the known susceptibility of HIBECs to lymphocyte-mediated damage.

This report describes the development and application of a flow-cytometric assay for examination of the effects of cytokine stimulation on lymphocyte adhesion to monolayer cultured endothelial and epithelial cells. This assay is potentially superior to conventional isotopic features as it is less dependent on the structure of target cell monolayers and allows simultaneous phenotypic characterization of the adherent cells.


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A novel flow-cytometric assay to assess neutrophil adhesion in whole blood
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Introduction
The adhesion of neutrophils to endothelium is a key event central to the process of cell migration and consequent tissue inflammation. Current assays of static neutrophil adhesion in the laboratory use purified neutrophils incubated on a cultured endothelial cell monolayer. The adherent cells are then quantified using isotopic or fluorescent markers or counted directly by using microscopy. Recently, neutrophil research has focused on the artifactual changes that occur in cellular activation during the process of neutrophil isolation and purification.

Conventional techniques to isolate neutrophils include Ficoll–Hypaque density centrifugation, two-layer centrifugation and dextran sedimentation. In addition, some protocols require hypotonic lysis of contaminating erythrocytes. The temperature changes, centrifugal force, hypotonicity and pH changes that occur during these processes have all been shown to be potent factors that cause neutrophil priming and activation [1,2]. This results in up-regulation of cell adhesion molecules, enhanced adhesion and increased cell locomotion and migration [3,4]. As conventional adhesion assays rely on cells prepared in this way, this suggests that the results of such assays do not closely reflect the original physiological adhesive potential of neutrophils in vivo.

The aim of the present study was to develop an adhesion assay able to use whole blood, thus avoiding the numerous stages in cell preparation at which artifactual cell activation can occur. We have adapted a flow-cytometric adhesion assay previously described by this laboratory [5] and demonstrate that fresh whole blood can be easily used in this system. In addition, this assay is able to quantify the changes in adhesion that occur when either endothelial cells or neutrophils are stimulated with various cytokines or bacterial products.

Methodology
Cell culture
The human umbilical vein endothelial cell line EAhy.926 was grown to confluence on 24-well plates in EAhy.926 cell culture medium [Dulbecco’s modified Eagle’s medium (DMEM)]
supplemented with 10% fetal calf serum, 2% HAT medium supplement, 1% glutamine and 1% penicillin/streptomycin) [6,7]. The confluent monolayers were stimulated with 100 units of tumour necrosis factor α (TNF-α) 48 h before the performance of an adhesion assay. Staining with anti-(von Willebrand's Factor) verified that more than 99% of the cells were endothelial.

Collection of blood samples
Peripheral venous blood from normal human volunteers with normal neutrophil counts was collected into sodium citrate using the Vacu-tainer system. Blood was used in adhesion assays immediately after collection.

Flow-cytometric adhesion assay
The endothelial cell monolayers were washed with warmed PBS to remove culture medium, cytokines and non-adherent cells. Fresh whole blood (1 ml) was added to each well before incubation at 37°C in 5% CO₂ for 1 h. After incubation the excess blood was aspirated from each well, and the wells were washed three times with PBS. The final two washes incorporated oscillation on a mechanical plate shaker at 100 rev./min to aid removal of non-adherent cells further. The remaining cells were then detached from the plastic and dissociated by a brief treatment with trypsin and EDTA. After the addition of DMEM with 20% fetal calf serum the cells were washed and labelled with FITC-conjugated CD45 and phycoerythrin-conjugated CD14. After a further wash the cells were resuspended and analysed by flow cytometry.

The flow-cytometer was set up to allow gating of the various leucocyte subpopulations and endothelial cells. Some 10000 cells were counted. The number of neutrophils and endothelial cells present was calculated by appropriate gating and analysis with Lysis II software. Results are expressed as neutrophil/endothelial cell ratio and are means ± S.E.M. for quadruplicate determinations.

Stimulation of whole blood
Whole blood was stimulated by adding lipopolysaccharide (LPS) in various concentrations or formyl-Met-Leu-Phe (fMLP) at 10⁻⁷ M. These bacterial products were added to each well in 10 μl of PBS before incubation of the wells at 37°C.

**Intercellular adhesion molecule 1 (ICAM-1) expression on EAhy.926 cells**
Cell surface expression of ICAM-1 on EAhy.926 cells was quantified by indirect immunofluorescence using an anti-ICAM-1 monoclonal antibody and sheep anti-mouse IgG. The cells were analysed by flow cytometry and the median equivalent of soluble fluorescence (MESF) for ICAM-1 was calculated by the use of calibration beads.

**Results**
We found that this flow-cytometric assay could easily be adapted for use with whole blood. The number of leucocytes adherent to the endothelial cell monolayers and the various subpopulations of cells were clearly identifiable on flow-cytometric analysis allowing an accurate quantification of neutrophil adhesion.

Figure 1 illustrates the results from 24 assays (six different subjects) with and without endothelial cell prestimulation with 100 units of TNF-α. These results show good reproducibility between subjects and assays.

Table 1 shows the effect of stimulation of either endothelial cells or neutrophils with various concentrations of TNF-α or LPS respectively. Associated with the increase in adhesion to TNF-α-prestimulated endothelium is a concomitant rise in ICAM-1 expression by these cells.

Figure 2 illustrates the effects of dual stimulation of both endothelium and neutrophils.
Table I

Adhesion of neutrophils from whole blood to EAhy.926

(1) Adhesion after endothelial cell prestimulation with various concentrations of TNF-α. (2) Adhesion in the presence of various concentrations of LPS (on EAhy.926 cells pretreated with 100 units of TNF-α). (3) ICAM-1 expression on EAhy.926 cells pre-treated with various concentrations of TNF-α. The ratios are expressed as means ± S.E.M.

<table>
<thead>
<tr>
<th>TNF-α pretreatment of endothelium (units/ml)</th>
<th>Neutrophil/endothelial cell ratio</th>
<th>ICAM-1 MESF</th>
<th>LPS (ng/ml)</th>
<th>Neutrophil/endothelial cell ratio</th>
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</table>

with TNF-α and fMLP. Treatment with both agents results in enhanced adhesion.

Discussion

We have demonstrated a novel flow-cytometric assay that can be applied to whole blood. This assay is reproducible and can detect changes in both endothelial cell and neutrophil activation.

The use of fresh whole blood in this assay overcomes the problems associated with cell purification and isolation that result in artifactual changes in neutrophil function. During our assay, blood cells are subjected to a minimum of physical and chemical stresses. The cell activation that may occur on venepuncture is unavoi-

able, but further activation is minimized by using sodium citrate as an anticoagulant [8]. By transferring the whole blood on to the endothelial cell monolayers straight away and commencing immediate incubation, the adverse effects of cooling on the cells are largely eliminated. The assay conditions also provide a more physiological environment in which adhesion can occur, as all the normal constituents of human blood are present.

This study has only examined neutrophil adhesion. However, a further advantage of our method is that adhesion of neutrophils, monocytes and lymphocytes can potentially be studied simultaneously as all these cells groups can be clearly identified on flow-cytometric analysis. The flow-cytometric method used here has previously been shown to be superior to more conventional adhesion assays as the ratio of cells binding to endothelium is measured rather than absolute numbers. This is beneficial as it takes into account the changes in monolayer morphology and integrity that occur on stimulation of endothelium with cytokines [5].

The potential applications of this assay are immense as it provides a unique opportunity to study static leucocyte adhesion in a laboratory model closer to physiological conditions than has previously been achieved with conventional assays.

U.K. Tumour Immunology Group

DNA vaccination as cancer immunotherapy

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Introduction
Molecular definition of potential tumour antigens is at last opening up the possibility of vaccination against cancer. In the case of tumours induced or promoted by a virus, preventative vaccination with viral antigens is likely to be of value. However, there remain many tumours that have no known viral aetiology, and preventative vaccination cannot be considered. For the vast majority of cancers, if vaccination is to have a role, it must be as a treatment of patients in remission. Recently it has become clear that non-viral tumour antigens exist in many cancers, and the idea of using these as vaccines is developing rapidly. One example of a candidate antigen present in a wide range of human cancers is the product of the ras proto-oncogene which accumulates mutations associated with tumorigenesis [1]. There are several additional categories of tumour antigen, including fusion proteins generated by chromosomal translocations, cryptic antigens and antigens of specialized cells, which are being actively investigated as possible vaccines [2,3]. A further tumour antigen that we have been concerned with is the idiotypic immunoglobulin (Ig) expressed by neoplastic B-cells of lymphoma and certain leukaemias [4]. Idiotypic antigens are true clonal markers since they arise from the variable regions of the heavy and light chains of Ig. The variable-region sequences are created via recombination and somatic mutational events, and are effectively unique to an individual B-cell. Since many of the principles of vaccine development may be shared by a range of tumour antigens, our data on idiotypic antigens, although focused on B-cell tumours, may have wider relevance.

Rationale for DNA vaccines
Idiotypic Ig protein has been investigated as a potential tumour antigen against B-cell lymphoma for some time [4]. In fact, in several mouse models, vaccination with idiotypic Ig protein with adjuvant induces clear protection against challenge with lymphoma [5,6]. In all cases, protection is mediated by anti-idiotypic antibody and is highly effective [4-6]. The results have been impressive enough to lead to a clinical trial of idiotypic vaccines in patients with low-grade follicular lymphoma [7].

The problem with idiotypic protein is that vaccines are individually based, and preparation of idiotypic Ig is expensive and technically demanding. To circumvent this, we developed the approach of using DNA vaccines, in which the variable-region gene sequences are assembled as single-chain (sc)Fv and incorporated into a plasmid for direct injection [8]. The rationale for DNA vaccines was based on successful vaccination against influenza in mice by using a plasmid containing the sequence for viral nucleoprotein [9]. After intramuscular injection, the encoding DNA is transcribed and translated, and antigenic protein presented to the immune system. The mechanism is not yet defined, but there is induction of both CD4+ and CD8+ T-cells, and production of antibody [9]. The consensus view of the sequence of events is

Abbreviations used: Ig, immunoglobulin; sc, single chain; APC, antigen-presenting cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; TT, tetanus toxin.

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