Generation of Signals Activating Neutrophil Functions by Leukocyte Integrins: LFA-1 and gp150/95, but Not CR3, Are Able to Stimulate the Respiratory Burst of Human Neutrophils

Giorgio Berton, Carlo Laudanna, Claudio Sorio, and Filippo Rossi

Institute of General Pathology, University of Verona, Verona, Italy

Abstract. To address the question whether leukocyte integrins are able to generate signals activating neutrophil functions, we investigated the capability of mAbs against the common β chain (CD18), or the distinct α chains of CR3, LFA-1, or gp150/95, to activate neutrophil respiratory burst. These investigations were performed with mAbs bound to protein A immobilized to tissue culture polystyrene. Neutrophils plated in wells coated with the anti-CD18 mAbs IB4 and 60.3 released H_2O_2; H_2O_2 release did not occur when neutrophils were plated in wells coated with an irrelevant, isotype-matched mAb (OKDR), or with mAbs against other molecules (CD16, β_2-microglobulin) expressed on the neutrophil surface at the same density of CD18. Four different mAbs, OKM1, OKM9, OKM10, 60.1, which recognize distinct epitopes of CR3 were unable to trigger H_2O_2 or O_2^- release from neutrophils. However, mAbs against LFA-1 or gp150/95 triggered both H_2O_2 and O_2^- release from neutrophils. Stimulation of neutrophils respiratory burst by both anti-CD18, and anti-LFA-1 or gp150/95 mAbs was totally inhibited by the microfilaments disrupting agent, cytochalasin B, and by a permeable cAMP analogue. While the capability to activate neutrophil respiratory burst was restricted to anti-LFA-1 and gp150/95 mAbs, we observed that mAbs against all members of leukocyte integrins, including CR3, were able to trigger neutrophil spreading. These findings indicate that, in neutrophils, all three leukocyte integrins can generate signals activating spreading, but only LFA-1 and gp150/95 can generate signals involved in activation of the respiratory burst. This observation can be relevant to understand the mechanisms responsible for the activation of neutrophil respiratory burst by tumor necrosis factor-alpha, which has been shown to be strictly dependent on expression of leukocyte integrins (Nathan, C., S. Srimal, C. Farber, E. Sanchez, L. Kabbash, A. Asch, J. Gailit, and S. Wright. 1989. *J. Cell Biol.* 109: 1341–1349).

THE β_2 subfamily of integrins is constituted by three heterodimeric transmembrane molecules that are all expressed by circulating leukocytes (9, 35). Expression of the individual members of the family, which are referred to as LFA-1 (CD11a/CD18), CR3 (CD11b/CD18), and gp150/95 (CD11c/CD18), varies in different cell types (35), neutrophils displaying the unique phenotype of expressing all the three leukocyte integrins, and CR3 at a higher density than LFA-1 and gp150/95 (6).

The peculiarity of CR3 is that to recognize a wide array of different molecules, a property that underlies its involvement in adhesion of phagocytes to endothelium (11, 17) and fibrinogen (45), uptake of parasites (8, 29-33, 42, 44, 46), and activation of the coagulation cascade (1, 2). Also, the recognition repertoire of both LFA-1 and gp150/95 indicate that these molecules certainly play a role in mediating neutrophil functions in the inflammatory site. LFA-1 has been established as the counter-receptor for ICAM-1 and ICAM-2 expressed by endothelial cells (19, 37). Recent data showed that gp150/95 also binds to a molecule induced on endothelial cells by IL-1 or endotoxin (36). Furthermore, both molecules, together with CR3, act as an endotoxin receptor (42), and bind *Histoplasma capsulatum* (7, 26). gp150/95 has been reported to bind fibrinogen (18) and possibly C3bi (14, 21).

Whether ligation of the three leukocyte integrins expressed by neutrophils is able to deliver a signal able to activate selective neutrophil functions is unknown. Studies performed on CR3 have indicated that this molecule, and hence possibly also LFA-1 and gp150/95, acts mainly as an adhesion molecule that does not trigger signals activating selective cell functions such as phagocytosis, release of toxic oxygen molecules, or secretion (25, 34, 40, 47). However, early studies on mononuclear phagocytes (reviewed in reference 41), subsequently extended to neutrophils (43), showed that uptake of C3bi-coated erythrocytes can be converted in a true phagocytic event. Furthermore, it has been demonstrated that the generation of hydrogen peroxide by adherent

neutrophils in response to tumour necrosis factor-alpha (TNF)[1] is strictly dependent on expression of leukocyte integrins (24).

Studies on the role of leukocyte integrins in generation of hydrogen peroxide in response to TNF did not allow one to conclude whether ligation of leukocyte integrins renders neutrophils responsive to TNF or TNF activates these molecules to generate a signal able to activate the respiratory burst (16, 22, 24). We therefore decided to address the question whether CR3, LFA-1, and gp150/95 per se are able to activate selective neutrophil functions by studying the effects of mAbs directed against distinct epitopes of the common β chain or the α chain of individual members of the family.

We show in this paper that two mAbs directed against the β chain of leukocyte integrins trigger neutrophil respiratory burst. Studies with mAbs directed against the α chain of CR3, LFA-1, and gp150/95 allowed us to establish that only anti-LFA-1 and gp150/95 mAbs are able to trigger neutrophil respiratory burst while mAbs directed against distinct epitopes of the CR3 α chain have no effect. Studies with inhibitors enabled us to demonstrate that signals generated by LFA-1 and gp150/95 require an intact cytoskeleton and are negatively regulated by an elevation of intracellular cAMP.

Materials and Methods

Binding of mAbs to Protein A Immobilized to Plastic

The list of mAbs used for these studies is reported in Table I. mAbs were kindly donated by Dr. S. D. Wright, The Rockefeller University (New York, NY) (IB4), Dr. P. Rao, Robert Wood Johnson Pharmaceutical Research Institute (Raritan, NJ) (OKM1, OKM9, OKM10), Dr. E. Plata, Oncogene (Seattle, WA) (60.3, 60.1), Dr. G. Trinchieri and B. Perussia, Wistar Institute for Anatomy and Cell Biology (Philadelphia, PA) (BB.M1, B66.6, 3G8), AIDS Research and Reference Reagent Program, National Institutes of Health (Bethesda, MD) (SIM 4), or obtained from commercial sources (see Table I). All the mAbs were used as purified preparations and immobilized to tissue culture plastic coated with protein A, purified from culture medium of a protein A secreting *Staphylococcus aureus* (Sigma Chemical Co., St. Louis, MO), or protein G, purified from group C *Streptococcus* (Sigma Chemical Co.). 96-well plates of tissue culture grade polystyrene (Nunc, Roskilde, Denmark) were treated for 30 min at room T with 50 μl of 0.1 mg/ml poly-L-lysine (Sigma Chemical Co.) in PBS, and, after washing with PBS, for 15 min at room T with 2.5% EM grade glutaraldehyde in PBS, as originally described by Michl et al. (20). 50 μl protein A or protein G at 0.1 mg/ml in PBS were added to poly-L-lysine-glutaraldehyde-treated wells and the plates left at 4°C for 5–8 h. Glutaraldehyde-free groups were quenched by overnight incubation with 2 mg/ml casein in PBS, and after washing with PBS, 50 μl mAbs suspended, at different concentrations, either in PBS or 1 M NaCl-50 mM Tris, pH 8, were added (see below for further details). After incubation for 2–3 h at 4°C with the mAbs solutions or PBS as control, plates were washed twice with PBS, incubated further for 2 h with 50 μl FCS, and finally washed three times with PBS. All the procedures described above were done with reagents diluted in endotoxin-free water for clinical use and in sterile conditions. Triplicate wells were prepared, as described, for assays of hydrogen peroxide (H_2O_2) or superoxide anion (O_2^-) release and another set, also in triplicate, for measuring binding of mAbs. The last step of quenching of the wells with FCS, after binding of the mAbs, was required to suppress the vigorous H_2O_2 release which occurs upon interaction of neutrophils with plain polystyrene (22) which was not suppressed by quenching with casein; previous studies showed that suppression of this neutrophil response can be achieved only with some serum or extracellular matrix proteins and not with albumin or elastin (22). Preliminary experiments were performed to define optimal and reproducible conditions of binding. Comparisons were made between binding of the different mAbs to protein A or protein G in PBS, to protein A in different buffers (PBS versus 1 M NaCl-50 mM Tris, pH 8), and to protein A in PBS at different concentrations. We observed that binding to protein A compared to protein G varied, but somehow independently of the theoretical expectancy on the basis of mAb subclasses, and that dilutions of mAbs in different buffers did not affect the extent of binding to protein A. However, binding to protein A of mAbs of different subclasses could be optimized by using different concentrations of mAbs. On the basis of these experiments we routinely used PBS solutions of 10, 15, or 50 μg/ml for mAbs of the IgG2a, IgG2b or IgG1 subclass, respectively. Comparison of binding to mock-treated wells where protein A was not immobilized showed that binding of the different mAbs used to casein-quenched poly-L-lysine-glutaraldehyde-derivatized polystyrene was not above 25% of the binding to protein A. Binding of mAbs was detected by ELISA with HRP-conjugated goat anti-mouse Igs (Amersham International plc, Amersham, UK) diluted 1/2,000 in PBS containing 1 mg/ml BSA, and revealed by using *o*-phenylene-diamine as substrate. Absorbance was read at 492 nm with an automated microplate reader (model EL34; Bio-Tec Instruments, Inc., Winooski, VT).

Table I. mAbs Used in This Study

mAb	Subclass	Specificity	References/Sources
IB4	IgG2a	CD18	44
60.3	IgG2a	CD18	3
OKM1	IgG2b	CD11b	38
OKM9	IgG1	CD11b	38
OKM10	IgG2b	CD11b	38
60.1	IgG1	CD11b	39
IOT16	IgG1	CD11a	Immunotech S.A.
IOM11c	IgG1	CD11c	Immunotech S.A.
OKDR	IgG2a	DR	Ortho Diagnostic System
SIM4	IgG1	CD4	AIDS Research and Reference Reagent Program.
OKT4	IgG2b	CD4	Ortho
B66.6	IgG1	CD4	28
3G8	IgG1	CD16	12
BB.M1	IgG2b	β_2-microglobulin	5

Binding of mAbs to Protein A–Sepharose or Staphylococcus aureus

25–50 μl of packed protein A–Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden) were rotated for 1 h at 4°C in 500 μl 0.15 M Na-phosphate buffer, pH 8.0, containing 50 μg 60.3, 60.1, OKM1 or purified mouse Igs. At the end of the incubation, beads were washed twice with the above buffer and resuspended in a final volume of 100 μl; 80 μl of this suspension were used to stimulate H_2O_2 release (see Fig. 2). 20 μl *Staphylococcus aureus* (Pansorbin, Calbiochem Co., San Diego, CA) were rotated for 1 h at 4°C in 500 μl PBS containing 40 μg purified mouse Igs or IB4, and 2 mg/ml BSA. At the end of the incubation, bacteria were washed twice with PBS and resuspended in 60 μl; 10 μl of this suspension were used to stimulate H_2O_2 release (see Fig. 2). Binding of mAbs to immunoadsorbents was controlled by SDS-PAGE analysis.

Isolation of Neutrophils and Assays of H_2O_2 and O_2^- Release

Neutrophils were isolated from buffy coats of healthy volunteers by dextran sedimentation and centrifugation over Ficoll-Hypaque (Pharmacia LKB Biotechnology). Contaminating erythrocytes were lysed by hypotonic saline, and then neutrophils washed with HBSS and finally resuspended in HBSS containing 0.5 mM $CaCl_2$ and 5 mM glucose. All the above procedures were done in sterile conditions and by using reagents prepared in endotoxin-free water for clinical use. Wells coated with mAbs were filled with 150 μl of Hanks'-$CaCl_2$-glucose containing 2 mM NaN_3, 0.8 mM homovanillic acid and 20 μg/ml HRP for assays of H_2O_2 release, or with the same volume of Hanks'-$CaCl_2$-glucose containing 2 mM NaN_3 and 100 μM ferricytochrome c for assays of O_2^- release. For assay of H_2O_2 release, plates were kept at 37°C in 95% air/5% CO_2 for 5 min and then 50 μl of neutrophil suspensions of 4×10^6 cells/ml were added, and the

1. *Abbreviations used in this paper*: CR3, complement receptor type 3; ICAM, intercellular adhesion molecule; LFA, leukocyte function-associated antigen; TNF, tumor necrosis factor.

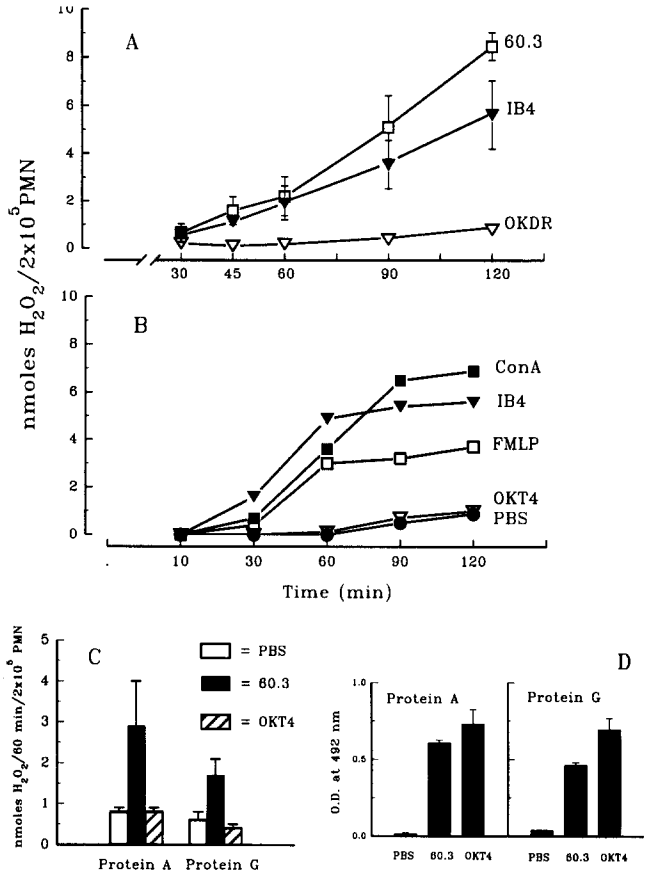


Figure 1. mAbs against CD18 trigger H_2O_2 release from neutrophils. (*A*) mAbs were bound to protein A-coated polystyrene plates as described in Material and Methods; the indicated mAbs were used at a concentration of 10 μg/ml. The mean results ± SD of three independent experiments are reported. (*B*) H_2O_2 release was assayed in the presence of 100 nM FMLP or 50 μg/ml ConA in polystyrene plates coated with protein A and mock-treated with PBS instead of IB4 or OKT4. One representative of three independent experiments is reported. (*C*) H_2O_2 release was assayed in polystyrene plates coated with protein A or protein G. Mean results ± SD of three independent experiments are reported. (*D*) Binding of 60.3 and OKT4 to protein A and protein G in the experiments shown in *C*.

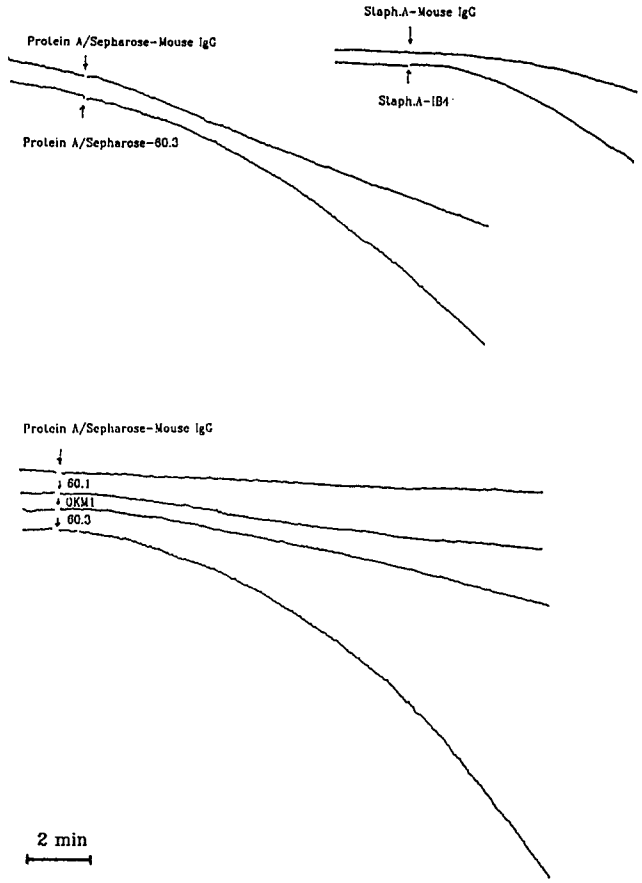


Figure 2. 60.3 bound to protein A-Sepharose and IB4 bound to *Staphylococcus aureus* trigger H_2O_2 release from neutrophils. 0.5 × 10^6 neutrophils in 2 ml of the reaction mixture used to measure H_2O_2 generation (see Materials and Methods) were maintained in suspension by magnetic stirring in the cuvette of a LS-5 luminescence spectrometer. Fluorescence changes were monitored before and after addition of immunoadsorbents coated with different antibodies.

incubation prolonged for different times in the same conditions. At the end of the incubation, 150 μl were withdrawn from each well, diluted in 2 ml of PBS, and read in a LS-5 Perkin Elmer luminescence spectrometer with an excitation wavelength of 315 nm and an emission one of 425 nm. The amounts of H_2O_2 produced were quantified from a standard curve done with known amounts of H_2O_2 diluted in the same volume of the reaction mixture described above and incubated in the conditions used for assays with neutrophils. For the measurement of O_2^- release, plates were incubated in an automated EL34 microplate reader (Bio-Tec Instruments) with the compartment T set at 37°C. Absorbance was recorded every 5 min and up to 90–120 min at wavelengths of 550 and 468 nm. Differences in absorbance at the two wavelengths were used to calculate nmol of O_2^- produced by using an extinction coefficient of 24.5 mM (4).

Photomicrographs

10-mm diameter glass coverslips were placed in 24-well plates and coated with different mAbs as above described for tissue culture plates. 1 × 10^6 neutrophils were plated on mAb-coated coverslips and after incubation for 60 min at 37°C in 95% air/5% CO_2, fixed by addition of cold formaldehyde at a final concentration of 3.7%. After 30 min at room T, cells were washed with PBS and dehydrated in alcohols. After clarification with benzene, coverslips were mounted up side down on glass slides in Entelan. Slides were analyzed at phase contrast with a Zeiss RA microscope equipped with a Zeiss MC 63C photomicrograph's apparatus.

Results

mAbs against the β Chain (CD18) of Leukocyte Integrins Trigger H_2O_2 Release from Neutrophils

To address the question whether leukocyte integrins can activate neutrophil respiratory burst, we first tested the effects of the two anti-CD18 mAbs, 60.3 and IB4. 60.3 and IB4 were bound to protein A immobilized to plastic to enhance the probability that the Ag-binding site of the mAbs was free to interact with the CD18 molecule and to reduce the probability that mAbs could interact with neutrophil through Fc receptors. The procedure used to bind mAbs of different subclasses to protein A is detailed in Materials and Methods.

As shown in Fig. 1, when neutrophils were plated in wells in which 60.3 or IB4 were bound to immobilized protein A, they released H_2O_2. H_2O_2 release was time-dependent, continued up to 120 min and then ceased (not shown). Similar

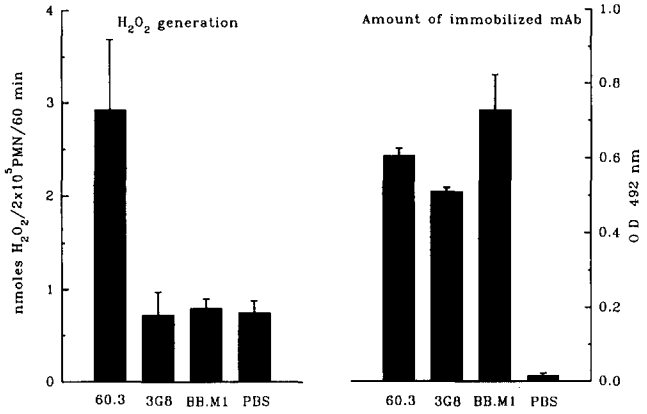


Figure 3. mAbs against CD16 and β2-microglobulin do not trigger H_2O_2 release from neutrophils. 60.3, BB.M1, and 3G8 were bound to protein A-coated polystyrene plates at concentration of 10, 15, and 30 μg/ml, respectively. The mean results ± SD of three independent experiments are reported.

results were obtained by assaying O_2^- generation (see, for example, Fig. 6). The isotype matched, IgG2a, anti-DR mAb, OKDR (Fig. 1 *A*), or the IgG2b mAb, OKT4 (Fig. 1 *B*), did not trigger H_2O_2 release, thus excluding any possible contribution of an Fc-dependent stimulation. Incubation of protein A-coated wells with 60.3 or IB4 at different concentrations showed that stimulation of neutrophil H_2O_2 depended on the amount of immobilized mAbs (not shown); a maximal effect was observed by treating protein A-coated wells with 50 μl of a 10-μg/ml solution, and this concentration was used in the experiments described below.

Interaction of neutrophils with immobilized anti-CD18 mAbs was accompanied by the release of quite elevated

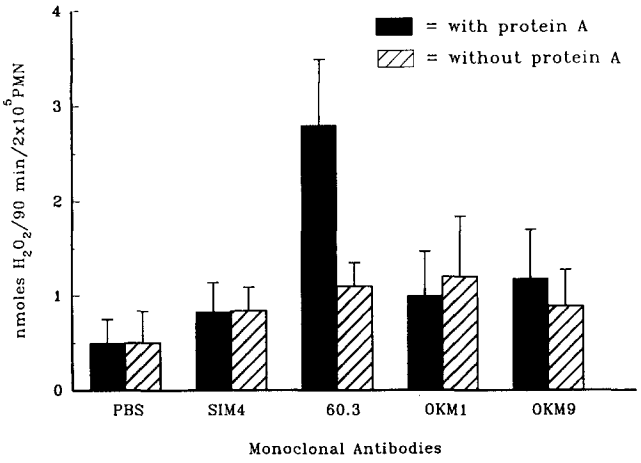


Figure 4. mAbs against the α chain of CR3 do not trigger H_2O_2 release from neutrophils. For binding to protein A- or mock(casein)-coated polystyrene plates, 60.3 was used at 10 μg/ml, OKM1 at 15 μg/ml, and SIM4 and OKM9 at 50 μg/ml. The mean results ± SD of three independent experiments are reported. Binding of mAbs was revealed in ELISA with HRP-conjugated goat anti-mouse Igs. Binding to protein A-coated wells or to casein-coated wells (values in parenthesis), in OD at 492 nm, was: 0.020 ± 0.010 (0.018 ± 0.010) for PBS; 0.529 ± 134 (0.106 ± 0.055) for SIM4; 0.538 ± 0.069 (0.163 ± 0.079) for 60.3; 0.550 ± 0.122 (0.288 ± 0.091) for OKM1; and 0.449 ± 0.168 (0.131 ± 0.068) for OKM9.

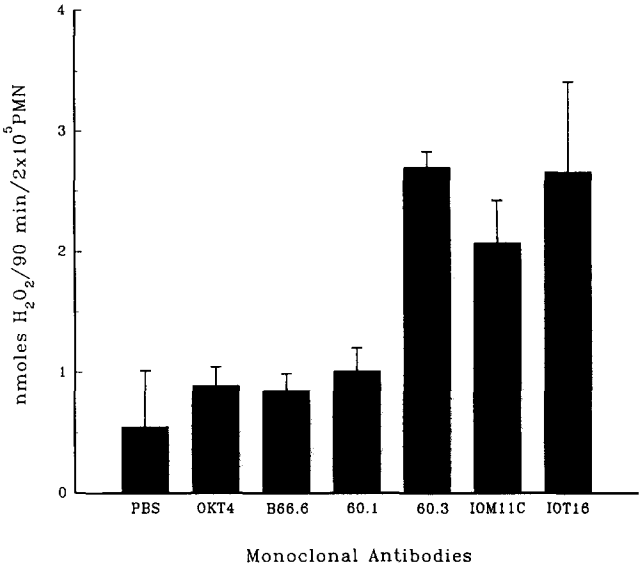


Figure 5. mAbs against LFA-1 and gp150/95 trigger H_2O_2 release from neutrophils. For binding to protein A-coated polystyrene plates 60.3 was used at 10 μg/ml and all the other mAbs at 50 μg/ml. The mean results ± SD of three independent experiments are reported. Binding of mAbs was revealed as described in Fig. 4; OD at 492 nm were: 0.018 ± 0.010 for PBS; 0.397 ± 0.035 for OKT4; 0.410 ± 0.050 for B66.6; 0.422 ± 0.045 for 60.1; 0.386 ± 0.042 for 60.3; 0.394 ± 0.041 for IOM1lc; and 0.400 ± 0.037 for IOT16.

amounts of H_2O_2. In the experiments presented in Fig. 1 (*A*), in assays, performed in parallel, with concentrations of PMA (10 ng/ml) that were maximally stimulatory in our experimental conditions, the release of H_2O_2 reached a maximal level of 20.2 ± 1.5 nmol. Therefore, release of H_2O_2 upon interaction with anti-CD18 mAbs reached 30–40% of that triggered by the most powerful stimulus of neutrophil respiratory burst. As shown in Fig. 1 (*B*), optimal concentrations of F-Met-Leu-Phe or Con A stimulated the production of amounts of H_2O_2 comparable to those produced in response to anti-CD18 mAbs. Fig. 1 (*C*) also shows that the anti-CD18 mAb, 60.3, was effective either if immobilized to protein A or protein G, the lower stimulation obtained when 60.3 was immobilized to protein G being due to the lower extent of mAb binding (Fig. 1 *D*).

The capability of anti-CD18 mAbs to trigger H_2O_2 release from neutrophils was also investigated by an alternative approach. As shown in Fig. 2, 60.3 and IB4 bound to protein A-Sepharose or *Staphylococcus aureus*, respectively, triggered H_2O_2 release from neutrophils; neither mouse control immunoglobulins, bound to both matrices, nor the anti-CR3 mAbs 60.1 or OKM1, bound to protein A–Sepharose, had any effect. The inability of anti-CR3 mAbs to trigger H_2O_2 release from neutrophils will be described in more detail in the following sections.

Comparison of the Ability of mAbs against CD18 and of mAbs against Other Surface Molecules to Trigger H_2O_2 Release from Neutrophils

The ability of anti-CD18 mAbs to trigger H_2O_2 release from neutrophils could depend on cross-linking of the CD18 molecule. Although mAbs have been widely used to identify a surface molecule as able to trigger selective cell functions,

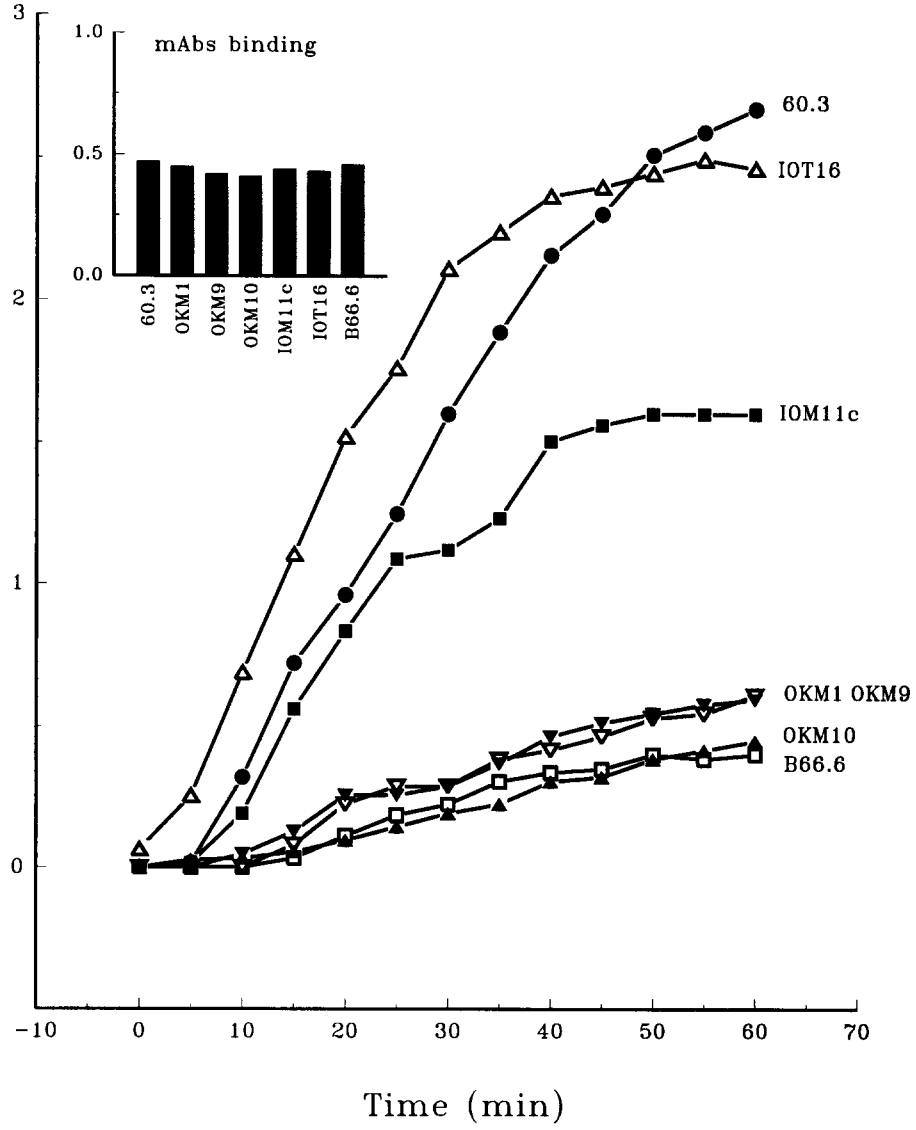


Figure 6. mAbs against LFA-1 and gp150/95 trigger O_2^- release from neutrophils. For binding to protein A–coated polystyrene plates 60.3 was used at 10 μg/ml; OKM1 and OKM10 at 15 μg/ml; and OKM9, B66.6, IOM11c, and IOT16 at 50 μg/ml. Binding of the different mAbs is shown in the inset of the figure, in OD at 492 nm. One representative of three experiments is shown.

one can not exclude that cross-linking of surface molecules by mAbs also causes nonspecific effects, including recruitment of other molecules able to generate activating signals. As a first approach to demonstrate the selectivity of anti-CD18 mAbs, we compared the capability of mAb 60.3 to trigger H_2O_2 release with that of other mAbs against neutrophils surface molecules.

We selected mAbs to be investigated on the basis of preliminary experiments on the extent of mAbs binding to the neutrophil surface. Cytofluorographic analysis showed that the anti-β_2 microglobulin mAb, BB.M1, and the anti-FcγR-III(CD16) mAb, 3G8, bound to neutrophils to an extent comparable to 60.3. Data of indirect immunofluorescence binding as mean channel fluorescence intensity in three independent experiments were 610 ± 84 for 60.3, 588 ± 10 for BB.M1, and 672 ± 106 for 3G8.

Fig. 3 shows that while 60.3 bound to immobilized Protein A triggered H_2O_2 release from neutrophils, BB.M1 and 3G8 did not cause any increase of H_2O_2 release above the background observed in wells coated with protein A and mock-treated with PBS.

These observations demonstrated that the ability of anti-CD18 mAbs to trigger H_2O_2 release is selective and ligation and/or cross-linking of two other surface molecules expressed on the surface at the same density are unable to trigger neutrophil functions.

mAbs against the α Chain of CR3 Are Unable to Trigger H_2O_2 Generation by Neutrophils

The evidence that anti-CD18 mAbs triggered H_2O_2 release from neutrophils prompted studies on the effect of mAbs against the α chain of CR3 since this is the member of leukocyte integrins expressed at the highest density on the neutrophil surface.

As shown in Fig. 2, experiments with the mAbs 60.1 and OKM1, which recognize different epitopes of the CR3 α chain, immobilized to protein A–Sepharose, already indicated that anti-CR3 mAbs are unable to trigger H_2O_2 release. Further experiments were performed with mAbs bound to protein A immobilized to tissue culture plates.

As shown in Fig. 4, neither OKM1 nor OKM9 were able to trigger H_2O_2 release from neutrophils. The lack of effect of the two mAbs was not due to a decreased binding of anti-CR3 mAbs; in fact, as reported in figure 4 legend, compara-

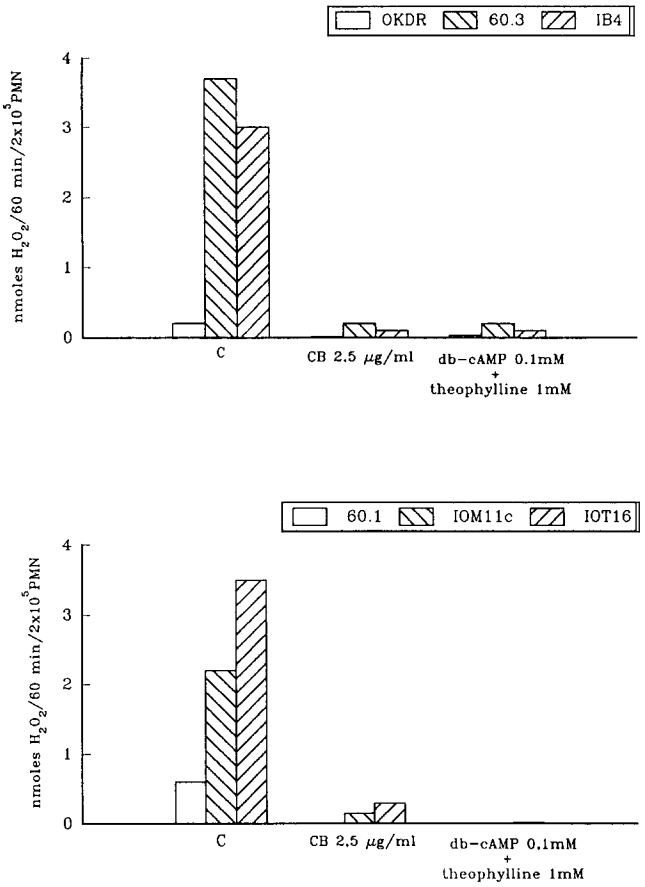


Figure 7. Stimulation of H_2O_2 release from neutrophils by anti-CD18 and anti-LFA-1 and gp150/95 mAbs requires assembly of microfilaments and is inhibited by a cAMP analogue. mAbs were bound to protein A-coated polystyrene plates as described in the legends to Figs. 4–6. Assays were performed as described in Materials and Methods in the presence of the indicated concentrations of inhibitors. One representative of three experiments is reported.

ble amounts of the different mAbs were bound to immobilized protein A.

In the experiments whose results are reported in Fig. 4, we also included another control to demonstrate that triggering of H_2O_2 release from mAb 60.3 is due to interaction of its Ag-binding site with CD18. In fact, immobilization of mAb 60.3 to plastic at random, i.e., in the absence of protein A did not cause any significant effect. The capabilities of OKM1 and OKM9 or the irrelevant mAb SIM4 to trigger H_2O_2 release were negligible either if immobilized through the Fc fragment to protein A or to plastic at random.

Also the effects of mAbs against other epitopes of the CR3 α chain were tested. As described in Figs. 5 and 6, neither 60.1 nor OKM10 had the capability to trigger H_2O_2 or O_2^- release, respectively.

mAbs against the α chain of LFA-1 and gp150/95 Trigger H_2O_2 and O_2^- Release from Neutrophils

The evidence that anti-CD18 mAbs but not anti-CR3 mAbs triggered H_2O_2 release, prompted studies to demonstrate whether the effects of anti-CD18 mAbs depended on the ligation of LFA-1 and gp150/95, the other two members of leukocyte integrins expressed, albeit at low density, by neutrophils.

The results presented in Figs. 5 and 6 clearly show that this is the case. As shown in Fig. 5, mAbs IOT16 and IOM11c, which are against the α chain of LFA-1 and gp150/95, respectively, triggered H_2O_2 release with the same efficiency of anti-CD18 mAbs. Again, an irrelevant, isotype matched, IgG1 mAb (SIM4), and an isotype matched, anti-CR3 mAb (60.1) had no effect. As shown in the Fig. 5 legend, the different ability of anti-CR3 or anti-LFA-1 and gp150/95 mAbs to trigger H_2O_2 release was not due to a different extent of binding to immobilized protein A.

Fig. 6 shows kinetics of O_2^- release from neutrophils plated in wells in which different mAbs had been bound to protein A. Clearly, anti-CR3 mAbs recognizing different epitopes of the α chain were unable to trigger O_2^- release. Both anti-LFA-1 and anti gp150/95 mAbs were effective in triggering O_2^- release.

Triggering of H_2O_2 Release from Neutrophils by Anti-CD18 and Anti-LFA-1 and gp150/95 mAbs Requires Integrity of Microfilaments and Is Blocked by Elevation of Intracellular cAMP

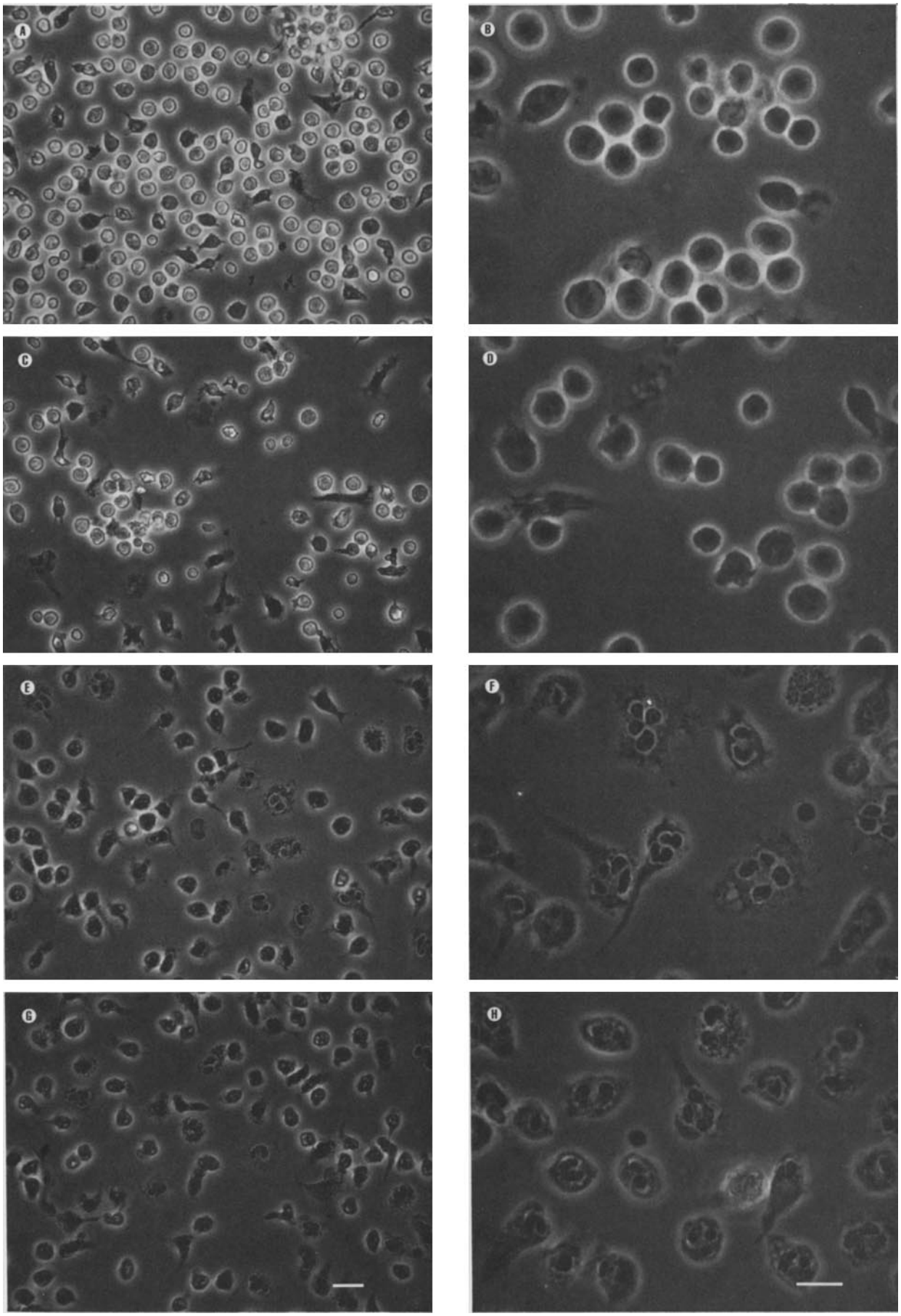
Activation of the neutrophil capability to produce H_2O_2 and O_2^- shows differential sensitivity to various drugs on the basis of the activating stimulus used. Recent studies on release of toxic oxygen molecules by neutrophils adherent to biological surfaces in response to TNF have shown that this type of stimulus displays an absolute sensitivity to the microfilaments' disrupting agent cytochalasin B, and to elevation of intracellular cAMP (16, 22, 23).

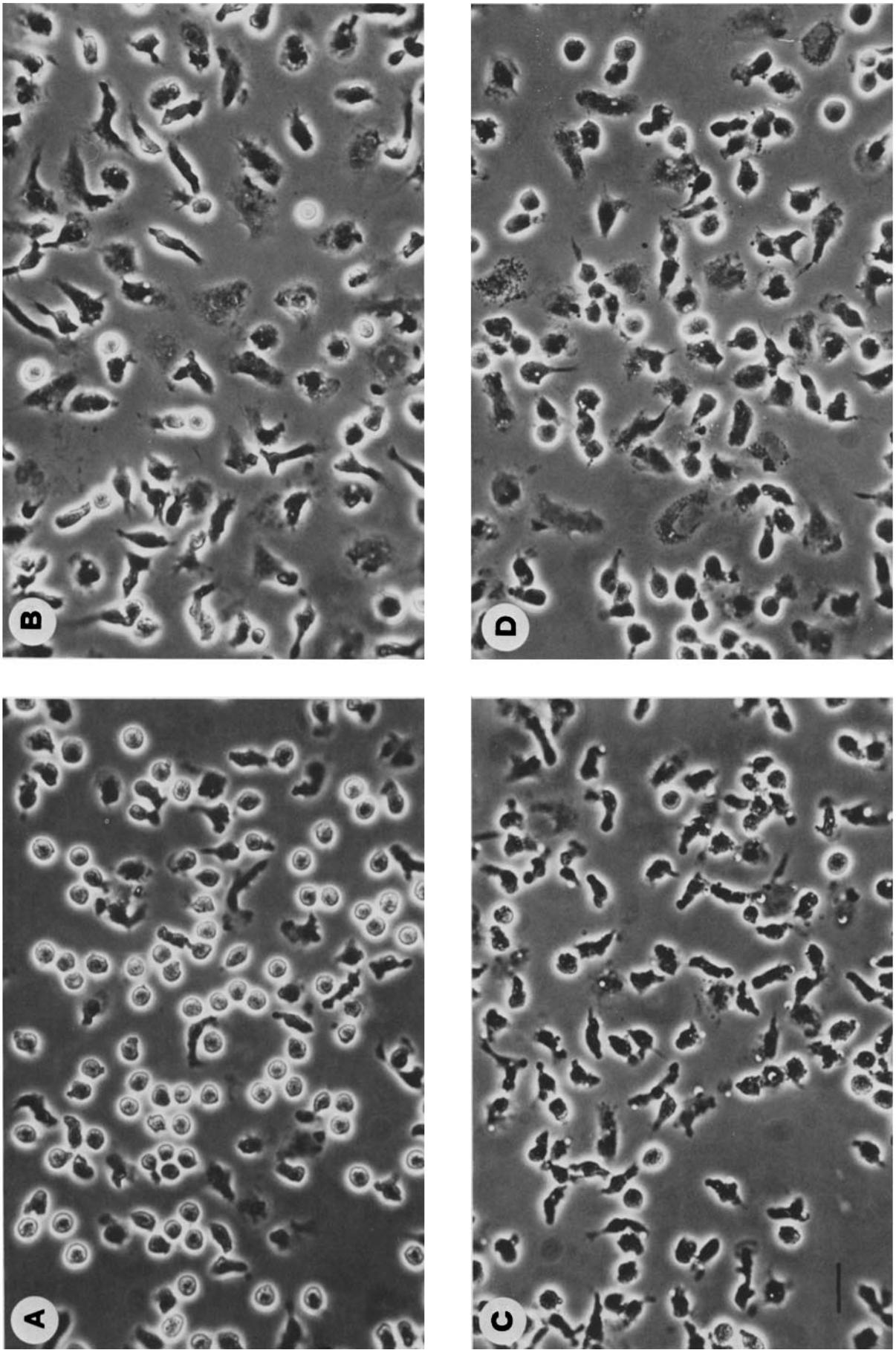
As shown in Fig. 7, triggering of H_2O_2 release by anti-CD18 and anti-LFA-1 and gp150/95 mAbs was completely blocked by cytochalasin B and dibutyryl cAMP in the presence of 1 mM theophylline. As expected from well established observations (see references 10, 23), in parallel experiments (not shown), cytochalasin B enhanced the response to F-Met-Leu-Phe, and dibutyryl cAMP did not affect the response to phorbol esters.

Interaction of Neutrophils with Antileukocyte Integrins mAbs Causes Cell Spreading

Fig. 8 and 9 show images of neutrophils plated on different mAbs. It can be seen that on protein A alone, as well as on an irrelevant mAb, cells appear rounded and simply settled on the bottom of the wells. In these conditions, neutrophils

Figure 8. mAbs against CD18 and CR3 trigger neutrophil spreading. Glass coverslips were coated with protein A and used to bind: *A* and *B* nil (PBS); *C* and *D*, SIM4; *E* and *F*, 60.3; and *G* and *H*, OKM1. mAbs were used at the concentrations indicated in the legends to Figs. 4–6. Bars, 10 μm.





can be easily detached from the bottom of the wells by washing.

Upon settling on an anti-CD18 mAb (Fig. 8, *E* and *F*), neutrophils underwent a marked spreading; spread cells appear mainly rounded with some of them showing extended pseudopods (Fig. 8 *F*).

As shown in Fig. 1 (*G* and *H*), the anti-CR3 mAb, OKM1, also induced spreading. Also, mAbs against other epitopes of the CR3 α chain, such as OKM9 and OKM10, had the same effect (not shown). Fig. 9 shows that spreading also occurred when neutrophils were plated in wells coated with anti-LFA-1 and anti-gp150/95 mAbs. On the basis of what we observed in the whole course of these studies, we believe that the extent of spreading on anti-CD18 mAbs is more pronounced than on anti-CR3, or anti-LFA-1 and gp150/95 mAbs; neutrophils spread on mAbs against distinct α chains of leukocyte integrins were more rounded and showed less elongated pseudopods (see Figs. 8 and 9).

These observations show that mAbs against all the three members of leukocyte integrins are able to trigger neutrophil spreading. Thus, the capability of antileukocyte integrins mAbs to trigger release of toxic oxygen molecules does not correlate in a simple way with induction of spreading; anti-CR3 mAbs generate signals able to activate cell spreading, anti-LFA-1 and gp150/95 generate signals able to activate both spreading and respiratory burst.

Discussion

By using mAbs recognizing epitopes of the common β chain or the distinct α chains of leukocyte integrins, we have demonstrated that ligation and/or cross-linking of LFA-1 and gp150/95, but not CR3, triggers signals activating neutrophil respiratory burst. Several observations allow us to conclude that the effects of anti-LFA-1 and gp150/95 mAbs are selective and due to binding of the Ag-binding site of the mAbs. In fact, (*a*) irrelevant mAbs (SIM4, B66.6) matched for the same subclass (IgG1) of the stimulatory anti-LFA-1 mAb, IOT16, and anti-gp150/95 mAb, IOM11c, were unable to activate neutrophil respiratory burst; (*b*) two anti-CR3 mAbs, 60.1 and OKM9, of the same subclass of the stimulatory mAbs were also ineffective; (*c*) two mAbs, IB4 and 60.3, recognizing different epitopes of the common β chain of leukocyte integrins were able to activate neutrophil respiratory burst while either an irrelevant, isotype matched (IgG2a), mAb, OKDR, or the anti-CR3 mAbs, OKM10 and OKM1, of the IgG2b subclass, were ineffective; and (*d*) mAbs 3G8 and BB.M1, against CD16 and β2-microglobulin, respectively, were unable to activate neutrophil respiratory burst.

The evidence that mAbs against epitopes of CR3 corresponding to distinct binding sites for different ligands are unable to activate neutrophil respiratory burst is in agreement with previous observations demonstrating that CR3 mediates binding of C3bi-coated particles without triggering ingestion, secretion, and respiratory burst (25, 34, 40, 47).

Our results show that LFA-1 and gp150/95 are able to deliver signals responsible for the activation of neutrophil respiratory burst. This observation is remarkable also in light of the evidence that, compared to CR3, LFA-1 and gp150/95 are expressed at a lower density on the neutrophil surface (6). To our knowledge, no study has been performed on the capability of gp150/95 to trigger signals possibly involved in activation of neutrophil or other leukocyte functions. However, evidence has been presented that anti-LFA-1 mAbs trigger phosphoinositides turnover and cytoplasmic calcium transients in lymphocytes subsets (27).

Elegant experiments have recently demonstrated that the cytoplasmic domain of the β subunit of LFA-1 is essential for the function of this molecule in adhesion and its activation by phorbol esters (13). This observation does not contradict our finding that ligation of CR3, which most of the β chain expressed by neutrophils is associated to, does not trigger a respiratory burst. In fact, we obtained evidence that signals involved in adhesion and spreading are likely distinct from those involved in activation of the respiratory burst. As shown in Fig. 8, anti-CR3 mAbs also trigger neutrophil spreading. This is in accord with the known adhesion function of CR3 in neutrophils and activation of CR3 to a molecule able to mediate phagocytosis. As discussed above, strong evidence has been presented that signals involved in phagocytosis and activation of the respiratory burst are distinct (40, 47). Taken together, these observations do suggest that the α chain of LFA-1 and gp150/95 can interact with signaling molecules involved in activation of respiratory burst. On the basis of comparison of CR3, LFA-1, and gp150/95 sequences, Springer and colleagues pointed out that CR3 and gp150/95 are more closely related to each other (61%) than to LFA-1 (35.7 and 37.4%, respectively) (15). It is, however, worth noting that the cytoplasmic tail of CR3 is shorter (19 amino acids residues) than that of gp150/95 and LFA-1 (29 and 53 amino acid residues, respectively). Furthermore, a short stretch of the cytoplasmic tail of LFA-1 (residues 1,113–1,123) and gp150/95 (residues 1,132–1,144) shows a high degree of homology, with four conserved residues, and the presence of two serines (LFA-1) and two serines and one threonine (gp150/95) (see 15). We believe that the possibility that these two homologous cytoplasmic domains are involved in the generation of signals by LFA-1 and gp150/95 should be considered.

The evidence that LFA-1 and gp150/95 can activate neutrophil respiratory burst may be relevant to understand the mechanisms underlying the acquirement of the capability to release toxic oxygen molecule in response to TNF by neutrophils adherent to endothelial cells, or serum and extracellular matrix proteins (16, 22, 24). It has been shown that this neutrophil response to TNF is strictly dependent on expression of leukocyte integrins (24). Experimental systems used to render neutrophils able to respond to TNF with release of toxic oxygen molecules require neutrophils adhere to surfaces coated with endothelial cells, fibrinogen, fibronectin, laminin, thrombospondin (22, 24). Endothelial cells express ligand for both LFA-1 and gp150/95 (19, 36, 37); fibrinogen is one of the ligands for gp150/95 (18). None of the members of leukocyte integrins are known to recognize other adhesive

Figure 9. mAbs against LFA-1 and gp150/95 trigger neutrophil spreading. Glass coverslips were coated with protein A and used to bind: *A*, nil (PBS); *B*, 60.3; *C*, IOM11c; *D*, IOT16. Bar, 10 μm.

proteins (fibronectin, laminin, thrombospondin) which capacitate neutrophils to respond to TNF. However, studies with neutrophils of LAD patients demonstrated that also on these proteins release of toxic oxygen molecules in response to TNF is strictly dependent on the expression of leukocyte integrins (24). We therefore believe it is conceivable to suggest that TNF activates LFA-1 and/or gp150/95 to generate signals responsible for stimulation of neutrophil respiratory burst. This suggestion is also legitimated by the results of studies with inhibitors. We observed that activation of the respiratory burst by both anti-CD18 and anti-LFA-1 and gp150/95 mAbs is suppressed by the microfilaments disrupting agent cytochalasin B, and by a cAMP analogue. A similar pattern of sensitivity to cytochalasin B and cAMP analogues has been observed for the TNF-stimulated respiratory burst by adherent neutrophils (16, 22, 23).

We wish to thank Miss Federica Calzetti for her excellent technical assistance.

This work was supported by grants from Consiglio Nazionale delle Ricerche (Comitato Nazionale Biotocnologie e Biologia Molecolare, grant 90.01260.CT14), from Ministero Università e Ricerca Scientifica (Fondi 40%), and from Associazione Italiana Ricerca sul Cancro (AIRC).

Received for publication 12 August 1991 and in revised form 6 November 1991.

References

1. Altieri, D. C., and T. S. Edgington. 1987. The saturable high affinity association of factor X to ADP-stimulated monocytes defines a novel function of the Mac-1 receptor. *J. Biol. Chem.* 263:7007–7015.
2. Altieri, D. C., J. H. Morissey, and T. S. Edgington. 1988. Adhesive receptor Mac-1 coordinates the activation of factor X on stimulated cells of monocytic and myeloid differentiation: an alternative initiation of the coagulation protease cascade. *Proc. Natl. Acad. Sci. USA.* 85:7462–7466.
3. Beatty, P. G., J. A. Ledbetter, P. J. Martin, T. H. Price, and J. A. Hansen. 1983. Definition of a common leukocyte cell-surface antigen (Lp150/95) associated with diverse cell-mediated immune functions. *J. Immunol.* 131:2913–2918.
4. Bellavite, P., P. Dri, V. Della Bianca, and M. C. Serra. 1983. The measurement of superoxide anion production by granulocytes in whole blood. A clinical test for evaluation of phagocyte function and serum opsonic capacity. *Eur. J. Clin. Invest.* 13:363–368.
5. Brodsky, F. M., W. F. Bodmer, and P. Parham. 1979. Characterization of a monoclonal anti-β_2-microglobulin antibody and its use in the genetic and biochemical analysis of major histocompatibility antigens. *Eur. J. Immunol.* 9:536–545.
6. Buckle, A. M., and N. Hogg. 1989. The effect of IFN-γ and colony-stimulating factors on the expression of neutrophil cell membrane receptors. *J. Immunol.* 143:2295–2301.
7. Bullock, W. E., and S. D. Wright. 1987. Role of the adherence-promoting receptors. CR3, LFA-1 and p150,95, in binding of Histoplasma Capsulatum by human macrophages. *J. Exp. Med.* 165:195–210.
8. Cain, J. A., S. L. Newman, and G. D. Ross. 1987. Role of complement receptor type three and serum opsonins in the neutrophil response to yeast. *Complement.* 4:75–86.
9. Detmers, P. A., and S. D. Wright. 1988. Adhesion-promoting receptors on leukocytes. *Curr. Opin. Immunol.* 1:10–15.
10. De Togni, P., G. Cabrini, and F. Di Virgilio. 1984. Cyclin AMP inhibition of fMet-Leu-Phe-dependent metabolic response in human neutrophils is not due to its effects on cytosolic Ca^{2+}. *Biochem. J.* 224:629–635.
11. Diamond, M. S., D. E. Staunton, A. R. de Fougerolles, S. A. Stacker, J. Garcia-Aguilar, M. L. Hibbs, and T. A. Springer. 1990. ICAM-1 (CD54): a counter-receptor for Mac-1 (CD11b/CD18). *J. Cell Biol.* 111: 3129–3139.
12. Fleit, H. B., S. D. Wright, and J. C. Unkeless. 1982. Human neutrophil Fcγ receptor distribution and structure. *Proc. Natl. Acad. Sci. USA.* 79: 3275–3279.
13. Hibbs, M. L., H. Xu, S. A. Stacker, and T. A. Springer. 1991. Regulation of adhesion to ICAM-1 by the cytoplasmatic domain of LFA-1 integrin β subunit. *Science (Wash. DC).* 251:1611–1613.
14. Hogg, N. 1989. The leukocyte integrins. *Immunol. Today.* 10:111–114.
15. Larson, R. S., A. L. Corbi, L. Berman, and T. A. Springer. 1989. Primary structure of the leukocyte function-associated molecule-1 α subunit: an integrin with an embedded domain defining a protein superfamily. *J. Cell Biol.* 108:703–712.
16. Laudanna, C., S. Miron, G. Berton, and F. Rossi. 1990. Tumor necrosis factor-α/cachectin activates the O_2^--generating system of human neutrophils independently of the hydrolysis of phosphoinositides and the release of arachidonic acid. *Biochem. Biophys. Res. Commun.* 166:308–315.
17. Lo, S. K., G. A. Van Seventer, S. M. Levin, and S. D. Wright. 1989. The leukocyte receptors (CD11a/CD18 and CD11b/CD18) mediate transient adhesion to endothelium by binding to different ligands. *J. Immunol.* 143: 3325–3329.
18. Loike, J. D., B. Sodeik, L. Cao, S. Leucona, J. I. Weitz, P. A. Detmers, S. D. Wright, and S. C. Silverstein. 1991. CD11c/CD18 on neutrophils recognizes a domain at the N terminus of the Aα chain of fibrinogen. *Proc. Natl. Acad. Sci. USA.* 88:1044–1048.
19. Marlin, S. D., and T. A. Springer. 1987. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1(LFA-1). *Cell.* 51:813–819.
20. Michl, J., M. M. Pieczonka, J. C. Unkeless, and S. C. Silverstein. 1979. Effects of immobilized immune complexes on Fc- and complement-receptor function in resident and thioglycollate-elicited mouse peritoneal macrophages. *J. Exp. Med.* 150:607–621.
21. Myones, B. L., J. G. Dalzell, N. Hogg, and G. D. Ross. 1988. Neutrophil and monocyte cell surface p150,95 has iC3b-receptor (CR_4) activity resembling CR_3. *J. Clin. Invest.* 82:640–651.
22. Nathan, C. F. 1987. Neutrophil activation on biological surfaces. Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. *J. Clin. Invest.* 80:1550–1560.
23. Nathan, C., and E. Sanchez. 1990. Tumor necrosis factor and CD11/CD18 (β_2) integrins act synergistically to lower cAMP in human neutrophils. *J. Cell Biol.* 111:2171–2181.
24. Nathan, C., S. Srimal, C. Farber, E. Sanchez, L. Kabbash, A. Asch, J. Gailit, and S. D. Wright. 1989. Cytokine-induced respiratory burst of human neutrophils: dependence on extracellular matrix proteins and CD11/CD18 integrins. *J. Cell Biol.* 109:1341–1349.
25. Newman, S. L., and R. B. Johnston, Jr. 1979. Role of binding through C3b and IgG in polymorphonuclear neutrophil function: studies with trypsin-generated C3b. *J. Immunol.* 123:1839–1846.
26. Newman, S. L., C. Bucher, J. Rhodes, and W. E. Bullock. 1990. Phagocytosis of Histoplasma capsulatum yeasts and Micronidia by human cultured macrophages and alveolar macrophages. *J. Clin. Invest.* 85:223–230.
27. Pardi, R., J. R. Bender, C. Dettori, E. Giannazza, and E. G. Engleman. 1989. Heterogeneous distribution and transmembrane signaling properties of lymphocyte function-associated antigen (LFA-1) in human lymphocyte subsets. *J. Immunol.* 143:3157–3166.
28. Perussia, B., S. Starr, S. Abraham, V. Fanning, and G. Trinchieri. 1983. Human natural killer cells analyzed by B73.1, a monoclonal antibody blocking Fc receptor functions. I. Characterization of the lymphocyte subset reactive with B73.1. *J. Immunol.* 130:2133–2141.
29. Relman, D., E. Tuomanen, S. Falkow, D. T. Golenbock, K. Saukkonen, and S. D. Wright. 1990. Recognition of a bacterial adhesin by an integrin: macrophage CR3 ($\alpha_M\beta_2$, CD11b/CD18) binds filamentous hemagglutinin of Bordetella pertussis. *Cell.* 61:1375–1382.
30. Ross, G. D., J. A. Cain, and P. J. Lachmann. 1985. Membrane complement receptor type three (CR_3) has lectin like properties analogous to bovine conglutinin and function as a receptor for zymosan and rabbit erythrocytes as well as a receptor for iC3b. *J. Immunol.* 134:3307–3315.
31. Ross, G. D., J. A. Cain, B. L. Myones, S. L. Newman, and P. J. Lachmann. 1987. Specificity of membrane complement receptor type three (CR_3) for β-glucans. *Complement.* 4:61–74.
32. Russel, D. G., and P. Talamas-Rohana. 1989. Leishmania and the macrophage: a marriage of inconvenience. *Immunol. Today.* 10:328–333.
33. Russell, D. G., and S. D. Wright. 1988. Complement receptor type 3 (CR3) binds to an Arg-Gly-Asp containing region of the major surface glycoprotein, gp63, of Leishmania Promastigotes. *J. Exp. Med.* 168:279–292.
34. Scribner, D. J., and D. Fahrney. 1976. Neutrophil receptors for IgG and complement: their roles in the attachment and ingestion phases of phagocytosis. *J. Immunol.* 116:892–897.
35. Springer, T. A. 1990. Adhesion receptors of the immune system. *Nature (Lond.).* 346:425–434.
36. Stacker, S. A., and T. A. Springer. 1991. Leukocyte integrin P150/95 (CD11c/CD18) functions as an adhesion molecule binding to a counter-receptor on stimulated endothelium. *J. Immunol.* 146:648–655.
37. Staunton, D. E., M. L. Dustin, and T. A. Springer. 1989. Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. *Nature (Lond.).* 339:61–64.
38. Talle, M. A., P. E. Rao, E. Westberg, N. Allegar, M. Makowski, R. S. Mittler, and G. Goldstein. 1983. Patterns of antigenic expression on human monocytes as defined by monoclonal antibodies. *Cell. Immunol.* 78: 83–99.
39. Wallis, W. J., D. D. Hickstein, B. R. Schwartz, C. H. June, H. D. Ochs, P. G. Beatty, S. J. Klebanoff, and J. M. Harlan. 1986. Monoclonal antibody-defined functional epitopes on the adhesion-promoting glycoprotein complex (CDw18) of human neutrophils. *Blood.* 67:1007–1013.
40. Wright, S. D., and S. C. Silverstein. 1983. Receptors for C3b and C3bi

promote phagocytosis but not the release of toxic oxygen from human phagocytes. *J. Exp. Med.* 158:2016–2023.
41. Wright, S. D., and F. M. Griffin, Jr. 1985. Activation of phagocytic cells' C3 receptors for phagocytosis. *J. Leuk. Biol.* 38:327–339.
42. Wright, S. D., and M. T. C. Jong. 1986. Adhesion-promoting receptors on human macrophages recognize Escherichia coli by binding to lipopolysaccharide. *J. Exp. Med.* 164:1876–1888.
43. Wright, S. D., and B. C. Meyer. 1986. Phorbol esters cause sequential activation and deactivation of complement receptors on polymorphonuclear leukocytes. *J. Immunol.* 136:1759–1764.
44. Wright, S. D., P. E. Rao, W. C. Van Voorhis, L. S. Craigmyle, K. Iida, M. A. Talle, E. F. Westberg, G. Goldstein, and S. C. Silverstein. 1983. Identification of the C3bi receptors of human monocytes and macrophages by using monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 80: 5699–5703.
45. Wright, S. D., J. I. Weitz, A. J. Huang, S. M. Levin, S. C. Silverstein, and J. D. Loike. 1988. Complement receptor type three (CD11b/CD118) of human polymorphonuclear leukocytes recognizes fibrinogen. *Proc. Natl. Acad. Sci. USA.* 85:7734–7738.
46. Wright, S. D., S. M. Levin, M. T. C. Jong, Z. Chad, and L. G. Kabbash. 1989. CR3 (CD11b/CD18) express one binding site for Arg-Gly-Asp-containing peptides and a second site for bacterial lipopolysaccharide. *J. Exp. Med.* 169:175–183.
47. Yamamoto, K., and R. B. Johnston, Jr. 1984. Dissociation of phagocytosis from stimulation of the oxidative metabolic burst in macrophages. *J. Exp. Med.* 159:405–416.