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SHORT REPORT

Increased *in vitro* neutrophil adherence in a case of chronic idiopathic neutropenia

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Summary. In this report we describe a patient with persistent neutropenia whose neutrophils showed increased adhesion in a microplate assay. In three separate assays, from 12·5% to 13·7% of the patient's blood neutrophils exhibited spontaneous (unstimulated) adhesion to fetal bovine serum-coated microplate wells, much higher than adhesion of cells from healthy controls (1·9% ± 2·5 SD, $n = 20$). The difference of spontaneous adhesion between the patient's and control neutrophils was even higher when cells from a skin-window exudate were examined (patient: 42·1–100% adhesion; control: 3·6% ± 3·5 SD, $n = 20$). Over 80% inhibition of the increased adhesion was produced by the 60.3 anti-CD 18 monoclonal antibody, suggesting an involvement of $\beta 2$-integrins.

Keywords: neutrophils, adhesion, idiopathic neutropenia.

Chronic idiopathic neutropenia in adults is an acquired disease of unknown aetiopathogenesis whose features are normal erythrocyte, lymphocyte and platelet counts, normal or increased blood monocyte counts, severe neutropenia and a cellular bone marrow with normal myeloid maturation but lacking segmented neutrophils. No chromosomal abnormalities, splenomegaly, malignant tumours or other causes capable of accounting for the neutropenia are present. *In vitro* colony formation is normal or only slightly reduced (Dale, 1990).

We describe a case of chronic idiopathic neutropenia in a 52-year-old man whose neutrophils *in vitro* showed a high spontaneous adhesiveness. Increased adherence has been observed in circulating neutrophils and in those obtained from an inflammatory exudate. Experiments with monoclonal antibodies indicated that the abnormality may be due to $\beta 2$-integrins.

CASE REPORT AND METHODS

The patient, a 52-year-old white man, was referred to our out-patient clinic in October 1991 for investigation of a neutropenia detected by chance in 1990; thereafter neutrophil blood counts were invariably below $0{\cdot}5 \times 10^9$/l ($0{\cdot}09$–$0{\cdot}45 \times 10^9$/l). Family and previous medical histories were unremarkable. The patient was a teetotaller and a non-smoker, was taking no drugs and was on a normal diet. Hepatic function and enzymes, erythrocyte sedimentation rate, fibrinogen, complement C3 and C4 fractions and C-reactive protein were normal. Tests for rheumatoid factor, anti-nuclear and anti-neutrophil surface antibodies were negative. The bone marrow was found to be normally cellular with abundant granulocytic precursors, but with few segmented neutrophils. The number of colonies (CFU-GM) which formed in marrow cultures was high. A chest X-ray and ultrasonography of the abdomen yielded normal findings.

Neutrophils were purified from blood and skin-window exudates from the patient and healthy subjects, after obtaining their informed consent. Blood neutrophils were prepared from ethylene diamine tetraacetate-anticoagulated blood by centrifugation over Percoll gradients (Pharmacia, Uppsala, Sweden) (Metcalf *et al*, 1986). Inflammatory exudates were obtained from a 1 cm^2 skin abrasion made on the forearm with a rotating sterile abrasive cylinder operated by a milling cutter. The abrasion did not cause bleeding as only the epidermis was removed and the wet transuding surface of derma was exposed. Exudate neutrophils were isolated using disposable chambers purchased from Far-Italia (Verona, Italy) as previously described (Biasi *et al*, 1993).

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Assays of metabolism and adhesion were carried out according to a microplate method whereby both adhesion of neutrophils and release of superoxide anion (O_2^-) can be evaluated (Bellavite *et al.* 1992). Briefly, sterile 96-well microtitre plates with flat-bottomed wells (Limbro type, ICN-Flow, Milan, Italy), were pre-coated with fetal bovine serum (ICN-Flow), in order to avoid non-specific adhesion of neutrophils to plastic. After washing out the coating medium, the microplate wells were supplemented with: (a) 25 μl of Hanks balanced salt solution (Gibco, Paisley, Scotland) containing 5 mM glucose, 0·2% human serum albumin, 0·5 mM $CaCl_2$ and 1 mM $MgSO_4$ (H-GACM), for assays on resting cells, or 25 μl of the stimulatory agent 4×10^{-7} M formyl-methionyl-leucyl-phenylalanine (fMLP, Sigma, St Louis, Mo.) dissolved in H-GACM, for assays on activated cells; (b) 25 μl of 0·45 mM cytochrome c (Boehringer, Mannheim, Germany) dissolved in H-GACM; (c) 50 μl of neutrophils, suspended in H-GACM at the concentration of 3×10^6/ml. When indicated, the anti-CD 18 monoclonal antibody 60.3 (kindly provided by Dr Dobrina, University of Trieste) was added to the neutrophil suspension at a final concentration of 10 μg/ml 5 min before the addition of the cells to the assay plates. Assays were done in triplicate for each experimental condition. Plates and cells were pre-warmed at 37°C and incubations were carried out for 40 min in a humidified thermostat at 37°C. The reduction of cytochrome c was measured with a microplate reader at 550 nm as a reference wavelength. Immediately after reading cytochrome c reduction, the plates were transferred to an automatic washer and subjected to two washing cycles with PBS. Adherent cells were quantitated by measuring the membrane enzyme acid phosphatase and the percentage adhesion was calculated on the basis of a standard curve obtained with known numbers of neutrophils from the same subject (Bellavite *et al.* 1992).

The quantitation of neutrophil levels of CD11/CD18 integrins was performed by fluorescence-activated flow cytometry on whole blood. 100 μl of blood were incubated with either 15 μl of fluoresceinated anti-human LFA-1b (CD18) or 15 μl of fluoresceinated control IgG1 monoclonal antibody (fluorescence conjugate antibodies from Becton Dickinson, San José, Calif.). Treatment with monoclonal antibodies was performed for 30 min at 4°C, then erythrocytes were lysed by NH_4Cl treatment (FACS lysing solution, Becton Dickinson). The cells were washed twice with PBS at 4°C and the fluorescence was measured using a Becton Dickinson FACS-can equipped with 488 nm argon laser. Analysis was performed on 5000 events on each sample, using software Lysys-II. When indicated, a maximum expression of neutrophil integrins was achieved by pre-incubation of blood with 0·1 μg/ml phorbol-myristate acetate (PMA, Sigma, St Louis, Mo.) for 10 min at 37°C.

RESULTS

As can be seen in Table I(a), the patient's neutrophils exhibited a spontaneous (unstimulated) adhesion which was much higher than that of neutrophils from a group of 20 normal subjects. This functional difference is significant in the unstimulated cells, as the adhesion of neutrophils from normal subjects to fetal bovine serum-coated culture wells is very low. Neutrophils isolated from peripheral blood showed an appreciable increase in adhesion after stimulation with the chemotactic peptide fMLP. Under the same experimental conditions the patient's neutrophils also showed a further increase in adhesion.

In order to investigate the adhesive capacity of cells present in an inflammatory site, which is a situation of physiological activation of their functions, we isolated leucocytes from a skin exudate. Exudate cells from the patient expressed very strong adhesive properties, with the result that the addition of fMLP did not produce any further increase in adhesion.

In the same experiments, in addition to adhesive activity, superoxide production was evaluated. As can be seen in Table I(b), the respiratory burst of the patient's neutrophils showed an elevated basic activity whereas the activity after stimulation with fMLP was normal. The basal over-production of O_2^- is probably due to the increased adhesion since the β2-dependent interaction of leucocytes with a surface can trigger signal transduction pathways leading to NADPH oxidase activation (Patarroyo, 1991; Berton *et al.* 1992).

Table I. Adhesion (a) and superoxide production (b) of neutrophils from normal subjects and from the patient.

	Blood cells		Exudate cells	
	Controls	Patient	Controls	Patient
(a) Adhesion (% of total cells)				
Unstimulated cells	1·94±2·5 (0–7)	12·54, 13·72, 12·5	3·6±3·5 (0–13·6)	100, 42·1
10^{-7} M fMLP	14·3±7·1 (5–34·2)	19, 41·8, 33·3	16·2±9·8 (3·6–39·8)	100, 58·9
(b) Superoxide (nmol/40 min/10^6 cells)				
Unstimulated cells	0·38±0·4 (0–1·2)	3·75, 0·95, 2·8	0·5±0·4 (0–1·2)	4·6, 1·7
10^{-7} M fMLP	8·2±0·6 (3·6–15·6)	10·75, 9·5, 15·1	21·4±8·6 (8·1–33·8)	21·7, 12·3

Control values are mean ±SD (and range) of subjects. Patient values are from three and two separate experiments for blood and exudate cells, respectively.

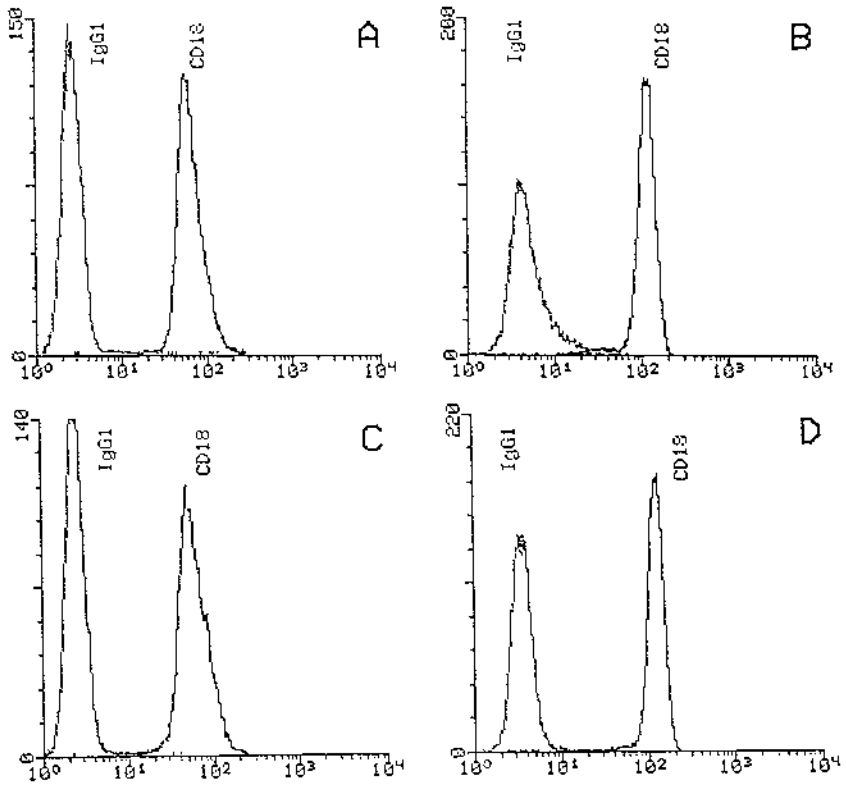

Fig 1. Cytofluorometric evaluation of CD11/CD18 integrins on neutrophils. Neutrophils from the patient (A and B) and from a normal subject (C and D) were stained with fluorescence conjugate anti-human LFA-1b (CD18) and a control monoclonal antibody of the same isotype (IgG1) as described in Methods. A and C: untreated cells; B and D: PMA-activated cells. Horizontal scale: fluorescence; vertical scale: number of cells. Five other normal subjects gave similar patterns of integrin expression (not shown).

An experiment was then performed including in the incubation mixture the 60.3 monoclonal antibodies, directed against β-chain (CD 18) of integrin adhesion molecules. Previous studies showed that 60.3 antibodies block the fMLP-stimulated neutrophil adhesion in this assay system (Bellavite *et al.* 1992). Monoclonal antibodies caused a significant inhibition of adhesion (patient: from 13·7% to 1·26% and from 42·1% to 7·4% in blood cells and exudate cells, respectively; control: from 4·8% to 0·34% and from 5·6% to 0·03% in blood cells and exudate cells, respectively). Control assays showed that no significant inhibition is caused by unspecific monoclonal antibodies of the same isotype.

Quantitative analysis of the expression of CD11/CD18 integrins in the patient's neutrophils did not show significant differences with control cells, either in the resting or in the activated state (Fig 1). These data rule out the hypothesis that the enhanced adhesion of the resting patient's neutrophils is due to quantitative up-regulation of integrins and suggest the involvement of changes in their activation and/or in the ligand affinity.

DISCUSSION

We describe a patient with an increased neutrophil adhesion which appears to be a primary condition associated with a severe neutropenia of unknown pathogenesis. Neutropenia was found over a 3-year period, while monocyte counts and bone marrow cellularity were normal, thus excluding cyclic neutropenia and marrow failure. On the basis of the criteria reported in the literature (Dale, 1990) he was therefore diagnosed as being affected by chronic idiopathic neutropenia. The possibility that the increased adhesion may have been caused by an activation secondary to systemic inflammatory processes can be ruled out on the basis of the clinical and laboratory investigations performed.

Blood neutrophils from the patient showed a 5-fold higher spontaneous adhesion than control neutrophils, and the difference was even higher when considering exudate cells. The finding of an increased adherence which was almost completely blocked by anti-CD 18 monoclonal antibodies could be very important, since it may reflect the molecular basis of this neutropenia. However, it should be pointed out that blocking of adherence by anti-CD 18 does not necessarily show that these molecules are of importance *in vivo*, whereas adherence to endothelial cells also involves other sets of adherence molecules.

The demonstration of cases of primary hyperadhesiveness may shed new light on the aetiology of neutropenias: a subset of chronic idiopathic neutropenias could be due to functionally or structurally abnormal adhesion molecules. This finding may be important for orienting therapeutic

approaches. In patients with primary increased neutrophil adherence, the therapeutic rationale should consider the use of drugs affecting the activation and up-regulation of integrins or, possibly, the use of specific antibodies or peptides (Makgoba *et al.* 1992).

It is worth noting that this patient has never had any severe infection and has lived a normal life. It is conceivable that a neutropenia due to increased adhesion may not be clinically severe for two main reasons: (a) the actual number of neutrophils available to the host defence at the inflammatory sites may be substantially greater than is indicated by the circulating granulocyte pool; (b) the quantitative or functional up-regulation of integrins, apart from causing an increased adherence, may facilitate phagocytosis, since these molecules may function as receptors for bacteria and opsonins (Springer, 1990). However, other long-term effects of increased neutrophil adhesiveness, related to the potentially toxic consequences of neutrophil activation, cannot be excluded.

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