.1–6 μg/ml Con A, 2 μg/ml Con A was shown to be an optimal concentration for lymphocyte proliferation in this assay system. After a 72-h incubation, [^{3}H]thymidine was added and blastogenesis was determined by the incorporation of [^{3}H]thymidine into replicating cells. After cell labeling for 6 h, the cells were harvested by suction filtration (Skatron, Liev, Norway) and the radioactivity bound to fiberglass filter paper was determined by β-scintillation counting (Beckman LS 7500, Beckman Instruments). Background radioactivity obtained from spleen cells cultured without Con A in the presence or absence of macrophages was substracted from each probe. The Con A-induced proliferation was determined according to the formula:

$$\% \text{ control cpm} = \frac{\text{cpm fract. spleen cells + macrophages}}{\text{cpm unfract. spleen cells}} \times 100$$

Unfractionated spleen cells still containing spleen macrophages were used for 100% accessory function.

Statistical analysis

Each experiment has been done at least in triplicate. SEMs were calculated for in vitro assays and differences were analyzed by means of Student's *t*-test.

Results

In vitro infection of mouse pulmonary macrophages

Cultures of AL and INT were exposed to RSV for 3 h, washed, and incubated for 24 to 96 h. During this time period no significant decrease in cell viability was observed at a multiplicity of infection (MOI) from 0.1 up to 1.0. Determination of infection was done by specific Ab staining, by measuring the PFU per cell, or by evaluation of the infectious virus released from infected macrophages into the medium detected by infection center assay.

To determine the permissiveness of pulmonary macrophages, equal numbers of AL and INT, and HEp-2 cells as positive controls, were exposed to RSV at a MOI of 0.1 to 2.0. The virus content of cell supernatants as well as the virus load within the cells was measured 24 to 96 h later by using the PFU assay. As shown in Figure 1, the infection of pulmonary macrophages did not lead to an increase in virus production during this time period, in contrast to the results for RSV infection of HEp-2 cells. To assess whether the observed PFU could be accounted for merely by RSV detached from the surface of the virus-exposed macrophages, we cultured free virus in buffer without addition of macrophages or HEp-2 cells. After 24 h of culture, infectious virus was no longer detectable by using the PFU assay. Preactivation of pulmonary macrophages with LPS reduced the amount of virus recoverable from the RSV-exposed cells and from cell supernatants.

To determine the PFU actually released from infected macrophages we used an infection center assay. Infectious virus was liberated from both types of pulmonary macrophages, but to a very low extent (Table I). INT not only contained more PFU per cell, but also released more infectious virus in comparision to AL. However, despite an initial RSV secretion the infection of pulmonary macrophages seemed to be nonproductive over a longer period of time. We followed the course of infection up to 14 days p.i. in vitro and were unable to detect any release of infectious virus after 4 days p.i. (data not shown). With increasing MOI we observed an increasing percentage of infected cells detected by specific Ab staining against the RSV fusion protein. However, the maximal portion of infected macrophages never exceeded 35% for both macrophage populations, even when increasing the MOI up to 5 indicating that RSV infection was not simply the result of phagocytosis. RSV Ag-expressing macrophages appeared 24 h p.i. The percentage of initially positive cells never increased during the next 3 days. Activation of macrophages before RSV infection resulted in a decreased percentage of infected cells, whereas mock infection of macrophages with UV-inactivated RSV did not induce anti-RSV antibody binding (data not shown).

Production of type 1 IFN and TNF by virus-infected macrophages

To address the question of whether the observed differences in virus susceptibility between INT and AL, as well as between unstimulated and preactivated macrophages, were attributable to difference in IFN production, we determined the amount of IFN released by these cells. As shown in Figure 2 supernatants of AL exposed to RSV contained substantially more IFN than supernatants of infected INT. Neither INT nor AL produced IFN spontaneously, and preactivation with LPS or mock infection induced only weak IFN production in both cell populations. As early as 4 to 10 h post-RSV exposure, maximal amounts of IFN were secreted. We observed a positive correlation between the degree of IFN released and the MOI used for infection (data not shown).

In contrast to IFN release, secretion of TNF appeared rather late. Whereas during the first 9 h p.i. comparatively low levels of TNF were measured in macrophage supernatants, increasing amounts of TNF were secreted by AL during the following 48 h after viral exposure. Depending on the infective dose of virus, maximal levels of TNF were observed 48 h after infection as shown in Figure 3. Mock infection of AL with UV-inactivated virus did not induce TNF secretion. As observed for type I IFN, the kinetics of secretion clearly differ from those of LPS-activated macrophages, which secrete peak levels of TNF 9 h after addition of LPS and reach values near zero at later time points.

Influence of RSV exposure on macrophage microbicidal activity

Pulmonary macrophages play an important role in maintaining a sterile environment within the lower respiratory

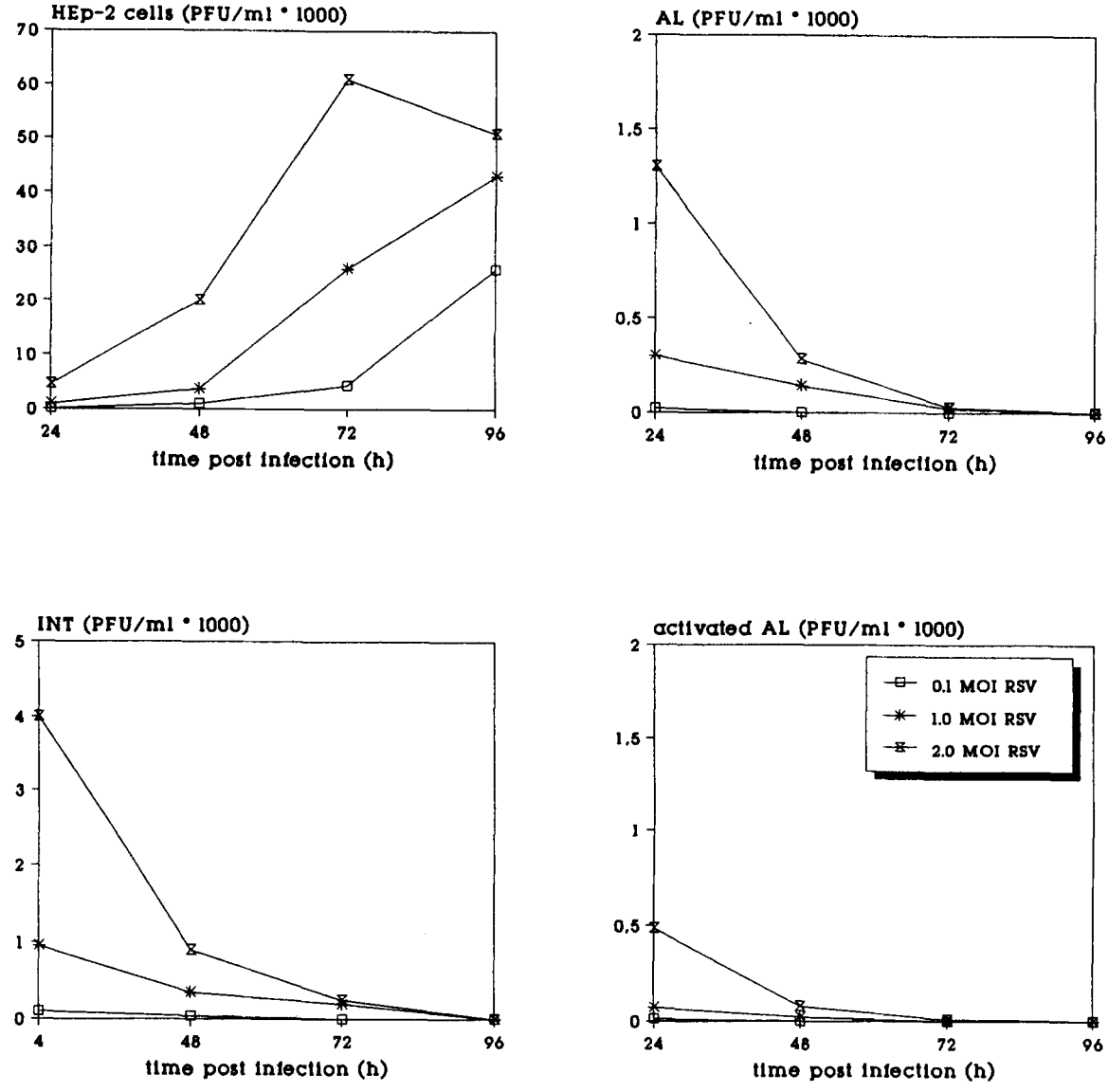

FIGURE 1. 5×10^4 cells were infected with RSV at the MOI indicated. At different time points p.i., cells were harvested and assayed for virus content on HEp monolayers as described in *Materials and Methods*. Some macrophage populations have been activated with LPS (2, 5 μg/ml) 24 h before infection. Results represent the means for five separate experiments. SEMs, usually < 6%, have, for clarity, been omitted.

tract because of their phagocytotic and microbicidal activity. The phagocytotic potential of AL was diminished after exposure to infectious RSV, as shown in Figure 4. The uptake of *Saccharomyces cerevisiae* was reduced for RSV-infected AL, whereas LPS-preactivated AL exhibited a higher phagocytosis rate as measured by flow cytometry. Along with the reduced phagocytotic activity, there was a significant reduction in the release of reactive oxygen intermediates (ROI). With increasing time p.i., the ROI production decreased, reaching maximal inhibition at 72 h p.i. (Fig. 5). At later time points p.i., inhibition of ROI production declined (data not shown). We observed a positive correlation between the amount of virus used for infection and the degree of ROI inhibition. In both assays only infectious virus was able to elicit the observed effects, because the addition of UV-inactivated virus had no influence on these macrophage functions.

To determine whether RSV infection also influences more complex microbicidal macrophage functions, we examined the cytotoxic activity of RSV-infected pulmonary macrophages against the protozoa *Leishmania donovani*. At different time points, RSV-exposed as well as control macrophages were coincubated with [^{3}H]thymidine-prelabeled *L. donovani* and their cytotoxic activity was determined. The results obtained are given in Figure 6. Infection of pulmonary macrophages with RSV resulted in an

Table I. *Release of infectious virus from pulmonary macrophages exposed to RSV at an MOI of 2*[a]

RSV (1×10^5 PFU) added to	PFU Measured	
	48 h p.i.	72 h p.i.
Cell-free medium	0	0
AL (5×10^4)	52 ± 8	50 ± 9
INT (5×10^4)	112 ± 11	108 ± 15

[a] Infection center assay was done 48 and 72 h p.i. of macrophages. Results represent the means ± of five independent experiments.

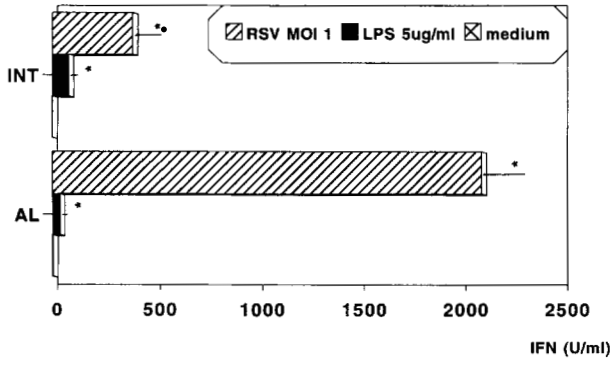

FIGURE 2. IFN release of pulmonary macrophages. 5×10^4 INT or AL were infected with RSV or stimulated with LPS, or as control incubated in medium alone. After 24 h the amount of IFN released into the supernatant was determined. Neutralizing Abs against RSV were added to the supernatants before testing in L929 assay. The results shown here represent the means of five experiments. * $p < 0.01$ control vs RSV or LPS.

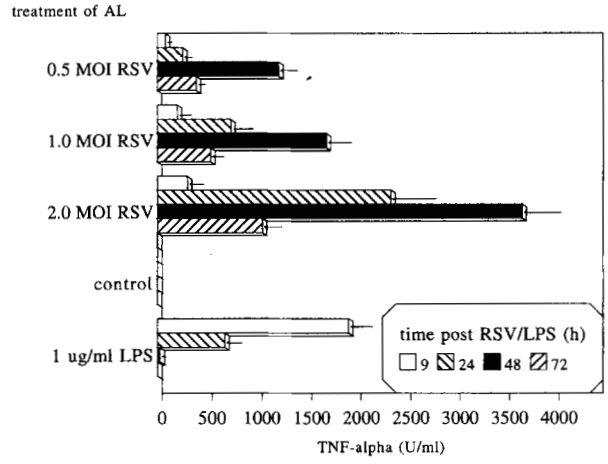

FIGURE 3. TNF secretion of infected or activated AL was evaluated over a period of 48 h. AL incubated in medium alone served as control. Neutralizing Abs against RSV were added to the supernatants before use in TNF assays. The results shown here represent the means of five experiments. Except for RSV (0.5 MOI, 9 h) and LPS (48 h, 72 h), all differences were significant, with $p < 0.05$, control vs RSV or LPS.

infectious-dose-dependent reduction of leishmanicidal activity. As observed for phagocytosis and for ROI production, the maximal inhibition of this macrophage function was observed 48 h after virus exposure. In control experiments, the addition of free infectious virus to leishmania organisms had no influence on the viability of these protozoa, and by using UV-inactivated virus for mock infection we could not detect any restriction of leishmanicidal function. To assess whether the inhibitory effects of RSV on macrophage microbicidal function could be accounted for by a PGE_2 feedback mechanism, macrophage supernatants were analyzed for PGE_2 using an ELISA. RSV induced an infectious-dose dependent release of PGE_2 into the supernatant with increasing amounts during the first 48 to 72 h p.i. (Fig. 7). Induction of PGE_2 secretion could not be observed with mock-infected AL. To verify whether the observed increase in PGE_2 synthesis could be responsible for the reduced microbicidal macrophage function, we added indomethacin (1 mg/ml), an inhibitior of prostaglandin synthesis, to the culture media before infection of macrophages. This treatment reduced the PGE_2 content of macrophage supernatants, even from infected AL, almost to background levels as measured with the PGE_2 ELISA. Inhibition of prostaglandin secretion resulted in a higher ROI production as well as in a slight increase of leishmanicidal activity of all macrophages, either infected or not. However, the difference observed between RSV-infected and noninfected AL remained the same. Therefore these results indicate that the reduction of microbicidal function observed after RSV infection of macrophages is not a result of increased PGE_2 secretion.

Changes in cytokine release and accessory cell function

Besides their important microbicidal activity, macrophages are able to act as accessory cells for the induction of specific immune responses. Several secretory products of macrophages take part in the interaction between macrophages and lymphocytes. Here we examined if the infection of pulmonary macrophages had any effect on release of cytokines essential for the induction of lymphocyte proliferation. Because we used biologic assay systems to measure cytokines secreted from infected macrophages, it was important to verify that infectious virus eventually present in the macrophage supernantants did not alter the cells used for the biologic tests. Therefore, before the assay, we added neutralizing Abs against RSV to all macrophage supernatants. This procedure has been shown to efficiently inhibit infection of virus-permissive HEp-2 cells as tested by preincubation of infectious virus with such Abs before infection of HEp-2 cells. The neutralizing serum itself did not alter the spontaneous or stimulated proliferation of the D10.G.4.1 or 7TD1 cells used for IL-1 and IL-6 assays nor did it lead to a change in cell viability of the L929 cells in the IFN and TNF assay.

Supernatants of AL were measured for their IL-1 and IL-6 content after varying time points p.i. with RSV or

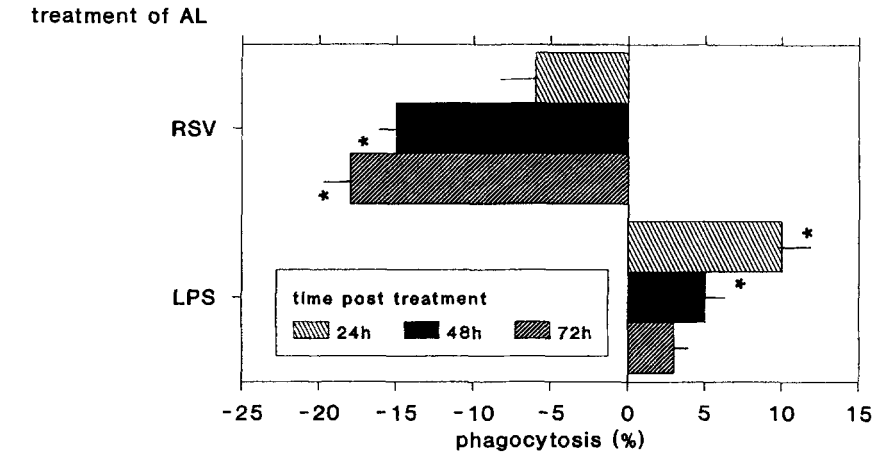

FIGURE 4. Phagocytotic activity of preactivated or virus-exposed AL measured by flow cytometry. Over a period of 30 min the percentage of macrophages that had ingested yeast particles was determined. Noninfected, nonstimulated AL served as control and showed a phagocytotic rate of 35% (± 2.8%). The influence of RSV infection or LPS stimulation is shown as increase above or decrease below the control rate (shown as zero). Data shown are means of four independent experiments. * $p <$ 0.05, control vs RSV or LPS.

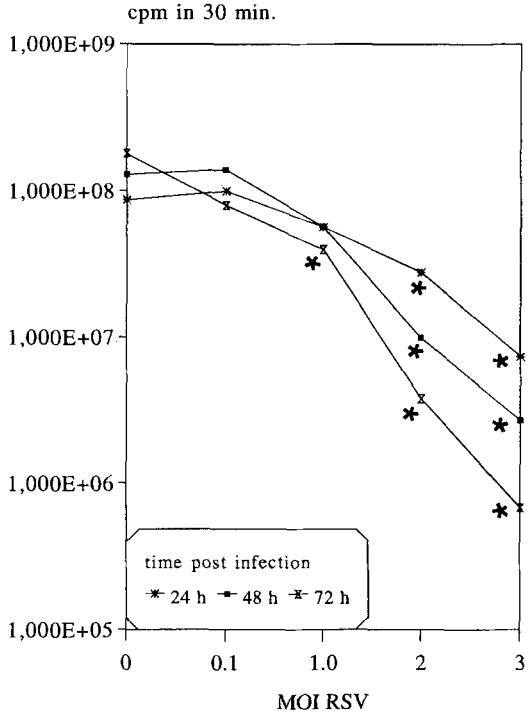

FIGURE 5. AL (5×10^4), either infected or not, were assayed for ROI production after incubation in medium for 24 to 72 h. Results are expressed as means of five separate experiments. SEMs, usually <5%, have, for clarity, been omitted. * $p < 0.05$, control vs RSV.

exposure to LPS. Both LPS and RSV stimulated secretion of IL-1 and IL-6, but with different kinetics. RSV exposure of AL induced a strong and very fast release of IL-6, with maximal amounts appearing as early as 4 to 12 h p.i., whereas LPS stimulation resulted in an enhanced IL-6 secretion reaching higher levels 72 h after LPS addition (Fig. 8). In contrast, LPS stimulation of AL induced the release of IL-1 into the supernatants with maximal levels 24 h later. Depending on the MOI of RSV used for macrophage infection, highest IL-1 levels were obtained between 24 and 48 h p.i. of AL. At higher infectious doses, RSV stimulation of IL-1 synthesis seemed to be comparable to stimulation with LPS (Fig. 9). Because some IL-1-dependent cells are known to be sensitive to increasing amounts of PGE_2, we examined the effect of exogenously added PGE_2 on the proliferation of both cell lines used for IL-1 and IL-6 determination. By adding PGE_2 in concentrations measured in supernatants of infected macrophages, we could not observe any change in proliferative response to cytokine standards (data not shown).

Increased IL-1 and IL-6 production has been associated with better accessory cell function. To prove whether these changes actually resulted in stimulation of lymphocyte proliferation, we incubated spleen lymphocyte preparations depleted of macrophages with AL. Here we used AL 24 h p.i. as well as noninfected AL and measured their stimulatory effect on mitogen-induced lymphocyte proliferation. Exposure of AL to RSV led to a slight increase in accessory cell function when higher percentages of macrophages were added (Fig. 10). Through intensive washing after infection of macrophages and application of neutralizing Abs against RSV to the lymphocyte cultures, we excluded the possibility of a direct infection of lymphocytes, which might interfere with their proliferative behavior.

Discussion

Although RSV seems to fulfill some replication cycles in human AL, the overall virus production is very low (16, 29). Here we observed a similar effect with murine AL and INT, the latter releasing higher amounts of infectious virus. In both populations the infection seemed to be abortive after some replication cycles. Nevertheless, we and others clearly observed expression of viral proteins on the surface of murine, ovine (30), human (31, 32), and bovine macrophages (20), and Panuska at al. (16) demonstrated

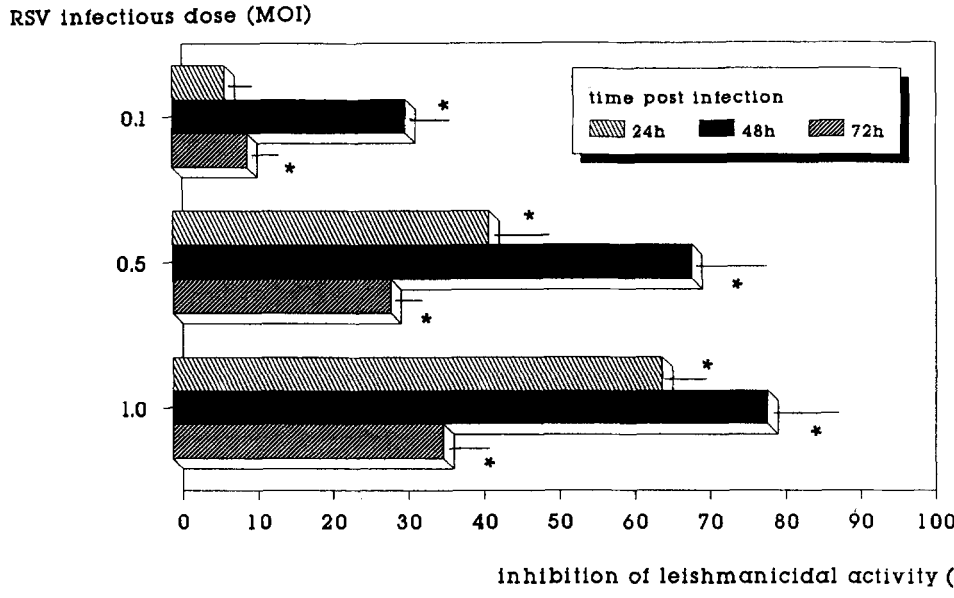

FIGURE 6. Inhibition of leishmanicidal activity of AL was determined at different time points after viral exposure. The influence of RSV infection on cytotoxicity of AL is shown as percent inhibition of the corresponding control cytotoxicity. Leishmanicidal activity of noninfected control AL increased during the culture peroid from 20% (±3), to 38% (±4.3) to 53% (±3.9). Data shown are means of triplicate samples of four independent experiments. * $p < 0.05$, control vs RSV.

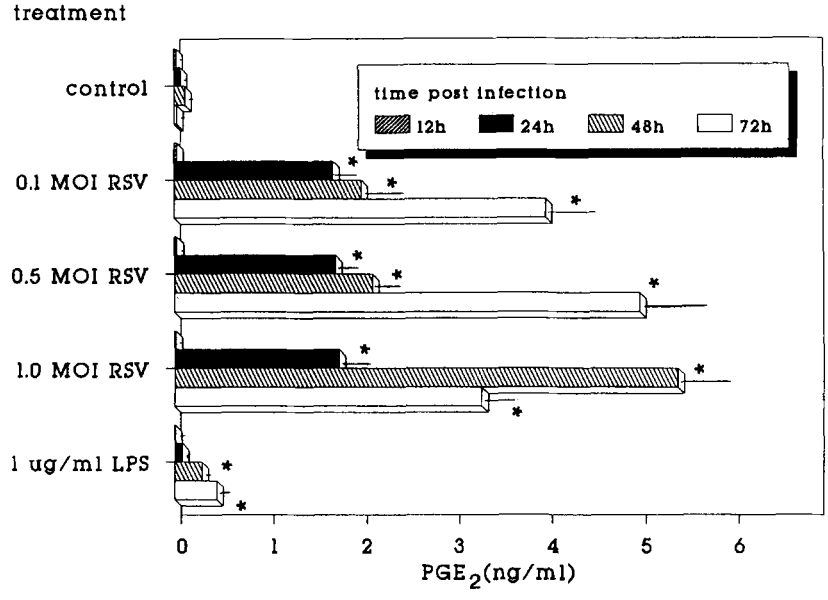

FIGURE 7. Kinetics of PGE_2 release was measured after infection of AL. 5×10^4 AL were either infected with RSV, stimulated with LPS, or incubated in medium alone. At different time points post-treatment, PGE_2 was determined by ELISA. Results represent the means of four similar experiments. * $p < 0.01$, control vs RSV or LPS.

viral mRNA in infected human AL using Northern blotting. In our experiments only a fraction of the lung macrophage population was permissive to RSV infection independent of the MOI used, an observation that has been made in species other than mice (16, 17, 20). Infection of lung macrophages appeared to be restricted to the initially infected macrophages, as indicated by the constant portion of virus-positive cells throughout the time course of in vitro infection. Apparently, RSV infection of AL is determined not only by the dose of infectious virus but also by factors intrinsic to the phagocytes. In general, the resistance of macrophages against viral infections seems to be determined not only by genetic conditions (33, 34) but also by the age of the host and the differentiation stage of the mononuclear phagoyctes used (35, 36). The mechanisms underlying the different rate of virus release between infected AL and INT are unknown, but may depend on the differentiation state of these pulmonary macrophages. INT are thought to represent an intermediate stage of macrophage maturation (37), whereas AL are considered to be an end-stage macrophage population (38). Such a relationship between the differentiation state and the degree of

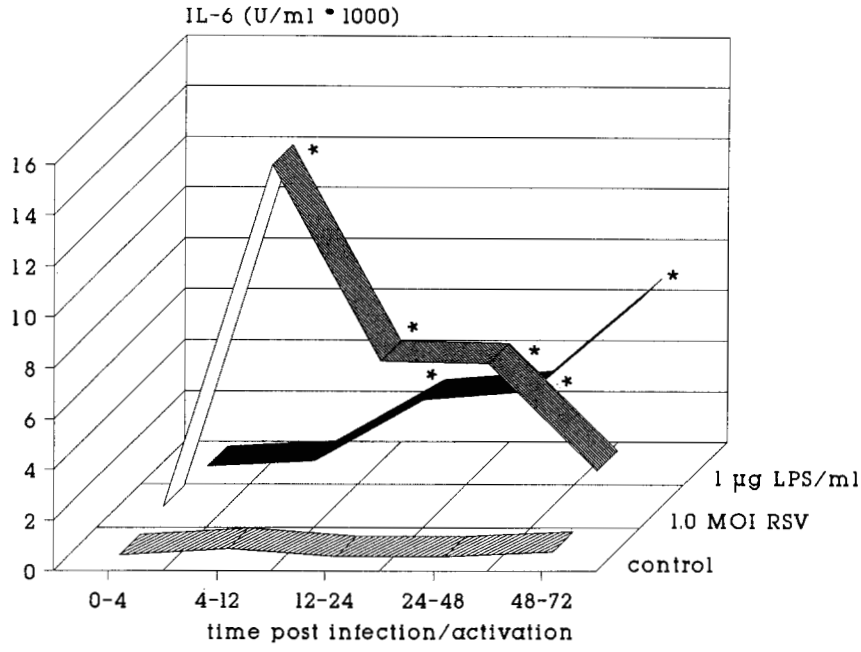

FIGURE 8. IL-6 content determined in supernatants of activated or virus-exposed AL. Supernatants were removed at different time points post-treatment and fresh medium was added. Neutralizing Abs against RSV were added to all supernatants before IL-6 testing. Noninfected AL incubated in medium alone were used as control. Data are expressed as means of five similar experiments. * $p < 0.01$, control vs RSV or LPS.

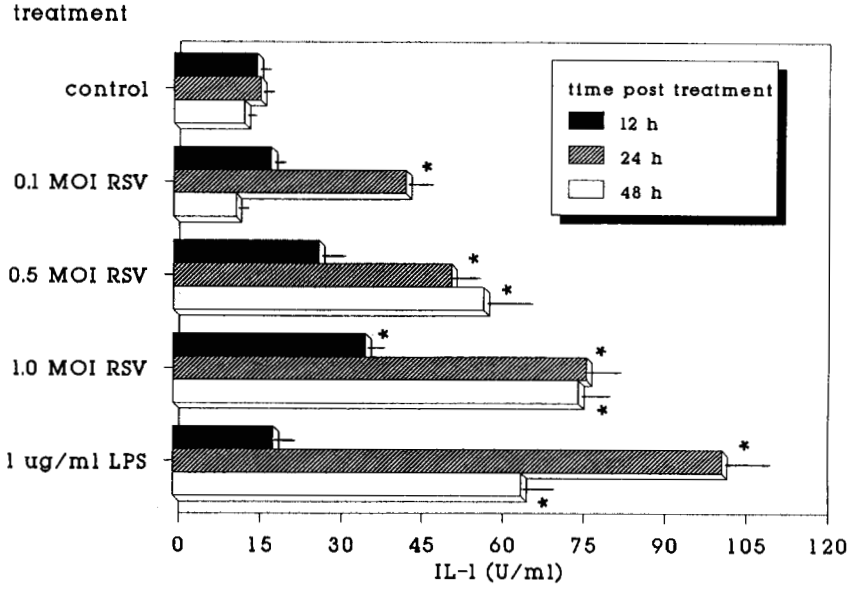

FIGURE 9. Release of IL-1 into the supernatant measured at different time points p.i. or post activation. For control, AL were incubated in medium alone. Before the IL-1-assay, neutralizing Abs against RSV were added to all supernatants. Results represent the means of five independent experiments. * $p < 0.05$, control vs RSV or LPS.

virus permissiveness respectively virus-production was also observed for infection of human blood monocytes and AL with RSV (31). Studies with RSV exposure of in vitro differentiated or undifferentiated U937 cells also support this observation (39). The different amounts of virus released by infected AL and INT might be in part explained by their distinct IFN production in response to RSV infection. A similar observation was made by Hanada et al. (40) using different cell lines for RSV infection in vitro. Concerning in vitro studies done with human cells, induction of IFN resulting from RSV exposure seemed to be minor in monocytes and especially in nonadherent monocytes (41, 39). One consequence of IFN synthesis is the induction of 2′-5′-oligoadenylate-synthetase activity, which leads to destruction of viral RNA. Interestingly, the amount of this intracellular enzyme increased with cell maturation (42) and may therefore contribute to the faster elimination of virus in the more differentiated AL in comparision to INT. Probably there are also other mechanisms that contribute to the varying permissiveness of macrophages to RSV infection.

An inhibitory effect caused by TNF induction would be conceivable because TNF has been shown to reduce RSV infection of human monocytes (31). One mechanism to

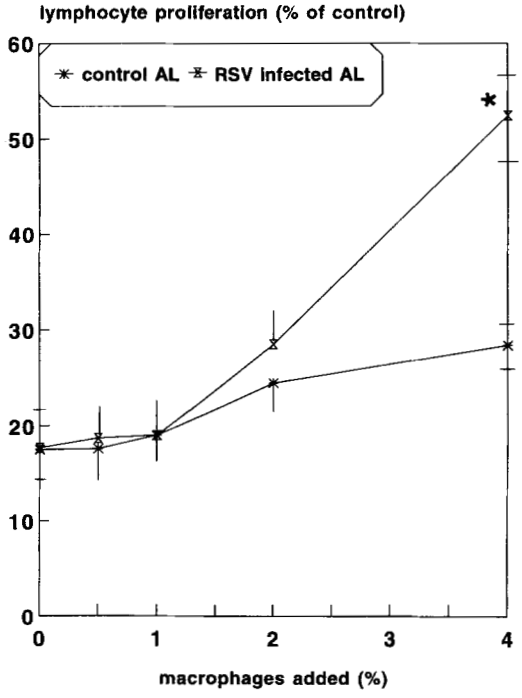

FIGURE 10. Accessory function of RSV-infected or control AL. AL were added at different ratios to Con A (2 μg/ml)-stimulated lymphocytes 24 h after RSV infection. Data shown are the means of three independent experiments. * $p < 0.05$, control vs RSV.

explain how TNF protects macrophages from viral infection may be its ability to stimulate type 1 IFN synthesis in macrophages.

Preactivation of macrophages with LPS resulted in a much lower initial virus load of AL, but induced only minimal amounts of antiviral IFN. In contrast, AL respond to stimulation with LPS with an early and strong production of TNF.

Together, these results indicate that the maturation stage and the state of activation may influence macrophage infection with RSV. Further investigations are needed to show exactly which factors are involved in permissiveness or resistance of mononuclear phagocytes to viral infection.

In spite of the fact that respiratory viral infections are frequently accompanied by bacterial infections, which is also described for RSV in different species (18, 19, 30) as well, little is known about the influence of viral infection on the microbicidal function of lung phagocytes. RSV infection did not result in a reduced viability of infected macrophages as was also shown for other species (21, 32). Therefore, secondary infection is unlikely to be the result of direct macrophage elimination as a result of viral infection in vivo. Our results indicate a suppressive effect of RSV infection on macrophage microbicidal activity. High inhibition as observed for phagocytosis, ROI secretion, and leishmanicidal activity was measured at later time points p.i. (48–72 h p.i.). Other groups demonstrated a similar effect on Fc receptor-mediated phagocytotic activity 3 days after infection of bovine AL with RSV (20). Because the phagocytosis event lead to the induction of ROI release (43), the reduced phagocytotic function might be responsible for the lower ROI production of infected macrophages in comparison to uninfected phagocytes. Killing of protozoa is considered to be dependent on the release of ROI and/or on the action of reactive nitrogen intermediates (44, 45). Therefore, the lower capacity of infected macrophages to release ROI may account for the observed reduction in leishmanicidal activity. Together, these results demonstrate an inhibition of the microbicidal activities resulting from RSV exposure of AL. The mechanisms that led to these functional changes are not yet clear and need to be characterized further. Inhibition of microbicidal activity as seen in our experiments could not be explained by a feedback mechanism through the action of PGE_2 released during viral infection. Interestingly, there seemed to be a difference between human and murine phagocytes with regard to PGE_2 release after RSV exposure. Panuska and coworkers (46) demonstrated no increased PGE_2 secretion after RSV infection of human AL or human monocytes at least at the time point tested (24 h p.i.).

However, all reduced macrophage functions observed are in some way associated with the phagocytosis event, which needs a functional cytoskeleton. Because paramyxoviruses use the host cytoskeleton for virus budding (47) and cytochalasin B treatment leads to prevention of virus release (48), one could speculate that the virus dependent suppression of phagocyte function might be related to an interference of RSV with cytoskeleton function. Despite a high probability that the observed functional changes in macrophage activity are caused by the infected macrophage population, we cannot rule out the possibility that noninfected macrophages are also influenced in some way. Therefore, secondary effects might contribute to the results observed with the whole macrophage population. Nevertheless, investigation of cell populations consisting of infected and uninfected macrophages in vitro seemed to resemble more the in vivo situation where both infected and noninfected macrophages are present together in the lung (17).

In contrast to the inhibitory influence of RSV on the microbicidal macrophage functions, RSV is able to stimulate release of cytokines. The few data known about the effect of RSV on IL-1 release are rather conflicting. In accordance with our data a recent publication showed induction of IL-1 secretion in RSV-infected human AL (17). Some studies demonstrated no net IL-1 activity in supernatants of infected human mononuclear phagocytes, which is explained by the induction of an IL-1 inhibitor (14, 15). A reason for these inconsistent results might be the use of different indicator cells for IL-1 bioassays, because D10.G.4.1 cells are more sensitive in bioassays to IL-1.

IL-1 and TNF are capable of stimulating the synthesis of each other (49). Therefore it is unknown whether the observed secretion of TNF or IL-1 is stimulated through the action of an other cytokine induced after viral exposure of AL or if their synthesis could be contributed to a direct inductive effect of RSV. This might happen through formation of dsRNA, because poly(I)-poly(C) has been shown to induce a TNF response (50). It is striking that the increase of TNF secretion after LPS stimulation is much faster than with RSV. Perhaps this is explained through the inhibitory effect of IL-6 (53), which is present in high amounts in macrophage supernatants early after RSV infection of AL but not after LPS stimulation. TNF induction is not restricted to the exposure of murine phagocytes to RSV because it has been demonstrated for other members of paramyxo- and orthomyxoviruses (51, 52) and for RSV infection of human AL (29, 46) as well. Apart from its cytotoxic activity, TNF acts also as a chemoattractant for T lymphocytes and thus might play a role in recruiting cells for the specific antiviral immune response in vivo.

Like other viruses (54, 55), RSV induced an early IL-6 response in infected murine macrophages, as has been shown for human AL (29). Secretion of TNF and IL-1, which are both known to stimulate IL-6 synthesis (56), did not increase significantly until IL-6 secretion reached maximal levels. Therefore IL-6 induction in response to virus exposure might be caused by a direct effect of the viral infection. The kinetics of IL-6 release observed after virus exposure is different from that seen with LPS stimulation. We and others observed higher amounts of IL-6 secretion at later time points after LPS stimulation (57). In contrast to some other investigators who used RSV infection of human mononuclear leukocyte populations (14), demonstrated an increase in accessory cell function after viral exposure of AL as demonstrated for isolated human AL as well (17). However, based on the high cytokine release observed after RSV exposure of AL, we expected a more pronounced increase in the Con A-induced lymphocyte proliferation. Perhaps mediators secreted by infected macrophages, such as IFN and PGE_2, which are able to inhibit lymphocyte proliferation, account for the overall accessory cell activity.

Comparison of our data with results obtained from experiments done with other species revealed many similarities between RSV-exposed murine and human macrophages. Both murine and human AL secrete IL-1, IL-6, and TNF and demonstrated an increased Ag-presenting function after RSV exposure in vitro (17, 29, 46). In contrast, infection of human monocytes with RSV did not lead to TNF induction nor enhanced Ag presenting function (46, 58). Therefore the induction of different functional changes resulting from RSV exposure may be more dependent on the type of phagocytes used for infection than on the species used for phagocyte isolation, at least in some cases. With respect to cytokine secretion, there are many studies, especially with human phagocytes, demonstrating pronounced functional differences between phagocytes of different maturation states even in the same species (57, 59–62) supporting this observations.

Assuming our data are at least in part transferable to the human system, these in vitro results indicate that recurrent infections with RSV observed in clinical situations might not be explained by an inhibitory effect of RSV on accessory cell function of AL. Furthermore, RSV infection of murine lung macrophages led to a reduction of the microbicidal capacity of AL in vitro, which might facilitate spread of secondary bacterial infections.

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