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Induction of neutrophil respiratory burst by tumour necrosis factor-alpha; priming effect of solid-phase fibronectin and intervention of CD11b-CD18 integrins

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SUMMARY

Human neutrophils, added to fibronectin (FN)-coated polystyrene wells and exposed to tumour necrosis factor-alpha (TNF-α), were found to exhibit a prolonged production of superoxide anion (O_2^-) after a lag period of approx 30 min. The O_2^- production, but not the cell adherence to FN, was completely inhibited by two MoAbs against CD18 and by a MoAb against CD11b, suggesting the involvement of CD11b-CD18 integrins in the neutrophil oxidative response. When neutrophils were induced to adhere to FN by incubation for 30 min on FN-coated surfaces and then washed to remove non-adherent cells, FN-anchored cells exhibited a rapid onset of O_2^- production in response to TNF-α. This suggests that FN primes neutrophils for the TNF-α-mediated respiratory burst. The O_2^- production by adherent neutrophils could be inhibited by anti-CD11b and anti-CD18 MoAbs only when the MoAbs were present both during the induction of adherence and during the subsequent exposure of FN-bound cells to TNF-α. The incapacity of MoAbs, added to neutrophils during the induction of adherence, to modify the characteristics of the subsequent neutrophil response to TNF-α suggests that the FN-mediated cell priming is independent of the interaction of CD11b-CD18 integrins with the FN substrate. The results are consistent with the intervention of three classes of cell receptors in the TNF-α-induced oxidative burst of neutrophils plated on FN: (i) neutrophil FN-binding sites, distinct from CD11b-CD18 and responsible for the cell priming; (ii) CD11b-CD18 integrins, absolutely required for permitting the cell triggering; and (iii) TNF-α receptors, responsible for switching on a rapid cell response in primed cells. The requirement of multiple classes of receptors for the full expression of the cell function can be envisaged as a natural precautionary measure to control the neutrophil responsiveness to TNF-α and, in turn, the TNF-α-dependent neutrophil-mediated oxidative injury at sites of inflammation.

Keywords neutrophils tumour necrosis factor-alpha integrins

INTRODUCTION

Tumour necrosis factor-alpha (TNF-α), a 17-kD protein preferentially produced by monocytes and macrophages, is presently considered a prototype of inflammatory cytokine [1]. Together with IL-1, it plays a pivotal role in induction of other endogenous inflammatory mediators [1] as well as in initiation of systemic responses to infections and inflammation [1]. One of the most relevant effects of TNF-α, that occurs at tissue sites of inflammation, is represented by the promotion of local neutrophil recruitment. In fact, TNF-α promotes the transendothelial migration of neutrophils by converting the venular endothelium to a proinflammatory-adhesive surface [2] and by activating various tissue cells for the production of neutrophil chemotaxins, mainly IL-8 [3]. Although incapable of stimulating the respiratory burst of neutrophils held in suspension, TNF-α has been shown to be a potent stimulus for oxidant production by neutrophils plated on surfaces coated with extracellular matrix proteins [4–6]. This reinforces the concept of TNF-α as a proinflammatory cytokine, rendering this protein a potential promoter of the neutrophil-mediated oxidative injury of inflamed tissues [7,8].

It has been found that neutrophils, incubated on polystyrene surfaces precoated with matrix proteins, secrete massive amounts of hydrogen peroxide in response to TNF-α after a lag period of more than 15 min [4]. The neutrophil response was shown to require binding of CD11-CD18 integrins to the solid-phase surface [9]. In fact, neutrophils from patients with genetic deficiency of CD11-CD18 integrins did not undergo a respira-

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tory burst in response to TNF-α when tested on surfaces coated with serum, fibrinogen, thrombospondin, laminin, vitronectin and fibronectin [9,10]. Moreover, the anti-CD18 MoAb IB-4, alone of 16 antibodies tested, induced a similar defect in normal neutrophils plated on surfaces coated with serum, fibrinogen, thrombospondin or laminin [9]. No defect was induced by the anti-CD18 IB-4 MoAb in normal neutrophils tested on vitronectin or fibronectin [9]. The relative contribution of TNF-α and CD11-CD18 integrins to the promotion of neutrophil oxidant release, i.e. the interplay between TNF-α and neutrophil–substrate interaction, was investigated primarily by using polystyrene surfaces coated with fetal calf serum (FCS) [4–10]. Under these conditions, the lag period does not appear to be the time taken for either TNF-α or the state of adherence to act as a priming factor in preparing the cells to display the response [4]. On the contrary, TNF-α appears to have an exceptionally prolonged period of signal transduction during which adherence-dependent signals, delivered by CD11-CD18 integrins [10], are required for the cell capacitation to undergo the respiratory burst [4].

The present study was undertaken to identify anti-CD11 and anti-CD18 MoAbs having inhibitory activity on the TNF-α-induced oxidative metabolism of neutrophils plated on surfaces coated with fibronectin (FN), and then to determine the role of the interaction between CD11-CD18 integrins and FN in the acquirement of the cell competence to exhibit the respiratory burst.

MATERIALS AND METHODS

Medium and reagents

Hanks' balanced saline solution with 1 mg/ml glucose and without phenol red (HBSS; ICN Biomed., Italy) mixed with Dulbecco's PBS (ICN Biomed., Milan, Italy) containing 1 mg/ml glucose (HBSS:PBS = 3:1), was used as incubation medium. Human lyophilized FN was purchased from Calbiochem (La Jolla; CA; lot 309352). Recombinant human TNF-α was from Genzyme Co. (Cambridge, MA). The following MoAbs were used: anti-CD18 MHM23 (Dako A/S, Glostrup, Denmark), anti-CD18 TS 1/18 (kindly provided by T. Springer, Boston, MA), anti-CD18 60.3 (kindly provided by J. Harlan [11]), anti-CD11a CLB-LFA 1/2 (kindly provided by R.A.W. VanLier, Amsterdam, The Netherlands), anti-CD11b 2LPM19C (Dako) and anti-CD11c KB90 (Dako). Superoxide dismutase (SOD, type I, bovine blood), ferricytochrome c (type VI, horse heart), phorbol myristate acetate (PMA), and N-formyl-met-leu-phe (FMLP) were from Sigma Chemical Co. (St Louis, MO). Other reagent-grade compounds were used as obtained from commercial suppliers. Endotoxin contamination of the reagents used was tested by a Limulus Amebocyte Lysate assay with a sensitivity of 10 pg/ml (Whittaker Co., Walkersville, MD). Only reagents free of detectable endotoxin were used. TNF-α was free of endotoxin as determined by Limulus assay performed by the manufacturer.

Neutrophils

Heparinized (heparin 10 U/ml) venous blood was obtained from healthy male volunteers. Neutrophils were isolated by dextran sedimentation and subsequent centrifugation on a Ficoll–Hypaque density gradient, as previously described [12]. Contaminating erythrocytes were removed by hypotonic lysis [12]. Neutrophils were then washed three times with the incubation medium and resuspended at appropriate concentrations. Final cell suspensions contained 98% or more neutrophils and more than 98% viable cells, as evaluated by the ethidium bromide-fluorescein diacetate test [13].

Superoxide anion assay

The production of superoxide anion (O_2^-) by neutrophils was measured by the SOD-inhibitable cytochrome c reduction, using a microplate reader (Titertek Twinreader Plus; Flow Labs, Irvine, UK). The assay was carried out in 96-well, flat-bottomed polystyrene plates (Primaria Plates; Falcon, Becton Dickinson, Oxnard, CA). The wells were pretreated with 50 μl of FN (1 μg/well) by incubation in 5% CO_2 at 37°C for 2 h. The plates were then flicked empty, flooded with normal saline and flicked empty again three times. Immediately after washing, 50 μl of incubation medium containing 15 nmol cytochrome c were added to each well and the temperature brought to 37°C in the microplate reader (usually within 15 min). Then, TNF-α was added (100 μl, 100 ng/ml final concentration) followed by 50 μl of cells (5×10^4 neutrophils). This number of neutrophils per well was on the linear portion of the dose-response curve for this system. When required, neutrophils were incubated for 30 min in FN-coated wells and washed to remove non-adherent cells before adding TNF-α. Experiments were carried out in triplicate, in the presence and absence of SOD. The reduction of cytochrome c was monitored (3–4 h) at intervals of time (5–15 min) by reading the plate at 550 nm. The amounts of O_2^- produced by neutrophils were determined from the OD_{550} of samples without SOD minus the OD_{550} of matched samples with SOD, using an extinction coefficient of $0{\cdot}0095 \times 10^6$ M^{-1} calculated according to Leslie [14]. Some experiments were performed with neutrophils in suspension (Falcon plastic tubes, 17 × 100 mm; Falcon Plastic, Oxnard, CA), using 1×10^6 cells and the classic SOD-inhibitable ferricytochrome c reduction method [15].

Adherence assay

Neutrophil adherence to FN was measured under conditions identical to those used for O_2^- production assay. Tests were carried out in triplicate. After incubation, non-adherent cells were removed by washing the plate twice with 0·15 M NaCl, using a Titertek microplate washer (Flow). Neutrophil adherence was detected by measuring alkaline phosphate (AP) activity of adherent cells [16]. Briefly, 100 μl of NaCl (0·15 M) plus 100 μl of a solution containing 1 mg/ml of sodium-*p*-nitrophenyl phosphate in diethanolamine buffer (1 M pH 9·8) were added to wells [16]. The optical density was monitored at intervals of time (5 min) by reading the plate for 30 min (37°C) at 405 nm (Titertek Twinreader Plus; Flow). The percentage of adherent cells was calculated using an appropriate standard curve, obtained by incubating known numbers of neutrophils with AP substrate in the same microplate.

RESULTS

Activation of O_2^- production by neutrophils plated on FN surfaces and exposed to TNF-α

When 5×10^4 neutrophils were added to FN-coated wells in the absence of stimuli, they failed to produce significant amounts of O_2^- throughout 4 h incubation (Fig. 1). The addition of TNF-α at the beginning of the assay resulted in substantial O_2^- production

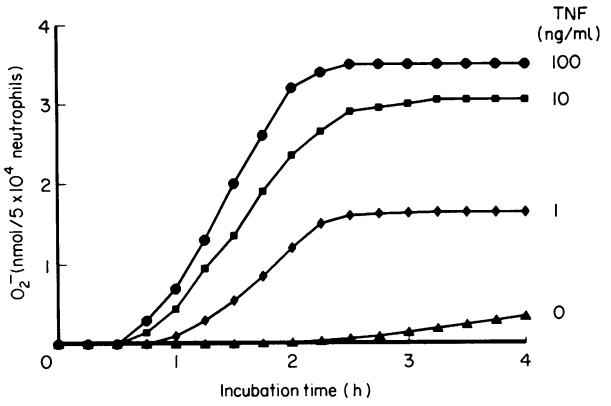

Fig. 1. Production of O_2^- by neutrophils plated in fibronectin (FN)-coated wells and exposed to different doses of tumour necrosis factor-alpha (TNF-α).

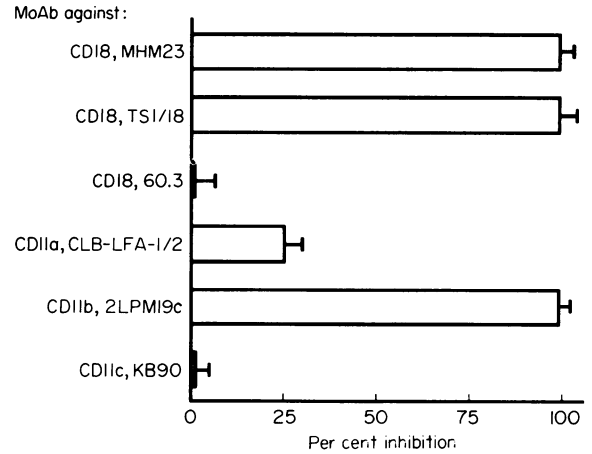

Fig. 2. Effect of anti-CD18 and anti-CD11 MoAbs on the production of O_2^- by neutrophils plated in fibronectin (FN)-coated wells and exposed to 100 ng/ml tumour necrosis factor-alpha (TNF-α). Each MoAb (2 μg/ml) was added at the beginning of the assay and immediately before the addition of TNF-α. The plateau levels of O_2^- production were chosen for comparison. Per cent inhibition was calculated in comparison with the control, carried out in absence of MoAbs, after substracting the O_2^- produced by neutrophils in the absence of TNF-α. The results are expressed as $\times \pm 1$ s.e.m. of four experiments.

(Fig. 1) after a lag period ranging from 30 to 40 min, depending on the neutrophil donor. Different doses of TNF-α induced different rates of O_2^- production, which continued for 1–2 h (Fig. 1). As the number of neutrophils/well was on the linear portion of the dose-response curve (data not shown), the cumulative quantities of O_2^- produced by neutrophils exposed to 100 ng/ml TNF-α were 50–85 nmol per 10^6 cells ($n=20$). The TNF-α dose of 100 ng/ml was chosen for subsequent experiments.

Effect of anti-CD11 and anti-CD18 MoAbs on the O_2^- production by TNF-α-triggered neutrophils

Each MoAb (2 μg/ml) was added together with TNF-α (100 ng/ml) immediately after neutrophil plating. As shown in Fig. 2, two of three anti-CD18 MoAbs completely inhibited O_2^- production. Similarly, an anti-CD11b MoAb was effective (Fig. 2). An anti-CD11c MoAb had no effect, whereas an anti-CD11a MoAb inhibited O_2^- production by approx. 25% (Fig. 2). The three MoAbs which permitted O_2^- production (60.3, CLB-LFA-1/2 and KB90) did not affect the duration of the lag period before the onset of the neutrophil response to TNF-α (data not shown). None of the MoAbs inhibited O_2^- production by neutrophils held in suspension and triggered by 10^{-7} M FMLP or 10 ng/ml PMA (inhibition $<5\%$). TNF-α could not be used because it was incapable of triggering the O_2^- production by cells in suspension. Therefore, the observed inhibitory effects of MoAbs (Fig. 2) do not seem to be due to inhibitory signals directly delivered by MoAbs. Finally, under the present conditions, more than 90% of the cells added to FN-coated wells and immediately exposed to TNF-α were adherent to the surface within 30 min of incubation. As all MoAbs used did not reduce the number of adherent neutrophils (inhibition $<7\%$), the suppression of the respiratory burst by anti-CD11b, anti-CD18 and, in part, anti-CD11a MoAbs was not due to inhibition of the cell anchorage to FN-coated surfaces. Taken together, these data suggest that neutrophil CD11b-CD18 (and in part CD11a-CD18) molecules and, by inference, their interactions with FN are strictly required for the cell response to TNF-α.

Analysis of the intervention of CD11b-CD18 in the promotion of neutrophil responsiveness to TNF-α

The lag period before the onset of O_2^- production does not appear to represent the time required for neutrophils to settle on FN surfaces, because the centrifugation of the microtitre plate immediately after the addition of the cells failed to affect the onset time of O_2^- production (data not shown). Also, the exposure of neutrophils to TNF-α for 30 min in tubes did not change the duration of the lag period, as detected when the same cell suspension was plated in FN-coated wells (data not shown). Therefore, it seems unlikely that the lag period simply reflects the time interval required for transducing the signal delivered by TNF-α. When neutrophils were incubated for 30 min on FN in the absence of stimuli, and then the wells were washed to remove non-adherent cells, nearly half of the neutrophils were found to be attached to the FN surface (per cent adherence $47{\cdot}2 \pm 3{\cdot}9, \times \pm 1$ s.e.m., $n=6$). The subsequent exposure of adherent cells to TNF-α resulted in the absence of lag period before the onset of O_2^- production (Fig. 3). The results suggest that prior contact of neutrophils with FN primes the cells for a rapid oxidative response to TNF-α. When the cells were allowed to adhere for 30 min (37°C) in the presence of anti-CD18 MoAbs (first step of the experiment, followed by washing away non-adherent cells and MoAbs), the subsequent induction of O_2^- production by TNF-α could be suppressed by re-adding MoAbs together with TNF-α (Fig. 4). When the cell adhesion to FN was carried out in the absence of MoAbs, the subsequent production of O_2^- could not be inhibited by MoAbs (Fig. 4), suggesting that the blockade of CD11b-CD18 integrins during the first step of the experiment is operative and absolutely required to inhibit TNF-triggering. As shown in Fig. 4, the addition of TNF-α (without MoAbs) to neutrophils, allowed to adhere for 30 min in the presence of anti-CD11b or anti-CD18 MoAbs and then washed to remove non-adherent cells and unbound MoAbs, resulted in a rapid onset of the O_2^- production. This suggests that the interaction of FN with CD11b-CD18 molecules expressed on the cell surface is not necessary for the acquirement of the cell capacity to mount a rapid respiratory burst to subsequent

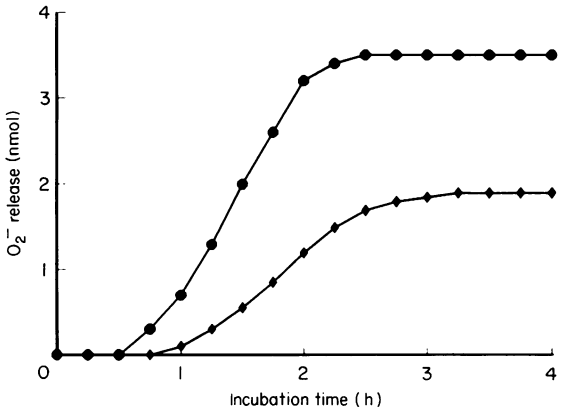

Fig. 3. Production of O_2^- by 5×10^4 neutrophils added in fibronectin (FN)-coated wells and immediately exposed to 100 ng/ml tumour necrosis factor-alpha (TNF-α) (●) and by adherent neutrophils exposed to 100 ng/ml TNF-α (◆). Adherent neutrophils were prepared by incubation of 5×10^4 cells in FN-coated wells (30 min, 37°C) followed by washing the wells twice.

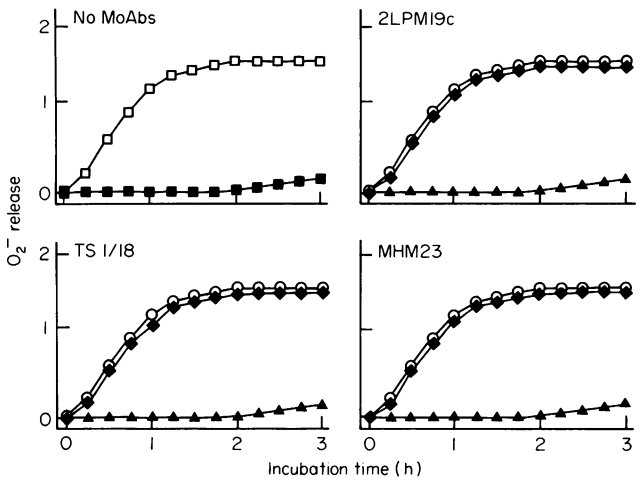

Fig. 4. Effect of the anti-CD11b MoAb 2LPM19c and the anti-CD18 MoAbs TS 1/18 and MHM23 on the production of O_2^- by adherent neutrophils exposed to 100 ng/ml tumour necrosis factor-alpha (TNF-α). Adherent neutrophils were prepared by incubation of 5×10^4 cells in fibronectin (FN)-coated wells for 30 min at 37°C (first step of the experiment), followed by washing the wells twice. The first panel (no MoAbs) shows O_2^- production by adherent neutrophils exposed (□) or not (■) to TNF-α. The other three panels show the effects of the indicated MoAb (2 μg/ml) on O_2^- production by adherent neutrophils: MoAb added during the first step of the experiment solely, i.e. during the induction of adherence (○); MoAb added together with TNF-α to adherent neutrophils, i.e. to cells induced to adhere in absence of MoAb (◆); MoAb added during the first step of the experiments and then re-added together with TNF-α (▲). The data are representative of four different experiments leading to similar results. Also, the results could be reproduced by plating 10^5 neutrophils/well and using 1 ng/ml TNF-α (data not shown).

stimulation by TNF-α. In other terms, the neutrophil priming for the cell response to TNF-α takes place independently of the CD11b-CD18 interaction with the FN surface.

DISCUSSION

The present study shows that neutrophils, plated on FN-coated surfaces and exposed to TNF-α, undergo a massive and prolonged respiratory burst after a lag period of approx. 30 min. This is in agreement with the results of other authors [4–9]. Also, we found that MoAbs against CD11b (2LPM19c) and CD18 (TS1/18 and MHM23) completely inhibit the TNF-α-induced oxidative burst without affecting the cell anchorage to FN surfaces. These data are consistent with observations made by using cells from patients with leucocyte adhesion deficiency (LAD), a genetic disease characterized by a decreased cell surface expression of CD11-CD18 integrins [17]. Neutrophils from LAD patients are indeed capable of adhering to FN surfaces [18,19] but do not secrete oxidants under these conditions [9]. Although our data do not exclude the intervention of CD11a-CD18 and/or CD11c-CD18 heterodimers, the interaction of the neutrophil CD11b-CD18 integrins with FN surfaces appears to be crucial in permitting the cell response to TNF-α. It has been previously reported that the anti-CD18 MoAb IB-4 and the anti-CD11b MoAb OKM1 do not inhibit the oxidative response of neutrophils, plated on FN and exposed to TNF-α [9,10]. Also, we found that one of three anti-CD18 MoAbs (60.3) is ineffective. It seems therefore possible that non-inhibitory MoAbs (OKM1, IB-4, 60.3) recognize epitopes of CD11b-CD18 distinct from that or those involved in the interaction with FN and obstructed by inhibitory MoAbs (2LPM19 c, TS1/18, MHM23). Alternatively, inhibitory MoAbs might bind CD11b-CD18 sites distinct from that or those involved in FN recognition, and consequently they might trigger conformational changes of integrin molecules leading to a reduced affinity for FN.

On the basis of various experiments carried out using neutrophils plated on FCS- and fibrinogen-coated surfaces, it has been suggested that the binding of CD11-CD18 integrins to the solid-phase substrate and the occupation of TNF-α receptors interact synergistically to promote the neutrophil respiratory burst [10]. The synergism between these two classes of neutrophil receptors appears to be a time-consuming process which covers at least the lag period before the onset of the respiratory burst [4,10]. Our results suggest another scenario, presumably as a consequence of the use of FN-coated surfaces. When neutrophils were incubated for 30 min on FN and then non-adherent cells were washed away, bound neutrophils underwent an immediate respiratory burst in response to TNF-α. This suggests that the neutrophil contact with FN promotes the conversion of the cells from a non-responsive to a TNF-α-responsive functional state, i.e. FN surfaces prime neutrophils for a rapid onset of the oxidative response to TNF-α. Moreover, when neutrophils were allowed to adhere in the presence of anti-CD11b or anti-CD18 MoAbs and then washed to remove non-adherent cells and unbound MoAbs, the exposure of adherent neutrophils to TNF-α plus MoAbs resulted in the complete absence of the oxidative response. This confirms the absolute requirement for CD11b-CD18 integrins in order to obtain the oxidative response to TNF-α. As the omission of MoAbs during the cell adhesion step of the experiment allowed FN-bound

neutrophils to mount the respiratory burst after the addition of TNF-α plus MoAbs, it appears that the presence of MoAbs during the time taken by neutrophils to settle and adhere, is operative and results in sufficient blockade of surface CD11b-CD18 molecules. Therefore, the addition of MoAbs during this step of the experiment can be used to test the role of CD11b-CD18 integrins in the adhesion-dependent priming of neutrophils. In this regard, neutrophils exposed to MoAbs during induction of adherence, and then washed to remove non-adherent cells and unbound MoAbs, displayed an immediate response to TNF-α. Consequently, it appears that: (i) the priming of neutrophils by FN is mediated by surface structures distinct from CD11b-CD18, possibly related to FN receptors [20,21]. Nevertheless, the identification of these structures with molecules involved in the promotion of the cell adherence to FN remains to be established; (ii) the activation of the respiratory burst of these adherent cells can be ablated by re-adding MoAbs together with TNF-α. Consistent with this finding, TNF-α has been shown to induce the surface expression of intracellular stores of CD11b-CD18 integrins [2,22], i.e. the expression of new CD11b-CD18 molecules on the neutrophil surface and their interaction with FN appears to provide a rapid and 'permissive' signal for an effective TNF-α triggering of already primed cells. The bulk of our data suggests the intervention of at least three types of neutrophil receptors in the TNF-α-induced respiratory burst of cells plated on FN surfaces. The first type of receptors is represented by FN-binding sites (distinct from CD11b-CD18), which interact with FN to promote the neutrophil priming during the lag period before the onset of the respiratory burst. The second type of receptors is represented by CD11b-CD18 integrins, able to interact with FN and deliver 'permissive' signals for the cell triggering. The third type of receptors is represented by TNF-α-receptors, whose occupation appears to rapidly switch on the oxidative response permitted by CD11b-CD18 in FN-primed cells.

This interpretation of our results does not contradict the above-mentioned dependence of the neutrophil respiratory burst on the simultaneous ligation of TNF-α receptors and CD11-CD18 integrins, showed by Nathan and co-workers using FCS- or fibrinogen-coated surfaces [4,10]. On one hand, model systems constructed with different biological substrates are likely to have individual features, and on the other hand, the differences in the results stress and contribute to the concept of individual extracellular matrix proteins as crucial determinants of the mode of response of the interacting cells [23,24]. In our setting, the interaction of neutrophils with FN in solid phase appears to result in the presentation of highly responsive cells to TNF-α. Although speculative, the intervention of at least two receptors, i.e. the aforementioned FN-binding sites and CD11b-CD18 integrins, in the acquirement of the neutrophil responsiveness, can be envisaged as a natural precautionary measure to control TNF-α-dependent neutrophil-mediated oxidative injury at sites of inflammation.

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