Comparative Effects of Antilactoferrin Antibodies and Tumor Necrosis Factor on Neutrophil Adherence to Matrix Proteins

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Received 21 September 1998/Returned for modification 5 November 1998/Accepted 26 January 1999

Neutrophil adherence to matrix proteins likely plays an important role in inflammatory responses. Antineutrophil cytoplasm antibodies may activate neutrophils in certain diseases. Using an in vitro method that allows simultaneous quantitation of neutrophil adherence and superoxide secretion, we compared the effects of antibodies against neutrophil granule proteins and tumor necrosis factor alpha (TNF-α), a known neutrophil agonist. Antilactoferrin antibodies but not antielastase or antimyeloperoxidase antibodies stimulated increased adherence to fibronectin and laminin similar in degree to that induced by TNF-α. This, but not the simultaneous superoxide secretion, was inhibited in the presence of anti-CD18 antibodies. Humoral immune responses to lactoferrin, likely expressed on the neutrophil surface, can activate neutrophils in proinflammatory responses that may be pathogenic.

The accumulation of neutrophils (polymorphonuclear leukocytes [PMNs]) in inflammatory sites may be due in part to interaction of PMNs with extracellular matrix proteins such as fibronectin, laminin, and collagen (4, 11). Several in vitro models for the assessment of such adherence have been described (3, 4). We have used a relatively simple method to reliably quantitate PMN adherence to plastic wells coated with individual proteins (20). With this approach, we have reported that stimuli such as tumor necrosis factor alpha (TNF-α) and the phorbol ester phorbol myristate acetate will stimulate increased PMN adherence to fibronectin, less adherence to laminin, but no increased adherence to collagen. Adherent PMNs appear to be more activated, with the release of some intracellular contents.

Such intracellular contents may be transported to the surface membranes of PMNs, particularly when these cells are activated by other stimuli (17). In such cases, these PMNs may be activated further following incubation with autoantibodies directed against certain intracellular components. In recent years, there has been considerable investigation of the possible activation of PMNs when they are exposed in the blood to autoantibodies against neutrophil cytoplasmic components (ANCAs) in certain diseases. In Wegener’s granulomatosis, the ANCAs are directed mainly against a tryptic proteinase called PR3 (12). In several types of vasculitis, the ANCAs more commonly react with neutrophil myeloperoxidase (MPO) (9, 12). In some individuals with inflammatory bowel diseases, the ANCAs may be autoantibodies against chymotrypsin and against lactoferrin, an iron-binding protein present in the secondary granules of PMNs (6, 10). In contrast, the ANCAs seen in the sera of some patients with rheumatoid arthritis appear to be predominantly antielastase antibodies (11).

Bartunkova et al. (2) have shown that zymosan-induced PMN chemiluminescence is enhanced by antibodies against the PR3 proteinase but is inhibited by antibodies against surface adhesion proteins CD16 and CD18. Elastase may be expressed on the surface of activated but not resting PMNs (7). However, it is not known whether there is similar transport of lactoferrin (Lf) or MPO to the PMN surface or whether antibodies directed against these components lead to increased adherence to matrix proteins. Such adherence could play an important role in the inflammatory reactions seen in the diseases in which serum ANCAs are found.

In the present study, we have investigated the effects of antibodies against (i) the neutrophil granule proteins Lf and MPO and (ii) the surface determinant CD18 on PMN adherence to matrix proteins. We have also assessed production of superoxide during this interaction of PMNs with matrix proteins as a marker of PMN activation.

MATERIALS AND METHODS

Cells. A granulocyte-rich fraction (over 95% PMNs) was obtained by density gradient centrifugation from the blood of a panel of healthy nonatopic donors receiving no medication, as described previously by us (17). PMNs were placed in replicate wells (10⁶ cells/well) of polystyrene, flat-bottom microtiter plates (Immunon-4; Fisher Scientific Co., Malvern, Pa.) coated with either human fibronectin (25 μg/ml; NV Blood Center, New York, N.Y.) or human laminin (25 μg/ml; Biomedical Technology, Inc., Stoughton, Mass.)

Incubation. To groups of four replicate wells each was added either (i) human TNF-α (2 × 10⁶ units/mg; R&D, Minneapolis, Minn.) at various concentrations; (ii) sheep anti-human Lf (anti-Lf) antibody (Dako, Carpinteria, Calif.) at various dilutions; (iii) sheep antielastase (anti-El) antibody (Binding Site, Inc., Stoughton, Mass.) at various dilutions; (iv) sheep anti-MPO antibody (Binding Site, Inc., Stoughton, Mass.) at various dilutions; (v) sheep anti-immunoglobulin G (anti-IgG) antibody (Binding Site, Inc.) diluted 1:100 (final concentration); and (vi) additional media instead of an agonist to assess spontaneously occurring PMN events (referred to as “cells alone” hereafter).

In some experiments, the effects of possibly modulation were investigated by incubating PMNs with the following combinations: (i) anti-Lf antibody plus TNF-α, (ii) anti-El antibody + TNF-α, (iii) anti-Lf plus anti-CD18 antibodies, (iv) anti-Lf plus anti-OX8 (isotype control) antibodies, (v) TNF-α and anti-CD18 antibody, (vi) anti-Lf antibody plus soluble Lf (10 μg/ml; Sigma, St. Louis, Mo.), and (vii) anti-Lf antibody plus soluble human IgG (10 μg/ml; Dako).

Cels and potential agonists were incubated in a medium of Hanks’ balanced salt solution containing cytochrome c and gelatin to permit assessment of superoxide secretion during the incubation (total reaction volume/well, 100 μl), as described previously by us (20). Superoxide secretion. After incubation at 37°C in 5% CO₂–air for 30 min, the reduction of the cytochrome c in the medium was assessed with a plate-reading...
spectrophotometer set at a wavelength of 550 nm as described previously by us (20). The superoxide levels in such wells were determined as the difference in cytochrome c reduction in the absence and presence of superoxide dismutase.

Cell adherence. The wells were then drained thoroughly and washed with warmed (37°C) buffered saline. Absolute methanol was added to each well to fix the cells adherent to the well surfaces. A solution of amido black was added to each well, and the plates were incubated at room temperature for 30 min and then the wells were drained. After thorough washing of the well 100 μl of a 10% solution of sodium dodecyl sulfate (SDS) was added to lyse the cells. The amount of released dye was quantitated by measuring the absorption by this dye at 595 nm on a plate-reading spectrophotometer.

In previous quality control studies, we had found that amido black is taken up only by adherent fixed cells and not by the matrix proteins or plastic. Quantitation of adherent cells by determination of the amount of amido black released after SDS lysis of the cells had been previously validated by comparing amido black levels in the well fluid with quantitation of amido black-containing adherent cells in replicate wells (not subjected to SDS lysis after 30 min of incubation with or without agonists at various doses) by computer-based image analysis (r = 0.95).

RESULTS

Studies in fibronectin-coated wells. (i) Adherence studies. As expected, TNF-α induced a dose-dependent increase in PMN adherence, as reflected in the increased amount of amido black released from such adherent cells after subsequent cell lysis (Fig. 1).

Incubation of other aliquots of these PMNs with anti-Lf antibody also led to a dose-dependent, significantly increased adherence compared to the level of spontaneous cell adherence (Fig. 2). In contrast, there was no increased adherence of PMNs incubated with antibodies against two other neutrophil cytoplasmic proteins (anti-El, anti-MPO) or anti-IgG antibodies; the last one was used as an antibody that might bind to IgG on the surfaces of the PMNs (Fig. 2).

(ii) Modulation of adherence. We had previously found that the increased PMN adherence to fibronectin induced by TNF-α was significantly inhibited in the presence of anti-CD18 antibody (5). Therefore, we compared the effects of adding anti-CD18 antibody to incubations of PMNs with TNF-α or anti-Lf antibody. As expected, TNF-α-induced adherence was almost completely inhibited in the presence of anti-CD18 antibody but was unaltered by similar concentrations of anti-OX8 (isotype control monoclonal antibody) (Fig. 3). A marked inhibition of the adherence-inducing effects of anti-Lf antibody was seen when anti-CD18 antibody was placed in the incubation medium (Fig. 4). Addition of the OX8 isotype control monoclonal antibody had no effect on the adherence induced by anti-Lf antibody.

The likelihood that the anti-Lf antibody was interacting with Lf on the PMN surface to induce increased PMN adherence is suggested by the observation that addition of soluble Lf (10 μg/ml) along with the anti-Lf antibody to the PMNs at the beginning of the incubation period almost completely blocked the increase in PMN adherence seen when anti-Lf antibody alone was added (Fig. 4). Addition of similar concentrations of human serum albumin and IgG to the anti-Lf antibody did not inhibit the adherence-promoting effects of anti-Lf antibody. The soluble Lf, albumin, and IgG did not stimulate adherence by themselves.

Previous studies suggested that prior stimulation of PMNs with agonists like TNF-α might induce translocation of cytoplasmic proteins such as Lf to the PMN surface, where they might be a target for interaction with anti-Lf antibody. Therefore, we compared the adherence of PMNs preincubated with TNF-α at 5 U/ml or buffered saline in siliconized tubes (to inhibit cell adherence) when these two cell populations were subsequently incubated in fibronectin-coated wells with anti-Lf
and anti-El antibodies. As shown in Fig. 5, the adherence of the PMN upon first exposure to TNF-α when anti-Lf antibody was added was somewhat (but not significantly) greater than the adherence of PMNs preincubated in buffered saline in response to added anti-Lf antibody. It turned out that these levels of adherence were similar to those in other PMN aliquots incubated with TNF-α alone. PMNs preincubated with TNF-α at 5 U/ml in siliconized tubes and then added along with anti-El antibody (diluted 1:100) to fibronectin-coated wells adhered to these wells to a degree similar to that seen when PMNs were incubated with TNF-α at 5 U/ml alone in fibronectin-coated wells (Fig. 5).

(iii) Superoxide secretion. As expected, TNF-α stimulated a dose-dependent secretion of superoxide (O$_2^-$) during the 30-min incubation period in the fibronectin-coated wells described above (Fig. 6). However, O$_2^-$ secretion was not inhibited in the presence of a concentration of the anti-CD18 antibody which had almost completely blocked PMN adherence (Fig. 6). There was a modestly increased secretion of O$_2^-$ by PMNs incubated with anti-Lf antibodies for 30 min (Fig. 6). Again, this increased O$_2^-$ secretion was not inhibited in the presence of anti-CD18 antibody. Anti-MPO and anti-El antibodies modestly stimulated O$_2^-$ secretion by suspended PMNs in siliconized tubes (2.1 ± 0.3 nM). However, such O$_2^-$ secretion was not significantly different when the incubation of these antibodies with PMNs was carried out in fibronectin-coated wells (2.3 ± 0.4 nM).

The extent of PMN adherence and O$_2^-$ generation by PMNs in the same wells was compared. There was no impressive correlation between adherence and O$_2^-$ secretion stimulated by either TNF-α (r = 0.35; P was not significant) or anti-Lf antibody (r = 0.35; P was not significant) (P values were determined by the Spearman rank order correlation).

Studies in laminin-coated wells. (i) Adherence. As expected, TNF-α stimulated increased PMN adherence to laminin-coated wells in a dose-dependent manner, although the increased adherence required TNF-α concentrations somewhat higher than those required for PMNs in fibronectin-coated wells. TNF-α at concentrations as low as 0.25 U/ml stimulated significantly increased adherence to fibronectin, with peak increased adherence (optical density, 0.30 ± 0.03) seen with TNF-α at 10 U/ml (Fig. 1). In comparison, significantly increased PMN adherence to laminin was stimulated by TNF-α, but only at concentrations of ≥5 U/ml, with peak adherence seen with TNF-α at 20 U/ml (optical density, 0.20 ± 0.03).

There was considerably increased adherence to laminin of PMNs incubated with the anti-Lf antibody diluted 1:100 (P = 0.001 versus the spontaneous adherence of these PMNs) (Fig. 7). A less prominent adherence was stimulated by a 1:500 dilution of the anti-Lf antibody, but this adherence was similar in degree to that induced by TNF-α at 5 U/ml (P = 0.01 versus spontaneous adherence). The adherence of PMNs incubated with anti-Lf antibody diluted 1:1,000 was not significantly greater than spontaneous adherence. Incubation with anti-El or anti-IgG antibodies did not induce increased PMN adherence to laminin-coated wells (Fig. 7).

(ii) Modulation of adherence. Addition of anti-CD18 antibody or soluble Lf to the incubation mixture significantly inhibited the adherence to laminin stimulated by anti-Lf antibody so that such adherence was not significantly different from the spontaneous adherence (Fig. 7).

(iii) Superoxide generation. Both TNF-α and anti-Lf antibody stimulated significantly increased the level of superoxide generation by PMNs during incubation in laminin-coated wells (3.8 ± 0.3 and 2.3 ± 0.2 nmol, respectively). This superoxide generation was not inhibited in the presence of anti-CD18 antibody. There was no significant correlation between the mean degree of adherence and the mean amount of superoxide generation in individual wells containing the agonists (r = 0.4 and 0.35, respectively; P was not significant).

DISCUSSION

The findings described here indicate that antibodies against Lf, a protein present in the secondary granules of the PMN cytoplasm, will stimulate resting neutrophils to adhere more readily to the matrix proteins fibronectin and laminin. The effect likely involves an immune interaction of the anti-Lf antibody with Lf on the PMN surface since the increased adherence is inhibited in the presence of soluble Lf. This increased adherence is likely mediated by increased expression of the beta-integrin CD11b/CD18 complex on the PMN surface since the increase in adherence was markedly inhibited in the presence of anti-CD18 antibody in a dose-dependent manner with an increase in adherence of 5 U/ml and then incubation in wells with anti-Lf antibody (diluted 1:100). There was a modestly increased secretion of O$_2^-$ by PMNs incubated in the presence of a concentration of the anti-CD18 antibody plus OX8 control antibody (A-Lf + OX8), and (iv) anti-Lf antibody (1:100 dilution) plus soluble lactoferrin (A-Lf + Lf).

![Figure 4](http://cvi.asm.org/)

**FIG. 4.** Effects of (i) anti-Lf antibody (1:100 dilution), (A-Lf), (ii) anti-Lf antibody plus anti-CD18 antibody (1:100 dilution) (A-Lf + A-CD18), (iii) anti-Lf antibody plus OX8 control antibody (A-Lf + OX8), and (iv) anti-Lf antibody (1:100 dilution) plus soluble lactoferrin (A-Lf + Lf).

![Figure 5](http://cvi.asm.org/)

**FIG. 5.** Adherence of PMNs to fibronectin-coated wells (i) alone, (ii) with TNF-α at 5 U/ml (TNF), (iii) with preincubation in TNF-α at 5 U/ml and then incubation in wells with anti-Lf antibody (1:100 dilution) (TNF + Anti-Lf), (iv) with anti-Lf antibody (1:100 dilution) (Anti-Lf), (v) with preincubation in TNF-α at 5 U/ml and then incubation in wells with anti-El antibody (1:100 dilution) (TNF + anti-Elast), and (vii) with anti-El antibody (1:100 dilution) (Anti-Elast).
ence of anti-CD18 antibodies. In contrast, antibodies against El and MPO, which are components of primary granules in the PMN cytoplasm, did not stimulate such increased adherence to fibronectin or laminin.

The increased adherence induced by anti-Lf antibodies was quite prominent, in the same range as that seen in PMNs stimulated by TNF-α, a potent PMN agonist. The TNF-α-induced adherence to fibronectin and laminin was also inhibited by anti-CD18 antibodies, suggesting similar pathways for the adherence stimulated by both agents.

Also, the adherence induced by both anti-Lf and TNF-α was accompanied by increased superoxide generation. Using the nitroblue tetrazolium assay for detection of intracellular superoxide, we have found that most of the cells containing formazan (the product formed by the reduction of nitroblue tetrazolium by superoxide) were predominantly in the PMNs adhering to fibronectin and not in the nonadherent PMNs after the 30-min incubation. Nevertheless, the superoxide generation induced by anti-Lf antibody or TNF-α was not altered when the added anti-CD18 antibodies blocked PMN adherence. It is unclear whether the superoxide generation occurs during initiation of PMN adherence or later during the 30-min incubation. Others have reported more prominent superoxide generation induced by TNF-α in PMNs previously plated on fibronectin-coated wells; this superoxide generation was not blocked by prior treatment of the adherent PMNs with an anti-CD18 antibody (15). No similar studies with antibodies against neutrophil cytoplasmic proteins have previously been reported. However, one report described increased chemiluminescence induced by anti-Lf antibodies in PMNs pretreated with the formylated tripeptide (but not in untreated PMNs); no results of adhesion studies were reported (16).

However, there is evidence that antibodies against some other cytoplasmic components of PMNs may activate PMNs. Several reports have described the generation of reactive oxygen species (ROS) by PMNs incubated with ANCA-positive sera, particularly when the PMNs were primed with stimulating concentrations of TNF-α (2, 7, 13). It was felt that this ROS production was due mainly to MPO activation (13). Previous studies have shown that anti-MPO antibodies can stimulate PMNs to damage human vascular endothelial cells in vitro (8). More recently, antibodies against the proteinase 3 found in PMN granules enhanced PMN chemiluminescence (3). ANCA-positive sera can also induce increased adherence of PMNs to the vascular endothelium and cause endothelial injury. Most ANCA-positive sera have been found to have antibody activities against proteinase 3 or MPO (9, 12). However, a minority of ANCA's have anti-Lf or anti-El activities (6, 14, 18). Therefore, our findings that anti-Lf antibodies but not
anti-El or anti-MPO antibodies stimulate PMN adherence and superoxide generation have potential clinical relevance.

The mechanisms underlying the stimulation by antibodies against proteins present in the PMN cytoplasmic granules have not been completely defined. Previous studies have shown reasonable amounts of proteinase 3 and cationic protein 57 and very small amounts of MPO on the PMN surface (7). The surface expression of these proteins is markedly increased on TNF-α-primed PMNs. Although there have been no analogous studies of the surface expression of Lf on PMNs, our preliminary flow cytometry studies with labeled anti-Lf antibodies suggest that Lf is constitutively expressed on the surface. To be sure that our process of separating the PMNs from the blood by density gradient centrifugation did not “activate” the PMNs to express more Lf on the surface, we repeated the studies on several occasions using leukocyte suspensions obtained after spontaneous gravity sedimentation in several experiments. Although we were dealing with a more heterogeneous leukocyte suspension in such experiments, there was evidence of increased adherence stimulated by anti-Lf antibodies.

It is perhaps not surprising that anti-El and anti-MPO antibodies did not stimulate increased PMN adherence to the matrix proteins. El and MPO, like other primary granule components, are secreted more into intracellular phagocytic vesicles than into the extracellular milieu (19). Therefore, there may be little constitutive El on the PMN surface. As noted above, impressive amounts of MPO were found on the PMN surface only after stimulation of the PMN by an agent like TNF-α (7).

Therefore, we can conclude from these findings that anti-Lf antibodies can stimulate PMN adherence and production of ROS as much as TNF-α, a potent agonist, can. Anti-Lf antibodies have been found in the sera of some patients with rheumatoid vasculitis, systemic lupus, ulcerative colitis, and sclerosing cholangitis (6, 14). Peen et al. (16) have described the activation of PMNs added with anti-Lf antibodies to Lf-coated endothelial cells. Therefore, it is possible that the proinflammatory activities of anti-Lf antibodies described here may play roles in the pathogenesis of these disorders.

ACKNOWLEDGMENTS

This work was supported by NIH grant RO1-AI-14332 and the Immunology Research and Education Fund.

The advice and collaboration of Steven Albelda and Paul Atkins in the initial development of the adherence assay used in the current study are greatly appreciated.

REFERENCES


