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Bovine respiratory syncytial virus infection influences the impact of α_4- and β_2-integrin-mediated adhesion of peripheral blood neutrophils

E. C. SOETHOUT*§, A. F. G. ANTONIS†, L. H. ULFMAN‡, A. HOEK*, R. G. VAN DER MOST*, K. E. MÜLLER§ & V. P. M. G. RUTTEN* **Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands, †Institute for Animal Science and Health (ID-Lelystad), Lelystad, the Netherlands, ‡Department of Pulmonary Diseases, University Medical Center, Utrecht University, Utrecht, the Netherlands, and §Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands*

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SUMMARY

Neutrophil migration into the airways and pulmonary tissue is a common finding in bovine respiratory syncytial virus (BRSV) infections. Although neutrophil trans-endothelial migration in general depends on β_2-integrins, alternative integrins such as the α_4-integrins have been implicated. In this study, rolling and firm adhesion of peripheral blood neutrophils isolated from healthy and BRSV-infected calves to tumour necrosis factor (TNF)-α activated pulmonary endothelium was investigated under flow conditions *in vitro*. For neutrophils obtained from healthy animals, inhibition of the β_2-integrin reduced firm adhesion to 63% and inhibition of α_4-integrin to 73% compared with untreated controls. Inhibition of both integrins reduced firm adhesion to 25%. Rolling velocity, which is used as a parameter for integrin involvement in neutrophil rolling, increased 1·7-fold by blocking β_2-integrin and was significantly augmented to 2·5-fold by blocking both α_4- and β_2-integrins. For neutrophils obtained from BRSV-infected animals, however, rolling velocities at 10 days after infection (p.i.) were not influenced by blocking adhesion of α_4- and β_2-integrins, indicating that these integrins did not support neutrophil rolling. In addition, the inhibition of firm adhesion by blocking both α_4- and β_2-integrins was reduced significantly 9 days post-infection, resulting in a residual 68% neutrophil binding at 9 days p.i. Non-blocked firm adherence was not reduced, indicating that binding was achieved by other mechanisms than through α_4- and β_2-integrins. These results demonstrate an important function for α_4- and β_2-integrins in rolling and firm adherence of bovine neutrophils, to TNF-α-activated endothelium and show the dynamic use of these integrins for adhesion and migration by neutrophils in the course of BRSV infection.

Keywords adhesion molecules animal models/cows bovine respiratory syncytial virus migration/traffic/circulation neutrophils

INTRODUCTION

Neutrophils are early emigrating leucocytes in response to proinflammatory signals and changes in the vasculature [1]. Neutrophil migration is accomplished in general by a sequence of steps. First, the leucocyte forms initial tethers and rolls along the postcapillary venules. This process is mediated largely by L-selectin, expressed on the neutrophil membrane. Then, the cell firmly adheres to and finally migrates through the endothelium [2]. It has long been accepted that for firm adhesion, neutrophils use exclusively β_2-(CD18) integrins, whereas other leucocytes such as lymphocytes and eosinophils make use of both α_4-(CD49d) and β_2-integrins [3]. Organ-specific differences occur, however, as the lung seems to allow neutrophil migration independent of β_2-integrin function [4–7]. These findings are supported by necropsy reports from humans as well as from calves deficient in expression of β_2-integrins that revealed extensive neutrophil infiltration into the broncho-alveolar lumen and connective tissue of infected lungs. Other tissues such as the intestines and the oral cavity were largely devoid of neutrophil infiltration, despite signs of extensive chronic inflammation and ulceration [8–11]. In addition to migration from the postcapillary venules, neutrophil migration in the lung appears to occur from the capillaries [12].

While neutrophils were considered originally to be devoid of β_1-integrins (including $\alpha_4\beta_1$), to date dynamic surface expression of $\alpha_2\beta_1$ – $\alpha_6\beta_1$ and $\alpha_9\beta_1$ has been detected on human and rodent neutrophils, primarily after emigration from the vasculature [13].

Correspondence and current address: E. C. Soethout, Netherlands Vaccine Institute, Department of Vaccine Research, internal postbox 92, PO Box 457, 3720 AL, Bilthoven, the Netherlands.

E-mail: ernest.soethout@nvi-vaccin.nl

Increased expression of the α_4-integrin was found on human neutrophils when stimulated *in vitro* [14], and *in vivo* in critically ill septic patients [15]. The α_4-integrin on these neutrophils as well as on *in vitro*-stimulated neutrophils is functionally active, as it binds to VCAM-1 [15,16] and tumour necrosis factor (TNF)-α-stimulated endothelium [16]. In particular, the $\alpha_4\beta_1$-integrin may mediate neutrophil extravasation, as shown in mouse and rat [17–22].

Recently, we reported that in calves, severely affected by respiratory inflammation, the α_4-integrin expression on peripheral blood neutrophils was increased compared to neutrophils from healthy animals [23]. However, data on the function of α_4-integrins in neutrophil emigration are lacking. In this study, we examined the role of α_4-integrin and β_2-integrin in neutrophil adhesion to pulmonary endothelial cells *ex vivo* and the effect of experimental bovine respiratory syncytial virus (BRSV) infection in this role.

MATERIALS AND METHODS

Animals and experimental design

Specific-pathogen-free (SPF) calves were obtained by caesarean section, deprived of colostrum and reared in isolation units. The calves were found to be free of bovine virus diarrhoea virus (BVDV) and of antibodies against bovine herpes virus 1, BRSV, BVDV and parainfluenza 3 at the start of the experiments.

In this study, we investigated the expression and function of α_4- and β_2-integrins for neutrophil adhesion. The study was performed using neutrophils from healthy calves ($n = 5$, 8–10 weeks of age) and neutrophils from BRSV-infected calves. In the infection experiments, we used neutrophils from calves ($n = 3$, 8–9 weeks) at days –1, 5, 8, 9 and 12 after BRSV infection (p.i.) and neutrophils from calves ($n = 4$, 28 weeks) 10 days after BRSV infection. Peripheral blood samples were obtained from the jugular vein in vacutainer tubes (Becton-Dickinson, San Jose, CA, USA) containing sodium citrate (0·38% final volume) as anticoagulant.

Virus shedding

BRSV infection was carried out by nebulization of 2 ml $10^{3\cdot9}$ TCID50/ml of BRSV, Odijk strain. A broncho-alveolar lavage (Bal) was performed at several days p.i. [24] to demonstrate development of infection by reverse transcription-polymerase chain reaction (RT-PCR) following the protocols described by Kuno [25]. Primers were designed for BRSV-N and BRSV-P, generating PCR-products of 1·1 kb and 0·7 kb, respectively, as follows: N 5′: GTTTAAACCATGGCTCTYAGCAAGGTC, N 3′: CARTTCCACATCATTRTCTTT, P 5′: GAAATTTCCATGGAAAAATTTGCACCTG P 3′: GAAATCTTCAAGTGATAGATCATTG, Y = C/T, R = A/G; degenerate as the BRSV–Odijk sequence was not known. Positive controls included plasmids containing the BRSV–Odijk N and P genes, as well as cDNA prepared from BRSV–Lelystad-infected cells.

Detection of cellular adhesion molecules on neutrophils in peripheral blood

The following monoclonal antibodies (MoAbs) were used for fluorescent staining: interleukin (IL)-A110 (antineutrophil granulocyte, kindly provided by Dr Naessens, ILRI, Nairobi, Kenya) [26], R15·7 (anticanine β_2-integrin, cross-reactive to its bovine homologue, kindly provided by Dr Rothlein, Boehringer Ingelheim Pharmaceuticals, Ridgefield, USA) [8] and BII218·1 (cross-reactive antisheep α_4-integrin, kindly provided by Dr A. Young, Basel Institute of Immunology, Switzerland) [27]; R73 (IgG1) specific for rat T cell receptor [28] served as isotype control MoAb. HUTS21 was kindly provided by Dr Sanchez-Madrid (Universidad Autonoma de Madrid, Spain). The MoAb recognizes an activation epitope on a regulatory region (355–425) of the human beta-1 chain [29], which is 95% identical to its bovine homologue (CD29 bos taurus, accession code NM_174368) by BLAST comparison.

Peripheral blood (50 μl) was incubated with MoAbs (0·25 μg/ml) for 30 min on melting ice, followed by two washes in FACS buffer (PBS, 0·1% azide, 0·5% bovine serum albumin), and incubated subsequently with saturating amounts of goat-antimouse FITC (Becton-Dickinson, San Jose, CA, USA). Peripheral blood leucocytes were purified by lysis of red blood cells in FACS-brand lysing solution (Becton-Dickinson, San Jose, CA, USA). Flow cytometry was performed on FACSCalibur (Becton-Dickinson, Brussels, Belgium). A minimum of 10 000 events was recorded for each sample. Neutrophils were identified on the basis of IL-A110 (antineutrophil) specific staining and light scatter profile. The expression index (EI) quantified the expression of cell adhesion molecules (CAM) on gated neutrophils and was defined as the quotient of specific MoAb and isotype control MoAb fluorescence.

Isolation and culture of bovine endothelial cells

Endothelial cells from the bovine pulmonary artery (BPAEC) were isolated from fresh lungs, obtained from 4–8-week-old calves at a local abattoir. The pulmonary artery was dissected and perfused with PBS (4°C) to remove blood clots. Both ends were ligated, and the dissected artery was injected with 0·05% (g/v) trypsin (Becton-Dickinson, San Jose, CA, USA) solution at 37°C for 15 s. The injected volume was recovered, pooled with an equal volume of bovine calf serum and centrifuged (500 ***g*** for 10 min at 4°C). Isolated cells were seeded into a 25-cm^2 flask, coated with 1% gelatin (Merck, Darmstadt, Germany), and cultured in Iscove's tissue culture medium (GIBCO BRL, Paisley, UK) supplemented with 10% fetal calf serum (FCS), 50 IU/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine (all from Sigma, Zwijndrecht, the Netherlands). After 2–3 days, single endothelial colonies were identified by typical cobblestone morphology [30] and subcultured. The subcultured endothelial cells were characterized by uptake of acetylated low-density lipoprotein labelled with fluorescent 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Dil-Ac-LDL, Sigma, Zwijndrecht, the Netherlands) and screened by fluorescence microscopy (data not shown) [31]. Cell-lines were found free of mycoplasma by PCR analysis on genomic DNA and on 2-day culture supernatant (data not shown) [32].

Isolation of neutrophils

During the complete isolation procedure, blood and neutrophils were kept on melting ice and centrifugation steps were performed at 4°C. Neutrophils were isolated by hypotonic lysis [33] and resuspended in neutrophil incubation buffer (20 mM Hepes, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 5 mM glucose, 1·0 mM $CaCl_2$, 0·05% (w/v) bovine serum albumin (Sigma, Zwijndrecht, the Netherlands), pH 7·4, before use in perfusion assays. Purity of neutrophils was >95%. The viability (>95%) was determined by trypan blue exclusion.

Perfusion assays

Neutrophil rolling adhesion and firm adhesion on endothelial cells under steady flow was investigated in a modified form of the transparent parallel plate perfusion chamber as described previously [34]. To simulate an immunologically activated pulmonary environment, BPAEC (third to fifth passage) were preactivated by human TNF-α for 7 h (100 U/ml, 37°C; Boehringer Mannheim, Mannheim, Germany) [35]. Activation of endothelial cells induces expression of VCAM-1 and increased expression of ICAM-1, which are major ligands for the leucocyte α_4- and β_2-integrins, respectively [36,37]. The perfusion equipment was placed in a 37°C temperature box. Neutrophils kept in suspension (2×10^6 cells/ml in neutrophil buffer) on melting ice until start of the experiment were allowed to adjust to 37°C for 20 min with or without blocking MoAbs (10 μg/ml) before the perfusions. The concentration of the blocking MoAbs was saturating as determined by FACS analysis (data not shown). The MoAbs were described as cross-reactive and function-blocking MoAbs specific for the bovine homologues of canine β_2-integrin (R15·7) [8,38] and human α_4-integrin (HP 2/1) [27,34,39]. Neutrophils were aspirated for 5 min from a reservoir through plastic tubing and the perfusion chamber with a Harvard syringe pump (Harvard Apparatus, South Natick, MA, USA), followed by buffer. The shear stress was set at 2·5 dynes/cm^2. Rolling and firmly adherent cells were detected using video recordings of at least 25 randomized high-power fields, representing a total surface of at least 1 mm^2 and analysed by customized software [34]. The percentage of rolling cells was detected in a sequence of 50 frames covering a 2-s period. At least 100 cells per experiment were investigated for rolling velocity. The cut-off speed for distinguishing rolling and static adherent cells was set at 1 μm/s. As the time-span for the experiments was limited by the functional life-span of the isolated neutrophils, controls were restricted to neutrophils without MoAbs. In a pilot experiment, adhesion of neutrophils (no. of adherent neutrophils/mm^2 ± s.e.m.), incubated with the MoAb W6/32 specific for human MHC I and cross-reactive to bovine MHC [40], was not different from adhesion of control neutrophils, 1206 ± 43 and 1232 ± 44, respectively ($P = 0{\cdot}735$, paired Student's *t*-test).

Statistical analysis

Results were compared by ANOVA, with Bonferroni correction for multiple comparison using the statistical software GRAPHPAD PRISM® and GRAPHPAD STATMATE™ (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at $P < 0{\cdot}05$. Results were expressed as mean ± s.e.m.

RESULTS

Expression and function of CAM on neutrophils from healthy cattle

Integrin expression on neutrophils. CAM on neutrophils, obtained from healthy calves ($n = 5$), were detected by flow cytometry and expression levels were converted into the EI ± s.e.m., which was 13·9 ± 1·8 for β_2-integrin and 2·8 ± 0·3 for α_4-integrin.

Perfusion experiments. The total number of adherent neutrophils (rolling and firmly attached) to 7 h TNF-α-activated BPAEC was determined (Fig. 1a). To investigate the role of α_4- and β_2-integrins, neutrophils were preincubated with blocking MoAbs. Co-administration of α_4- and β_2-integrin blocking MoAbs inhibited adherence to 47% of the control value ($P < 0{\cdot}001$).

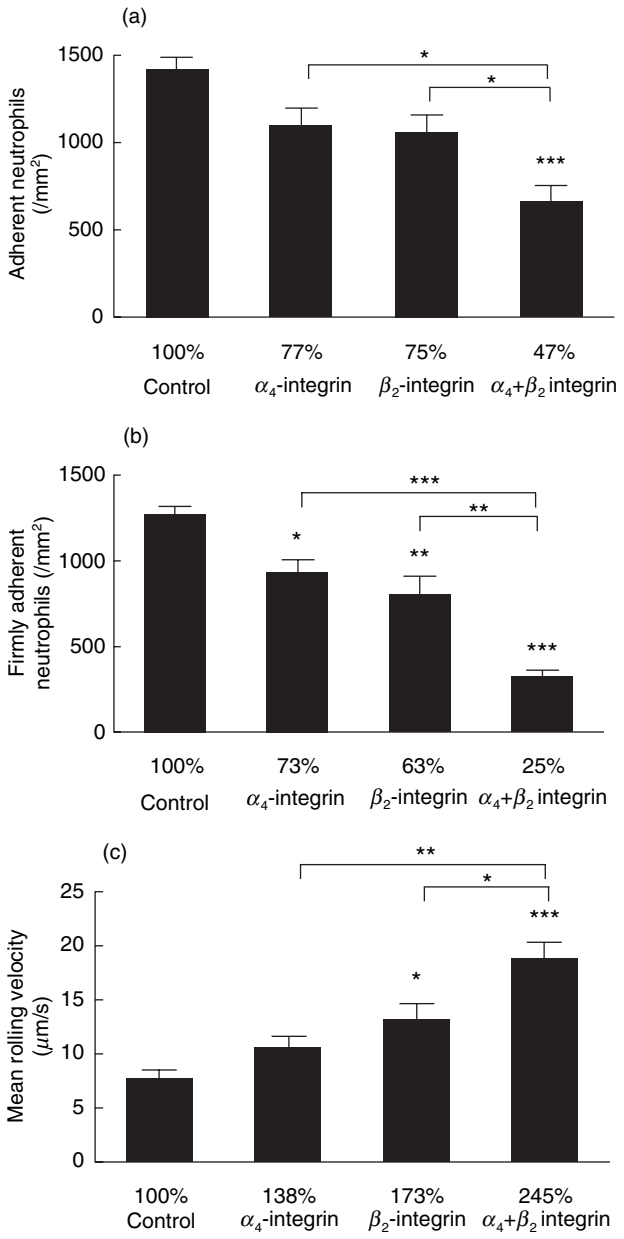

Fig. 1. Effect of blocking α_4- and β_2-integrins on the interaction of neutrophils to TNF-α-activated BPAEC. Neutrophils (2×10^6/ml) preincubated with or without blocking MoAbs (10 μg/ml, 20 min, 37°C) were perfused at a shear stress of 2·5 dynes/cm^2. After 5 min, microscopic images were recorded on video and the number of adherent cells – rolling or firmly attached – were determined in at least 25 images per perfusion experiment. (a) The effect of blocking MoAbs specific for α_4- and β_2-integrins on the number of adherent cells (rolling and firmly attached) to BPAEC. (b) The effect of blocking MoAbs on the number of firmly attached cells as percentage of the total number of adherent cells. (c) The effect of blocking MoAbs on mean rolling velocity of neutrophils. The rolling velocities of at least 100 cells per experiment were determined. Means are plotted for five animals per group ± s.e.m. The mean percentages for each group compared to the control group have been indicated. The statistically significant effects of the blocking MoAbs compared to the control situation or between different treatments (as indicated in the figure) were determined by ANOVA with Bonferroni correction for multiple comparison, $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

The percentage of rolling neutrophils increased by a factor 4·8 ($P < 0·001$) by the combination of blocking antibodies compared to control values. Treatment of neutrophils with blocking MoAbs specific for α_4-integrin or β_2-integrin reduced the number of firmly adherent neutrophils to 73% ($P < 0·05$) and 63% ($P < 0·01$), respectively (Fig. 1b). When these MoAbs were used in combination, firm adhesion reached up to 25% of control levels ($P < 0·001$).

Rolling velocity with and without preincubation of neutrophils with blocking MoAbs was used as a parameter for integrin-mediated rolling. Involvement of integrins in neutrophil rolling resulted in increased neutrophil speed of MoAb pretreated neutrophils. Mean rolling velocities of neutrophils on TNF-α activated endothelium increased by a factor of 1·7 ($P < 0·05$) by blocking β_2-integrins, whereas blocking of α_4-integrins did not significantly enhance neutrophil speed. Co-administration of α_4- and β_2-integrin-specific MoAbs significantly enhanced neutrophil velocity 2·5-fold compared to unblocked controls ($P < 0·001$) and to singly blocked neutrophils (Fig. 1c).

Together, these results indicate that both α_4- and β_2-integrins mediated firm adhesion and supported rolling adhesion, despite differences in level of integrin expression.

Expression and function of CAM on neutrophils from BRSV-infected cattle

BRSV infection experiments were set up to monitor neutrophil adhesion *in vitro* during pulmonary infection. Infection developed similarly in all animals. In the first infection experiment ($n = 3$), production of viral RNA in BALF was determined daily, from days 5–9 p.i. Viral RNA production peaked at day 7 p.i. (data not shown). Similarly, in the second experiment ($n = 4$), viral RNA production was detected at day 7 p.i.

Integrin expression on neutrophils. Expression levels of α_4- and β_2-integrins were measured by flow cytometry. In the first infection, both the α_4- and β_2-integrins did not change significantly from day –1 (EI 2·6 ± 0·5 and 13·9 ± 3·4, respectively) until day 10 p.i., but increased at day 12 p.i. (EI 3·7 ± 0·7, $P < 0·05$ and 38·7 ± 5·4, $P < 0·001$, respectively) (Fig. 2). In the second infection, expression levels of α_4- and β_2-integrins before and 8, 9 and 10 days after infection were similar to the results found in the first infection experiment. In addition, expression of an activation epitope for β_1-integrins (HUTS21) was determined. The expression decreased in all animals from 1·27 ± 0·17 at day –1 to a minimum of 1·005 ± 0·01 at day 8 and 1·038 ± 0·05 at day 10 p.i. Mean expression levels of HUTS21 were, however, not significantly different ($P = 0·1053$).

Perfusion experiments. During the first infection, neutrophil perfusion experiments were performed before (day –1) and at several days after (days 5, 8, 9 and 12) BRSV infection. The overall frequency of adherent cells (without blocking antibodies) remained constant, whereas the frequency of rolling neutrophils increased ($P = 0·0020$) from 10·6% ± 3·6 before infection to 36·7% ± 6·0 at day 12 p.i. The frequency of firmly adherent cells tended to decrease ($P = 0·092$) in the course of infection.

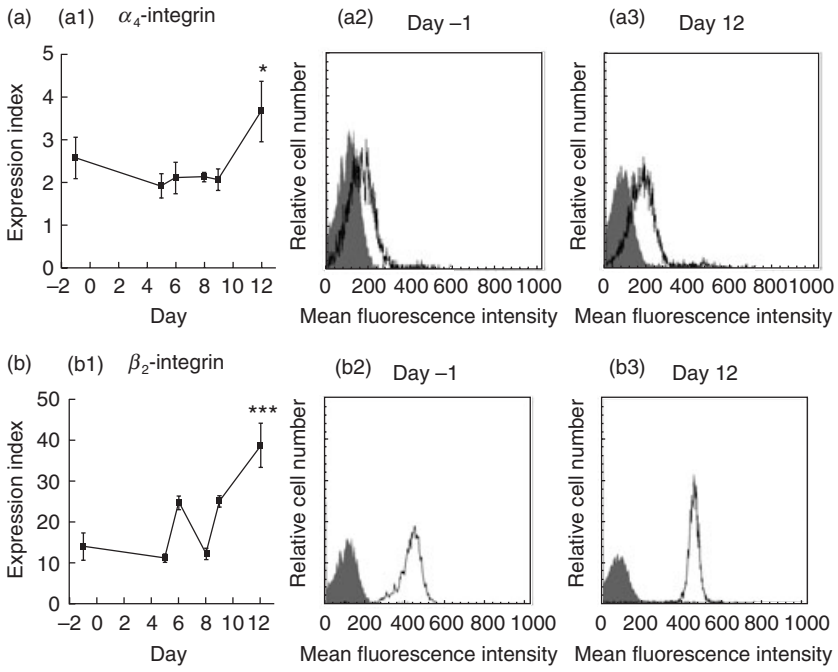

Fig. 2. CAM expression on peripheral blood neutrophils of calves ($n = 3$) in the course of BRSV infection. The mean fluorescence intensities of gated neutrophils stained with CAM-specific MoAbs (BII218·1 for α_4-integrin and R15·7 for β_2-integrin) or isotype control MoAb (R73) were measured. The expression index (EI), defined as the quotient of specific MoAb and isotype control MoAb fluorescence, quantified the expression of CAM. (a) Expression of α_4-integrin: (a1) expression indices before and in the course of infection; (a2) mean fluorescence intensities of α_4-integrin (line) and its isotype control (grey area) at day –1; (a3) mean fluorescence intensity of α_4-integrin (line) and its isotype control (grey area) at day 12. (b) Expression of β_2-integrin: (b1) expression indices, before and in the course of infection; (b2) mean fluorescence intensities of β_2-integrin (line) and its isotype control (grey area) at day –1; (b3) mean fluorescence intensities of β_2-integrin (line) and its isotype control (grey area) at day 12. Means are plotted ± s.e.m. Histograms of one animal, representative of three, are shown. Significant differences in EI levels compared to day –1 are indicated. *$P < 0·05$; ***$P < 0·001$.

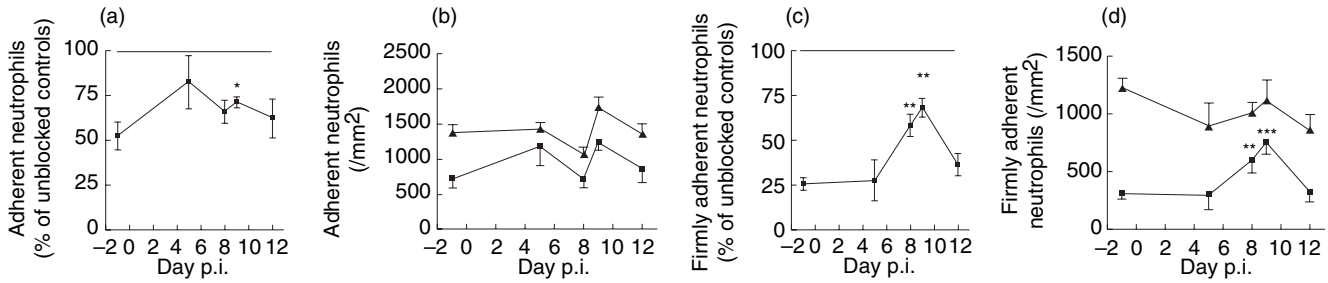

Fig. 3. Effect of inhibition of α_4- and β_2-integrins on the interaction of neutrophils, isolated in the course of BRSV infection, to TNF-α-activated BPAEC. Neutrophils were isolated from calves ($n = 3$) 1 day before and 5 ($n = 2$), 8, 9 and 12 days after BRSV infection. Adhesion of isolated neutrophils was investigated in the presence of α_4- and β_2-integrin-blocking MoAbs (Hp2/1 and R15·7, respectively) using neutrophil flow experiments. The numbers of adherent neutrophils on 7-h TNF-stimulated BPAEC were determined by computer-assisted image analysis using at least 25 video recordings. (a) α_4- and β_2-integrin-blocked adherent neutrophils (firm and rolling) as percentage of controls ($P = 0{\cdot}0495$). (b) Absolute number of adherent neutrophils (firm and rolling) using α_4- and β_2-integrin-blocked (squares, $P = 0{\cdot}0053$) and control (triangles, $P = 0{\cdot}016$) neutrophils. (c) α_4- and β_2-integrin-blocked, firmly adherent neutrophils as percentage of controls ($P = 0{\cdot}0022$). (d) Absolute number of firmly adherent neutrophils using α_4- and β_2-integrin blocked (squares, $P = 0{\cdot}0006$) and control (triangles) neutrophils ($P = 0{\cdot}27$). Means are plotted ± s.e.m. Significant differences at one time-point compared to day −1 are indicated. $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

Blocking either α_4- or β_2-integrins separately did not result in significant differences before and after infection (data not shown). The number of α_4- and β_2-integrins doubly blocked cells per mm^2, adherent to activated endothelial cells (TNF-α 100 U/ml, 7 h), changed significantly in the course of infection and was determined in absolute numbers and as a percentage of adherent, control (untreated) neutrophils on that day. The percentage of adherent (which equals the entire number of firmly adhered and rolling cells), doubly blocked neutrophils increased after BRSV infection. At day −1 it was 52 ± 8% of control values. At day 9 p.i., it was 71 ± 3% ($P < 0{\cdot}05$). The biological significance of this finding is limited, as the absolute number of doubly blocked adherent neutrophils was not significantly different at this time-point (Fig. 3a,b).

The percentages of rolling cells, incubated with α_4- and β_2-integrin blocking antibodies did not change significantly in the course of infection.

The number of firmly adherent, doubly blocked neutrophils increased during infection. Before infection, blocking both α_4- and β_2-integrins reduced firm adhesion to 25 ± 4% of control values. In the course of infection, blocking the α_4- and β_2-integrins was less effective, resulting in higher residual binding ($P = 0{\cdot}0022$, Fig. 3c). At day 8 p.i., 58 ± 6% ($P < 0{\cdot}01$, compared to d -1) and at day 9, 68 ± 5% ($P < 0{\cdot}01$) of the neutrophils still adhered firmly despite the presence of blocking MoAbs. The rise in firmly adherent cells that had been pretreated with α_4- and β_2-integrins blocking MoAbs was also significant in absolute numbers. This indicates that the effect was due to an increase in the number of firmly adherent neutrophils and not to a decrease in the total number of adherent cells (Fig. 3d).

A non-significant trend suggesting a reduced influence of α_4- and β_2-integrins blocking MoAbs on rolling speed in the course of infection was detected (data not shown).

In the second infection experiment, neutrophil perfusions were performed at day 10 p.i. No significant effect was found by blocking α_4- and β_2-integrins on the total number of rolling and firmly adherent cells at this time-point (Fig. 4a). Co-administration of MoAbs enhanced the percentage of rolling neutrophils 2·3-fold ($P < 0{\cdot}01$). Firm adherence of neutrophils isolated from BRSV-infected animals was inhibited by co-administration of α_4- and β_2-integrins blocking MoAbs to 51% of the adherence of control neutrophils ($P < 0{\cdot}01$) and to 60% of the adherence reached after β_2-integrin blocking of neutrophils ($P < 0{\cdot}05$, Fig. 4b).

Rolling velocities of neutrophils isolated from BRSV infected animals were not affected significantly by blocking MoAbs (Fig. 4c).

These data show that 8, 9 and 10 days after BRSV infection the contribution of α_4- and β_2-integrins to neutrophil firm adhesion decreased significantly. In addition, neutrophil rolling at day 10 p.i. functioned independently of α_4- and β_2-integrins. An increase in expression of α_4- and β_2-integrins was detected at day 12 p.i. Simultaneously, the dependency of neutrophil adhesion to the function of both integrins was restored. The decrease in expression of HUTS21 may indicate that the β_1-integrin, which forms a heterodimer with the α_4-integrin chain, falls back in the inactive conformation.

DISCUSSION

In healthy cattle, expression of α_4-integrin on neutrophils was relatively low compared to β_2-integrin. Despite the differences in expression between α_4- and β_2-integrins, adhesion to activated pulmonary artery endothelial cells seemed to depend on both integrins. The present experiments illustrate that the α_4-integrin, although expressed at a low level on neutrophils, may contribute significantly to leucocyte-endothelial adherence.

The function of α_4- and β_2-integrins in neutrophil rolling was investigated by measuring rolling speed. This velocity is influenced by differences in the 'on' or 'off' rate of each CAM that binds to its endothelial ligand. Because blocking of β_2-integrins increased rolling speed, while the effect was augmented by co-administration of α_4-integrin-blocking antibodies, both the α_4- and β_2-integrins may support neutrophil rolling. These contributions of α_4- and β_2-integrins in neutrophil rolling were also acknowledged recently by intravital microscopy studies in mice [20]. For integrin $\alpha_L\beta_2$ (LFA-1), it was reported previously that the inactive (closed) conformation of the integrin dimer supports the rolling phase, while the activated (open) conformation mediates leucocyte firm adhesion [41]. Firm adhesion of neutrophils isolated from healthy cattle was mainly mediated by α_4- and β_2-integrins. The inability of doubly blocked neutrophils to enter the

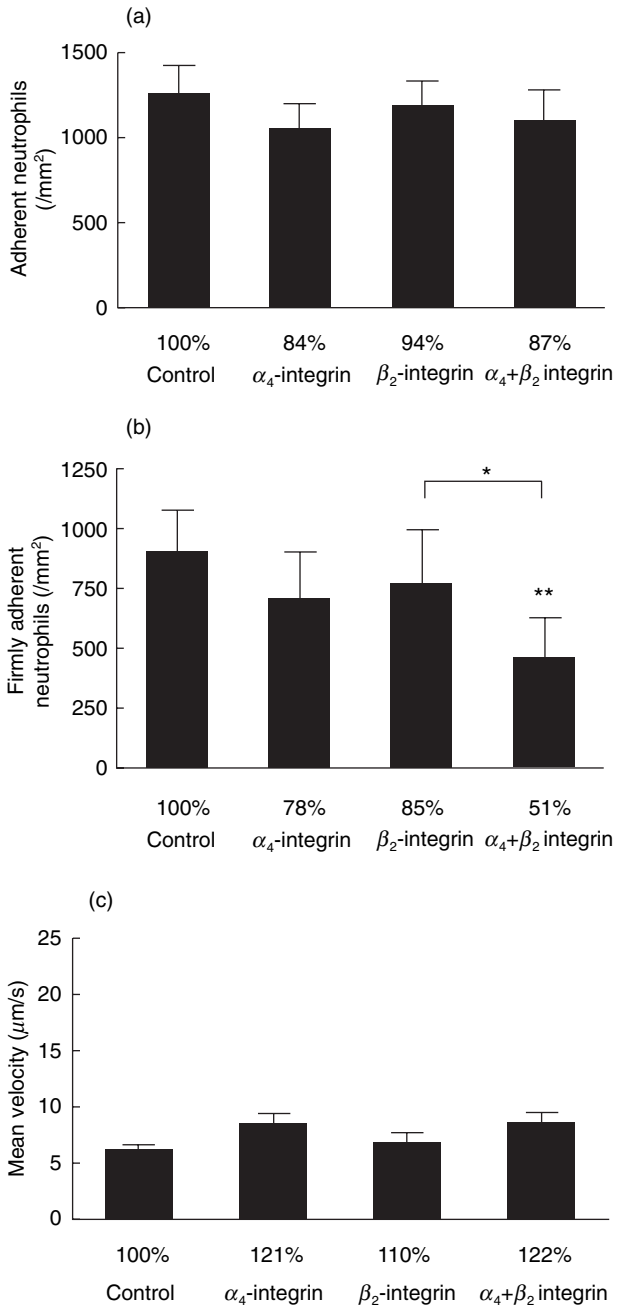

Fig. 4. Effect of blocking α_4- and β_2-integrins on the interaction of neutrophils isolated from 10-day BRSV-infected calves to TNF-α-activated BPAEC. Neutrophils (2×10^6/ml) preincubated with or without blocking MoAbs (10 μg/ml, 20 min, 37°C) were perfused at a shear stress of 2·5 dynes/cm^2. After 5 min, microscopic images were recorded on video and the number of adherent cells – rolling or firmly attached – were determined in at least 25 images per perfusion experiment. (a) The effect of blocking integrins on the number of adherent cells (rolling and firmly attached) to BPAEC. (b) The effect of blocking integrins on the number of firmly attached cells as percentage of the total number of adherent cells. (c) The effect of blocking integrins on mean rolling velocities of at least 100 rolling cells. Means are plotted for four animals per group ± s.e.m. The mean percentages for each group compared to the control group have been indicated. The statistically significant effects of the blocking MoAbs compared to the control situation or between different treatments (as indicated in the figure) were determined by ANOVA with Bonferroni correction for multiple comparison, $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

stage of firm adhesion was shown by a reduced number of firmly adherent neutrophils and a rise in rolling neutrophils.

In experimentally induced BRSV pneumonia, increased integrin expression on neutrophils was detected at 12 days p.i. These findings are consistent with our earlier work that reports higher levels of α_4-integrin expressed on neutrophils from severely pneumonic cattle compared to healthy cattle [23]. Similarly, humans with sepsis were found to have elevated levels of α_4-integrin expressed on peripheral blood neutrophils [15]. Furthermore, in human cases of RSV infection, increased expression of the β_2-integrin Mac1 ($\alpha_M\beta_2$) was detected [42].

A peak in virus production in the lungs 7 days p.i. preceded a period of 5 days, displaying two remarkable phenomena. First, α_4- and β_2-integrin expression did not increase up to 10 days p.i. Interestingly, BRSV production at day 7 p.i. is associated with proinflammatory cytokine production in the following days [43]. Despite this proinflammatory environment, increased expression of α_4- and β_2-integrins was detected several days later, after day 10 p.i.

Secondly, an α_4- and β_2- integrin-independent type of adhesion occurred. This was marked by the fact that blocking the α_4- and β_2- integrins did not influence neutrophil rolling 10 days p.i. (Fig. 4c). The numbers of rolling cells and rolling velocities observed in the α_4- and β_2-integrin-independent adhesion of neutrophils from infected animals at this time-point were less than from uninfected animals. This would suggest that the function of these integrins in supporting rolling is unique for neutrophils from healthy calves and is taken over by other rolling receptors in the course of BRSV infection. A similar phenomenon was detected in neutrophil firm adhesion, i.e. the inhibiting effect of blocking antibodies on firm adhesion decreased significantly 8, 9 and 10 days p.i (Figs 3c,d and 4b). Together, these findings show that BRSV infection does not increase integrin expression on neutrophils up to 10 days p.i., but may prime circulating neutrophils from day 8 to day 10 p.i. to start extravasating in a α_4- and β_2-integrin-independent manner. In this time-frame there seems to be redundancy in the CAM that may be used for neutrophil adherence.

The reduced involvement of α_4- and β_2-integrins in neutrophil rolling might have been compensated for by increased function of other CAM, such as L-selectin, as the number of rolling neutrophils (non-blocked) increased in the course of infection. Other CAM that may be involved in leucocyte firm adhesion, and possibly mediate α_4- and β_2-integrin independent neutrophil adhesion, are β_3-integrins such as $\alpha_V\beta_3$. This integrin is expressed constitutively on neutrophils and associated possibly with transendothelial migration [44]. Expression of the activation epitope on the β_1-chain tended to decrease, suggesting a shift of the active conformation to the non-active conformation during infection. This would suggest that β_1-integrins are not the missing adhesion factor in the infected animals. Alternatively, non-integrins such as ICAM-1 may be induced after BRSV infection. An indication for the possible involvement of ICAM-1 is that its expression was up-regulated on human neutrophils after RSV infection [42].

The use of a β_2-integrin-independent type of adhesion may be a general characteristic of pulmonary inflammation [45]. It was reported previously that neutrophils from acutely infected patients used a β_2-integrin-independent pathway, whereas neutrophils from chronically infected patients used a β_2-integrin-dependent type of migration *in vitro* [45]. Alternatively, α_4- and

β_2-integrin-independent migration may be a specific aspect of syncytial virus infections. Several reports on human RSV indicate a direct or indirect influence of the virus on leucocyte function. Direct incubation of viable or inactivated RSV with human neutrophils seemed to exert an activating effect as it induced production of proinflammatory chemokines [46]. In addition, the RSV G-protein – which is produced in a soluble form by bovine and human RSV [47,48] – was shown to influence IL-8 production by neutrophil granulocytes in a concentration-dependent fashion [49].

In conclusion, the results of the present study show that, to a large extent, neutrophils from healthy calves use α_4- and β_2-integrins for adhesion to activated pulmonary endothelial cells *in vitro*. The majority of firm adhesion interactions are mediated by these integrins. In addition, both integrins support neutrophil rolling. BRSV infection of the lungs induces an α_4- and β_2-integrin-independent type of adhesion in circulating neutrophils. Only at 12 days after infection do α_4- and β_2-integrins tend to reclaim their prominent role in adhesion, which is associated with an increase in expression of both integrins. These results demonstrate a dynamic use of α_4- and β_2-integrins by neutrophils for adhesion and migration, which is induced in the course of BRSV infection.

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