Dynamic blood cell contact with biomaterials: validation of a flow chamber system according to international standards

M. OTTO*, C. L. KLEIN, H. KÖHLER, M. WAGNER, O. RÖHRIG, C. J. KIRKPATRICK
Institute of Pathology, Johannes Gutenberg-University, D-55101 Mainz, Germany

The increasing number of patients requiring prosthetic substitution of segments of the vascular system strongly supports the need to optimize a relevant, standardized testing panel for new materials designed for synthetic vascular prostheses. The ISO gives the standard requirements for testing biomaterials provided for implantation. Our primary interest was the establishment of a reliable *in vitro* panel as a useful and relevant screening system for vascular implant devices to evaluate blood/device interactions under flow conditions. The aim of the present study was to evaluate influences of different flow conditions on blood cell–biomaterial interactions with special emphasis on the interactions of human granulocytes (PMN) and polymeric surfaces. PMN were isolated and vital cells were quantified by flow cytometrical analysis directly before, as well as immediately after the experiments. The viscosity of the final cellular suspension was analysed by using a computerized cone-plate rheometer. As reference materials we used FEP-teflon, PVC-DEHD, PU, PP and PE. Dacron and ePTFE synthetic vascular protheses were tested in a comparative way to those references. The adhesion processes were observed over a period of 40 minutes under arterial (shear stress 0.74 Pa) and venous (shear stress 0.16 Pa) flow conditions in a parallel plate flow chamber system under highly standardized conditions and laminar flow. The cells were observed with the help of inverse light microscopy. Cell behaviour was recorded and analysed in both analogue (video) and digital (imaging system) modes. Samples of the cell suspensions were obtained at regular time intervals and analysed by enzyme linked immuno sorbent assay (ELISA) to quantify LTB_4 release. Irrespective of the material, approximately 3 to 4 times more PMN adhered to the biomaterial surfaces under venous flow conditions compared to the arterial. Shear intensity did not influence the running order of biomaterials with respect to cell numbers. This response in descending order at the end of the experiments was as follows: PU, PVC-DEHD, PP, PE and ePTFE. The biochemical analyses indicate that in the system used only a weak effect on LTB_4 release induced by the different materials could be determined. A significant effect caused by flow conditions was not observed. Further experiments, both static as well as dynamic, must be performed for multiple, relevant parameters of haemocompatibility, for potential biomaterials as well as those currently in use in vascular prostheses.

1. Introduction

Vascular diseases are the major cause of mortality and morbidity in technologically and economically developed countries. Hypertension, coronary artery disease, cerebrovascular and peripheral vascular diseases, as well as venous thromboembolism give a higher annual lethality than all other diseases together [1]. As a result of this situation an increasing number of patients are coming to operation for substitution of parts of their vascular system. In the last few years the development and testing of potential biomaterials for vascular grafts have focused on the need for standardized methods which are relevant for their application. In order to establish a commonly accepted method of test procedures for biomaterials the International Standard Organisation (ISO) inaugurated the ISO 10993 directive [2]. Part 4: "Selection of tests for interaction with blood" and part 5: "Testing cytotoxicity" presented guidelines on flexibly designed testing panels for the evaluation of biomaterials. The

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* Author to whom correspondence should be addressed.

ISO 10993 directive includes the fundamental principles for the *in vitro* evaluation of blood-contacting devices. The testing systems should be appropriate models which simulate the conditions of blood contact with the devices during clinical applications. Important parameters include the duration of contact, sterile environment, as well as flow conditions. As a parameter for the reproducibility of the tests, standardized controls should be used, for example the reference materials of the European Community, as well as materials which have been established in clinical practice, such as ePTFE and Dacron. In accordance with the recommendations of the ISO our primary interest was focused on the establishment of a reliable *in vitro* panel as a useful screening system for testing biomaterials for vascular implant devices to evaluate blood/device interactions under reproducible and defined flow conditions.

In the last few years various research groups have used different models for the investigation of haemocompatibility under well-defined *in vitro* conditions [3, 4].

The aim of the present study was to evaluate the influences of different flow conditions on blood cell-biomaterial interactions, with special emphasis on the interaction of human granulocytes with polymeric surfaces. Up until now more attention has been given to platelet–biomaterial interaction, which is of course an essential step in thrombosis development [5]. Nevertheless, blood granulocytes often play an important role in the acute reaction following biomaterial implantation and express various molecules which can influence platelet and monocyte function.

2. Materials and methods

2.1. Isolation of PMN

The isolation of the PMN was carried out by using a modification of the methods described by Dooley *et al.* [6]. Freshly obtained citrated human blood was centrifuged at 180 g for 10 min. Plasma was removed and the blood was remixed. The blood was carefully applied to a 55–70% discontinuous percoll gradient and centrifuged at 354 g for 20 min at room temperature. The PMN phase was aspirated and resuspended in phosphate-buffered saline (PBS) in a concentration of 20 mM at pH 7.2, in a ratio of 1 : 5 v/v. After washing, the PMN suspension was centrifuged at 180 g. Erythrocyte contamination was minimized by lysis in a buffer containing 9% NH_4Cl, 1% $KHCO_3$ and 0.037% EDTA. After additional washing, PMN were counted using a computerized cell counting system (Schärfe, Germany). PMN were identified as cells with a mean diameter between 7.5 μm and 12 μm. The concentration of PMN was set at 10^6 cells/ml in RPMI 1640 without serum substitutes.

2.2. PMN vitality testing by flow cytometrical analysis

In order to investigate the vitality of the isolated PMN, the cells were analysed with the help of flow cytometry directly before and at the end point of the experiments according to the methods of Ormerod [7]. The vitality tests were based on a double labelling method with fluorescein diacetate (FDA) and ethidium bromide (EB). 1 ml of the PMN suspension was incubated with 50 μl FDA (100 ng/ml) solution and 50 μl EB (100 ng/ml) for 10 min at 20 °C. The flow cytometric analysis of the labelled samples was carried out by the analysis of red ($\lambda > 630$ nm) and green ($\lambda = 520$ nm) fluorescence after gating by forward as well as orthogonal light scatter. In the experiments a Coulter Epics XL MCL flow cytometry system was used. Data evaluation was performed with the help of the Coulter Listmode software. By using this method the number of vital cells, which incorporate and metabolize FDA, as well as the dead cells, which accumulate the EB due to loss of their membrane integrity, could be quantified.

2.3. Analysis of rheological properties of the PMN suspension

The apparent viscosity [8] of the final cellular suspension was analysed with the help of a computerized cone-plate rheometer, LVDV-III (Brookfield Engineering Labs., USA), based on a CP-40 spindle and a RHEOCALC analysis software package (V.1.2). The rheometer was calibrated with the viscosity standards for fluids of low viscosity at 25 °C. The specimens consisted of standard RPMI 1640 cell culture medium with and without PMN (10^6 ml^{-1}) and were analysed according to the standard test methods of the American Society for Testing and Materials (ASTM D 2556-91) [9]. The measurement was carried out under standardized temperature conditions (37 °C) and shear rates between 37.5 and 1500 s^{-1} into two steps, first the ascending shear rate and second the descending shear rate loop. The shear stress and the apparent viscosity of the specimens were quantified and the results were corrected to eliminate the effects of the decrease of temperature during the analysis.

2.4. Biomaterials

Polyvinylchloride (PVC-DEHP), polyurethane (PU), polypropylene (PP) and polyethylene (PE) from the concerted action EUROBIOMAT-Hemocompatibility, i.e. medical grade according to the regulations of the USP XXI, US-Federal Standard 209B, and DAB 9 [10] were the standard references in our experiments. As clinically used materials we analysed Dacron and ePTFE (Meadox Medicals Inc. & Newtec Square Products Limited, USA) synthetic vascular protheses. Chamber size-adapted biomaterial samples were washed with the help of a strong detergent solution (2% RBS 35 (Perstop Analytical, The Netherlands)) in distilled water with ultrasound and rinsed in distilled water in order to remove the detergent. Final sonication in distilled water followed. In order to obtain stable conditions in the chamber system without elastic tension effects, the biomaterial films were fixed on standard glass slides (Frenzel, Germany-ISO 8037).

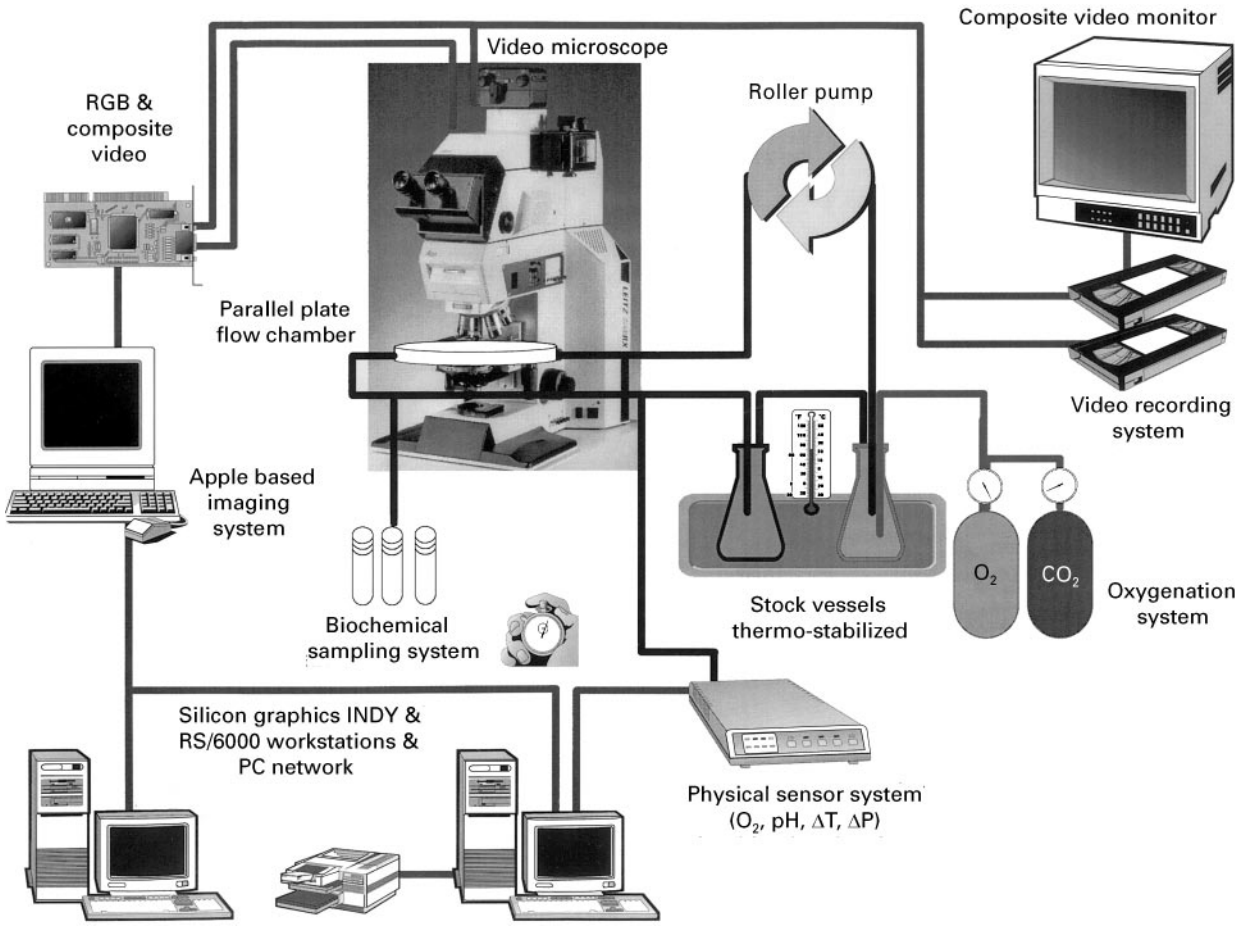

Figure 1 Flow system with directly associated components for environment modification as well as morphological, physical and biochemical analysis.

2.5. Flow chamber system

The flow chamber system consisted of three basic modules: the parallel flow chamber itself [11], the flow loop and the image analysis system. The flow chamber, consisting of a polycarbonate-based frame, contained two parallel plates, one polycarbonate surface and an opposite sample-material surface. The area exposed to the blood cell suspension was 637 mm^2 with a chamber height of 100 μm.

The quality of the flow was analysed in latex particle experiments (mean size 10.2 μm, Coulter, USA) at concentrations from 10^5 to 10^{10} ml^{-1} in PBS. Additional studies were performed on culture media including RPMI 1640. The flow abilities of latex particles were studied with the help of conventional videomicroscopy as well as with laser scanning microscopy by direct measurement of the particle speed in the different layers. The flow was recognized to be laminar. The flow loop (Fig. 1) consisted of a computer-controlled roller pump (Ismatec, Germany) and silicon tubes with an inner diameter of 1.81 mm and a length of 480 mm, which gave a contact area of 1235 mm^2. The tubes formed a complete circuit with a reservoir consisting of a teflon tube with a volume of 12 ml. The system temperature was thermostatically controlled at 37 °C. The system pressure was adjusted to 1 cm H_2O. The chamber was observed by the use of an inverse microscope Fluovert (Leitz, Germany) with a position which was observed continuously using a three-chip CCD camera (SONY, Japan). During defined periods of time, RGB-pictures were grabbed automatically, using a Data Translation frame grabber controlled by an Apple McIntosh IIci imaging system (Fig. 1) including the software package NIH-Image 1.54. The adhesion processes of the PMN (Fig. 2) were observed over a period of 30 min under arterial and venous flow conditions (shear stress of 0.16 and 0.74 Pa, respectively). The number of adherent cells, as well as the PMN-occupied area on the different biomaterial surfaces were counted every two minutes using the imaging program.

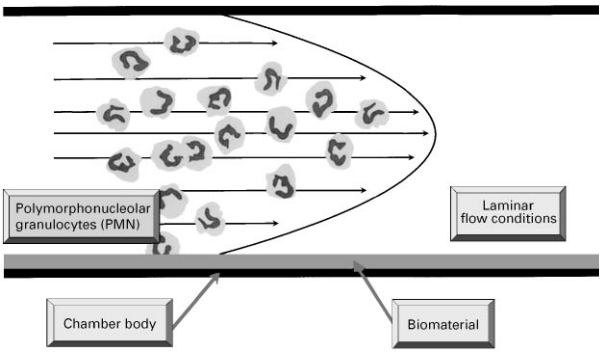

Figure 2 Schematic representation of flow chamber in cross-section with adhesion surface and chamber body.

2.6. Determination of the LTB_4 release by PMN under shear stress conditions

The samples for biochemical analysis were obtained by aspiration directly after the passage of the chamber. The samples (250 µl) were diluted 1 : 1 in 100% icecold methanol to stop further lipoxyoxygenase activity.

The samples were centrifuged (15 000 rpm, 2 min, 4 °C) for cell separation. LTB_4 was concentrated with the help of C18-reversed phase chromatography, including washing procedures with methanol (pH3 and pH5) and a chloroform/hexane solution (ratio: 65 : 35, v/v), in a vacuum separator. Drying of the samples was realized with the help of a 'speed vac' concentrator (Bachofer, Germany) after elution with methyl formiate [12]. For the assay procedure ELISA Kits for 96-well microtitre plates (Cayman Chemical, USA) were used to determine LTB_4 following standard protocols. The lower test limit of the ELISA was determined to be 6 pg/ml [11, 12].

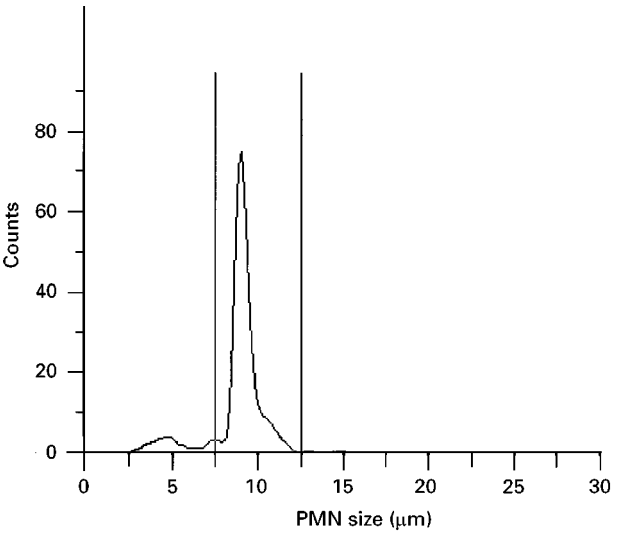

Figure 3 Determination of the PMN-size using Casy Cell Counter, counting limitation: 7.5 to 12.5 µm.

2.7. Scanning electron microscopy of adherent PMN on biomaterials

Directly after flow experiments the biomaterials were removed from the flow chamber and fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer solution at 37 °C for 2 h and washed five times with the same buffer. Postfixation was performed with 1% osmium tetroxide and 1.5% $K_4Fe(CN)_6$ in 0.1 M cacodylate buffer at room temperature. Subsequently, the samples were washed three times for 10 min in 0.1 M cacodylate buffer at room temperature. The specimens were then dehydrated using an ascending acetone series. A 'critical' point drying method and sputtering was performed before specimens were analysed in a scanning electron microscope (Zeiss 962, Germany) at an accelerating voltage of 15 kV.

2.8. Evaluation and statistics

Statistical analysis of the data was performed using the internal statistical module of Excel (Microsoft, USA). In Student's *t*-test and *F*-test, *p*-values of less than 0.05 were considered to be statistically significant. The statistical testing was performed using a software package with standardized statistical testing procedures for multivariate data analysis. Initially the statistical sample mean, the sample standard deviation and the confidence intervals from all independent samples were evaluated [13]. In the next step the samples were tested for abnormally deviating values using the testing method described by GRUBBS with a significance level of $p = 0.01$. The following test, described by David *et al.* [14] is used to evaluate the "normal" distribution of the comparable specimens using a *p*-value of 0.1. Depending on the results of this test the normally distributed specimens would be further analysed using the classical *F*-test procedure to compare the variance of the subpopulations with a *p*-value of 0.05. To compare the samples, for an equal variance the Students *t*-test was used as final testing method with a significance-level of $p = 0.05$, whereas for different variances the *t*-test modified by Welch was used with a *p*-value of 0.05 [15]. If the test for normal distribution gave a further non-normal distribution, the distribution-free equivalent of the *t*-test, the U-test procedure from Mann and Whitney [16] with a *p*-value of 0.05, was used for statistical assessment.

3. Results

3.1. Flow cytometrical determination of PMN vitality

On average, 15.6×10^6 PMN from 20 ml native citrated blood were isolated, as shown in Fig. 3 (CASY-counter data). Flow cytometrical analysis of PMN demonstrated defined populations of PMN, which could be differentiated by both their EB and/or FDA uptake. Before starting the flow experiments, 86% of the cells gave FDA positivity and EB negativity. 12% elicited FDA incorporation as well as EB-positivity. 2% were determined to be only EB positive. At the end of the experiments, 96% of the cells gave FDA positivity, only 2% of the PMN elicited both FDA as well as EB incorporation. Only 2% of the PMN which were used in the flow chamber experiments gave isolated EB positivity (Fig. 4).

3.2. Viscosimetrical studies

RPMI 1640 without cells at 37 °C has an apparent viscosity of 0.7363 mPa s (standard deviation 0.0501). The analysis of the relationship between shear stress (τ) and shear rate ($\boldsymbol{S}$) suggested linear correlation. The correlation between the fluid's apparent viscosity (η) and the shear rate indicated a constant ratio with good comparability between the values during the ascending and descending shear rates. These two profiles are typical for 'Newtonian fluids'. The analysis of the viscosity of the PMN suspension gave a viscosity of PMN suspensions (10^6 cells/ml in RPMI 1640) of 0.7483 mPa s (standard deviation 0.0488). Analysis of the relationship between shear stress and shear rate (Fig. 5) as well as viscosity and shear rate suggested

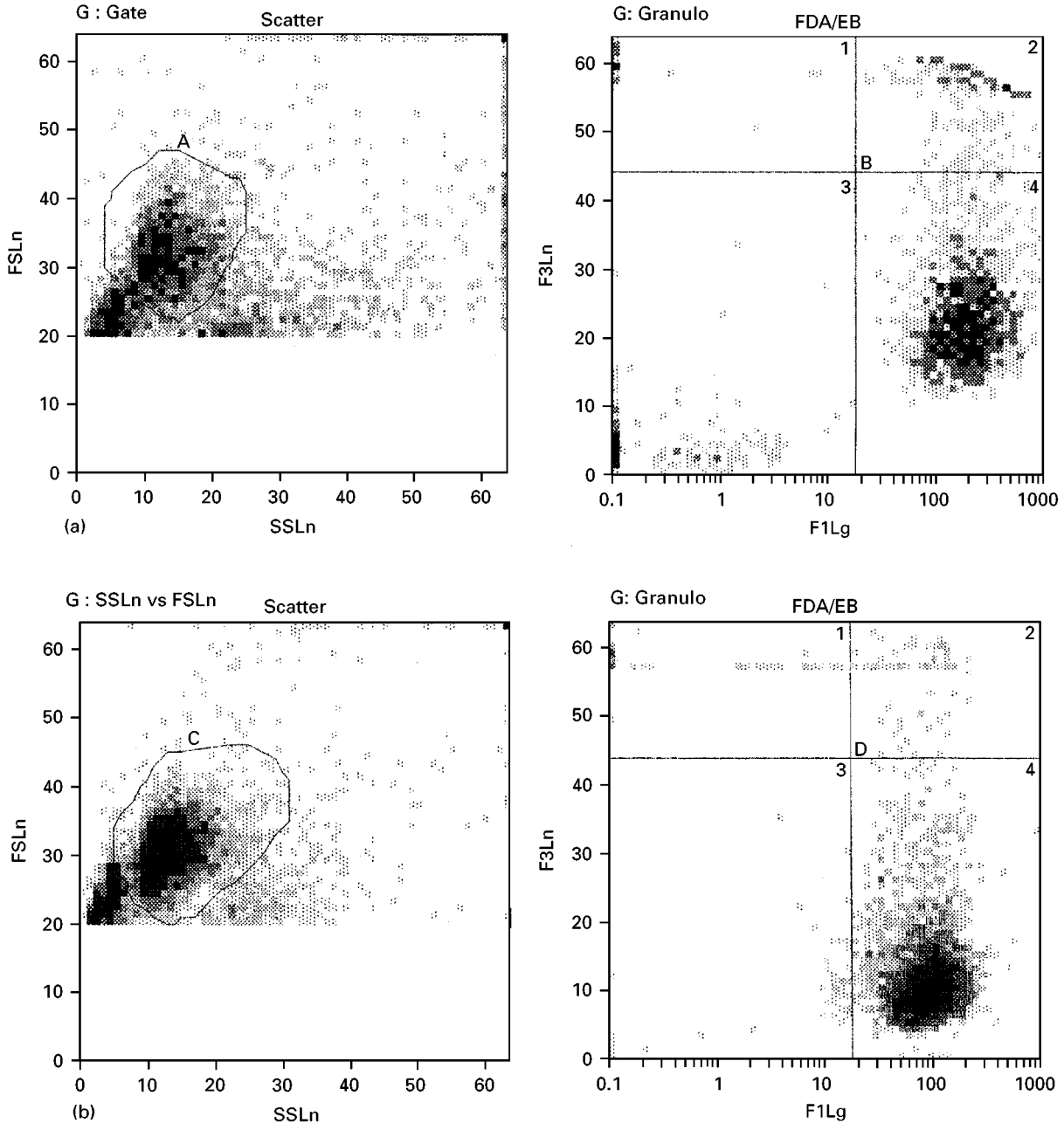

Figure 4 Flow cytometric vitality test of (a) the freshly isolated PMN in comparison to (b) PMN after the biomaterial (FEP) contact (obtained from the contact media) using combined FDA/EB staining.

this cellular suspension to be a classical 'Newtonian fluid' [8].

3.3. Computerized morphometry

Quantitation of the adherent PMN on the different biomaterials using the imaging program showed that the adhesion process started immediately and reached a maximum approximately after 15 min under venous and after 8 min under arterial flow conditions (Figs 6 and 7). Clear differences in response were observed for different materials, although the adhesion profile of the number of adherent cells compared to the profile of the PMN-occupied area gave no significant differences (results not shown). At the end of the experiments under arterial flow conditions we found the highest amount of adherent PMN on the ePTFE surface ($max_{art} = 88\,PMN/mm^2$) whereas the smallest number of adherent PMN was detected on the PU surface ($max_{art} = 34\,PMN/mm^2$). Similar results were obtained under venous flow, with the highest amount of cells on ePTFE ($max_{ven} = 346\,PMN/mm^2$) and the lowest on PU ($max_{ven} = 116\,PMN/mm^2$). Shear intensity did not influence the running order of biomaterials with respect to cell numbers. This response in ascending order at the end of the experiment was as follows: PU, PVC-DEHD, PP, PE and ePTFE. Irrespective of the material, approximately three to four times more PMN adhered to the biomaterial surfaces under venous flow conditions.

The analysis of time dependency using time plots of the adhesion process of the PMN showed that under arterial flow conditions the adhesion of a solitary PMN was stable over the time of the experiment. Thus, approximately 80% of the adherent PMN adhered longer than 20 min. Under venous flow

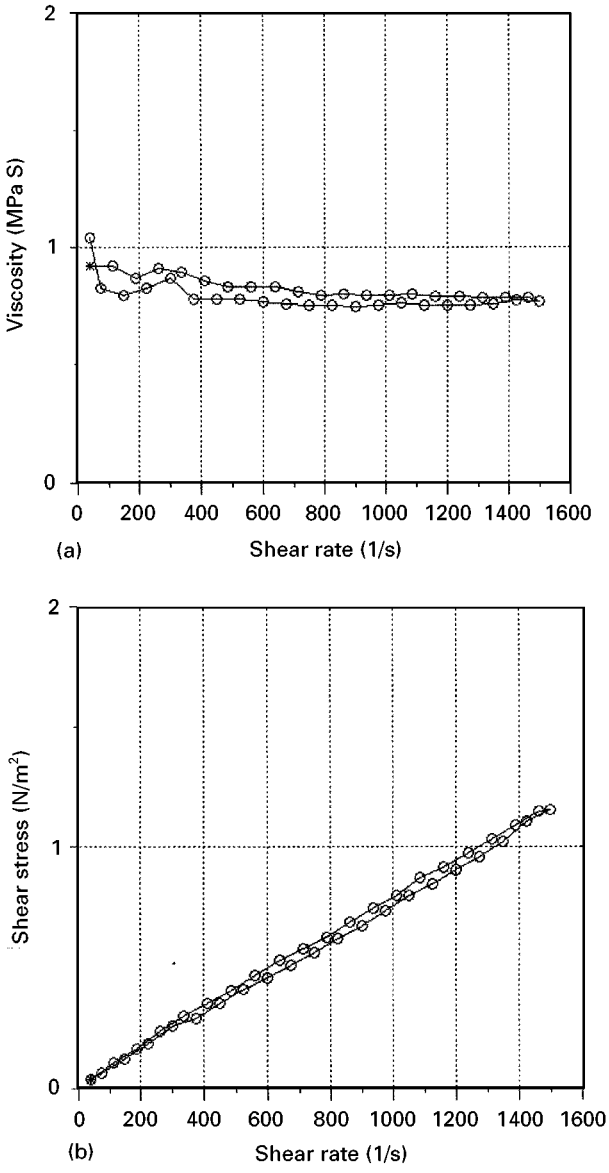

Figure 5 Results of viscosity measurement of the PMN suspension (10^6/ml) in RPMI using a Brookfield rheometer. (a) shows the viscosity versus the shear rate and (b) shows the shear stress versus the shear rate.

conditions we found a higher turnover of the adherent PMN, that is, more cells became adherent to the biomaterial surface, and also more cells became detached than under arterial flow conditions. Only 25% of the adherent PMN adhered longer than 20 min (Figs 6 and 7).

3.4. Morphological analysis using phase contrast and scanning electron microscopy

Morphological analysis of the adherent PMN on the different biomaterials was performed using conventional scanning micrographs. Analysis of the morphological properties of the PMN showed that, dependent on the tested material, the adherent cells exhibited different morphologies. On the surface of the reference materials we found predominantly single, isolated adherent PMN and some small groups of adherent PMNs. On the irregular surface of the ePTFE material, multiple large groups similar to

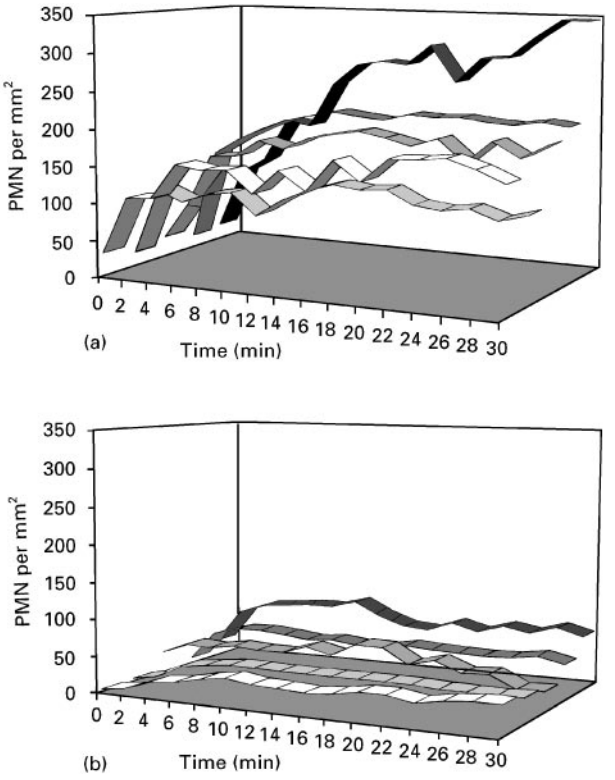

Figure 6 PMN adhesion to different biomaterial surfaces under (a) venous and (b) arterial flow conditions with respect to time. ■ PVC-DEHD; □ PU; ■ PP; ■ PE; ■ ePTFE.

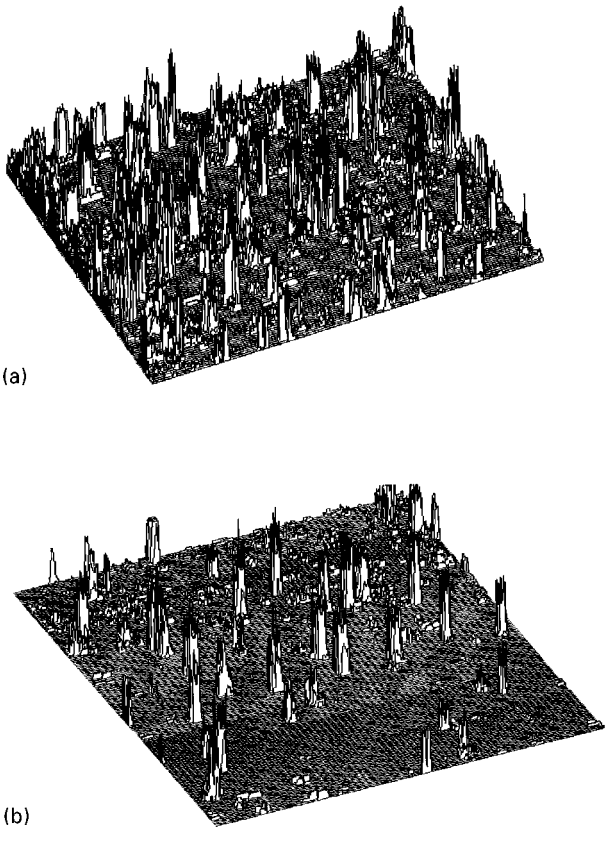

Figure 7 Time plots of PMN adhesion on the PU surfaces under (a) venous and (b) arterial flow conditions.

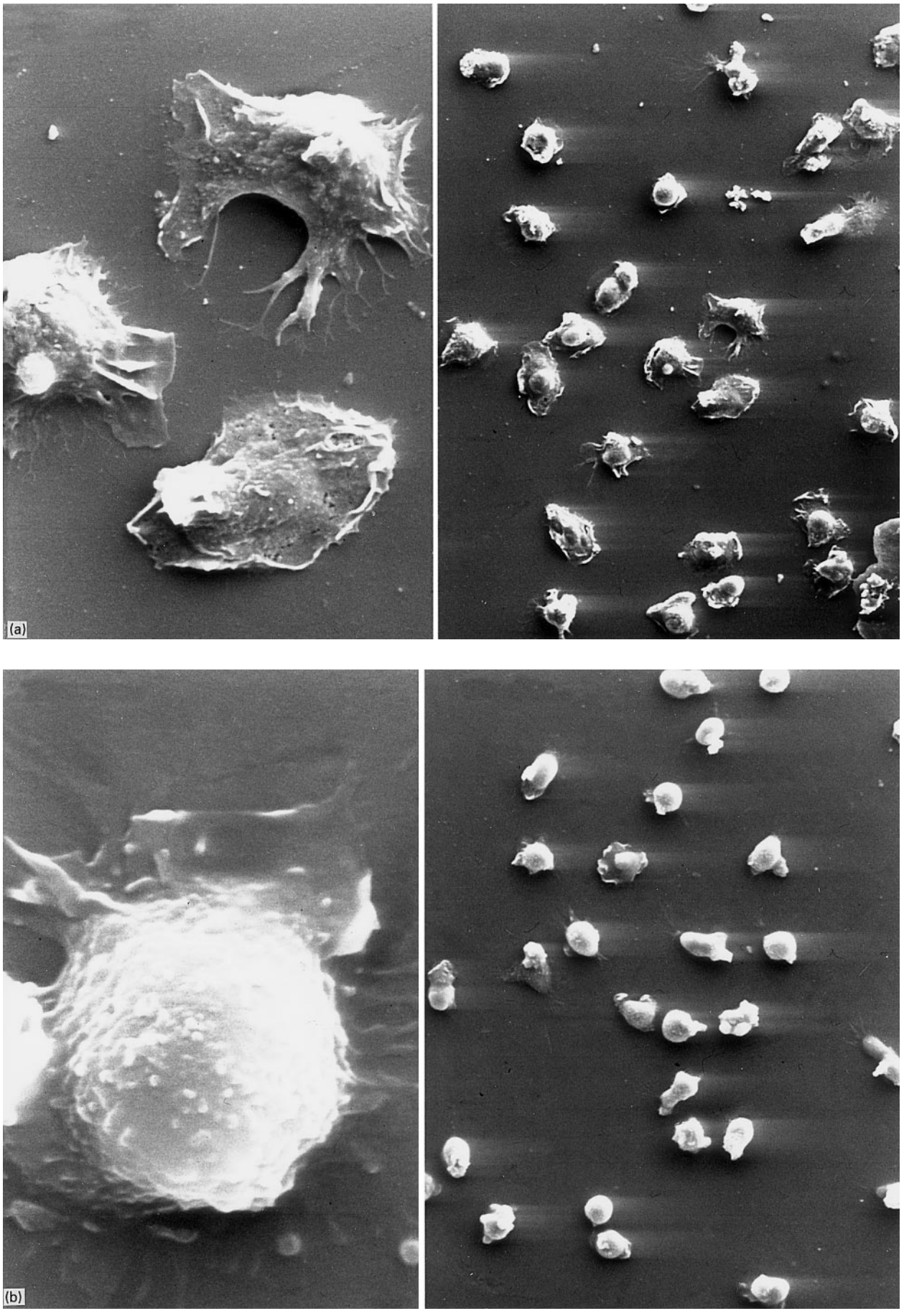

Figure 8 Scanning electron micrographs of adherent PMN on the surface of PE (a) and on the PVC surface (b) at the end of the flow experiment, both under venous flow conditions.

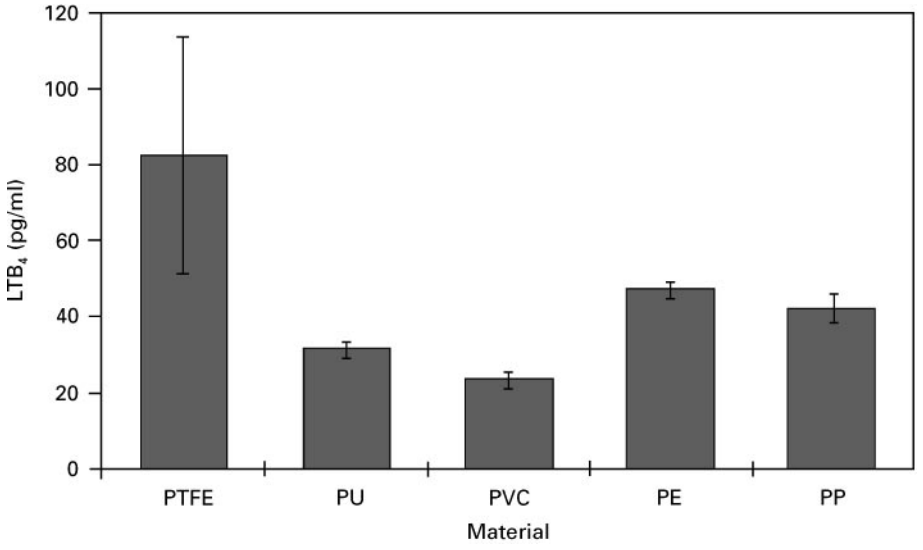

Figure 9 Mean LTB_4 release from PMN over the timespan of the flow experiment under arterial flow conditions ($\tau = 0.74$ Pa) on various exposed materials.

grapes were observed and only rarely isolated adherent PMNs.

PMNs demonstrated a spectrum of morphological appearance, dependent on the material used. As shown in Fig. 8. for PMNs on PE and PVC, it can be seen that on the PE surface PMN gave a well-spread and amoeboid cell shape. The cell surface was relatively smooth, with residual ridges and partly with small buds. Cells with broad cytoplasmatic or multiple dendritic extensions were rarely detected (Fig. 8a).

On the PVC surface, PMNs elicited a relatively spherical appearance with small spread cytoplasmic extensions (Fig. 8b). The cell body shows multiple small ruffles mostly without larger ridges and folds.

The wall shear stress also had a limited influence on cellular morphology. Under arterial flow conditions we often found more widely spread dendritic projections on the biomaterial surface and also a prolongation of the cellular body with multiple ridges, and ruffles on the surface.

3.5. LTB_4 release by PMN under different flow conditions

LTB_4 release was quantified for all materials under arterial flow conditions. The mean total release over the time of the experiment (Fig. 9), i.e. over 30 min, resulted in the following sequence, the lowest release for PVC ($c_{max} = 23.3$ pg/ml), followed by PU, PE, PP, and the highest release for ePTFE ($c_{max} = 82.1$ pg/ml). The differences between the material-induced, mean LTB_4 release were statistically significant. The release for the ePTFE specimens gave a higher, significant (F-test) variability compared to the other specimens.

LTB_4 release over the time period did not give significant differences for each time interval (Fig. 10). Samples from ePTFE showed the largest fluctuation of the LTB_4 release, with the highest variability.

To compare the influences of the flow conditions, samples from PP were analysed for venous and arterial flow conditions. A significant difference between

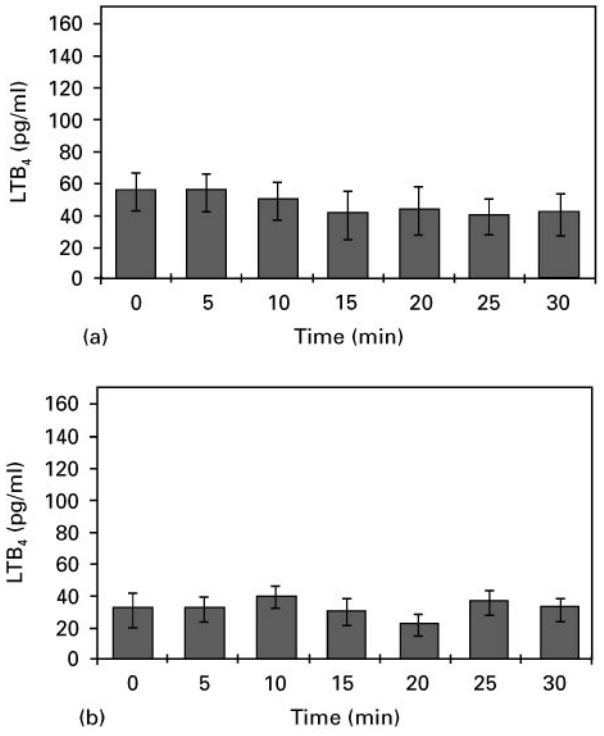

Figure 10 LTB_4 release over the timespan of the flow experiment under arterial flow conditions ($\tau = 0.74$ Pa) for (a) PE and (b) PU.

the LTB_4 release under these flow regimes could not be demonstrated during the short-term exposure. The mean LTB_4 release was quantified to be $c_{max/ven} = 45.65$ pg/ml for venous shear stress and $c_{max/art} = 47.72$ pg/ml for arterial shear stress (Fig. 11).

4. Discussion

The results presented provide evidence that the parallel plate flow chamber [11, 17] is a useful tool for the evaluation of blood cell adhesion under well-defined, experimental *in vitro* flow conditions. The model has multiple advantages for practical use. The flow conditions in the system can be regulated in a practically

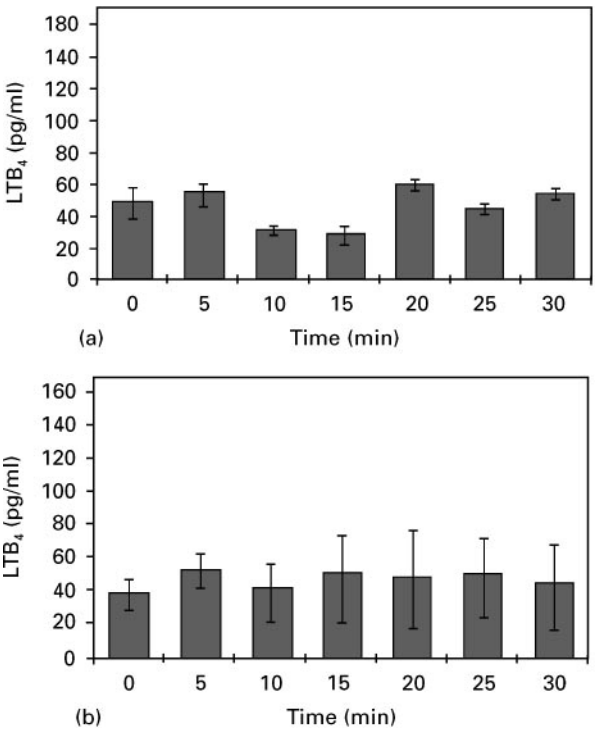

Figure 11 LTB_4 release over the timespan of the flow experiment under (a) arterial ($\tau = 0.74$ Pa) and (b) venous ($\tau = 0.16$ Pa) flow conditions for PP.

unlimited range of shear stress between 0.015 Pa and 100 Pa. In addition, the adhesion process can be directly and continuously visualized with the help of different microscopical techniques, such as inverted light microscopy under conventional or darkfield conditions. The model also offers multiple possibilities to manipulate the experimental conditions, such as continuous or discontinuous flow, quick regulation of the system temperature, modification of the system pressure, modification of the oxygenation status, coculture experiments and long-term experiments with approximately unlimited length, tested for 14 days. Many other modifications of the experimental protocol are available. The technical limitations of the model are the inflexibility of the chamber wall, an important quality of the blood vessels and some limitations of the detection of the adhesion process depending on optical phenomena, such as polarization and light bending.

The study of the adhesiveness of PMN is important for the evaluation of material properties according to the ISO 10993 [2]. The highest standards must be required for the haemocompatibility testing of materials which are designed for use as implant devices, such as vascular prostheses. In the studies to be carried out the importance of different material attributes, whether for potential or already established biomaterials, for the induction and progress of the adhesive contact with circulating immunocompetent cells must be investigated with respect to the effective flow conditions.

PMN adhesion, as studied in our experiments, plays a less important role in the ISO standards for test methods. On the other hand, PMN adhesion is highly relevant to biomaterial degradation induced by reactive oxygen species [18, 19]. Furthermore, PMNs are of significance due to their interaction with platelets [20]. Activated PMNs induce the expression of P-selectin on platelet membranes via the PMN-derived cathepsin G [21], and this adhesive glycoprotein modulates cell–cell contact and transcellular metabolism of arachidonic acid and induce large mixed cellular aggregates. Human leucocyte elastase enhances platelet activation induced by cathepsin G [22] to form combined aggregates. Platelet activating factor (PAF) also induces PMN–platelet adhesion [23] and PMN–endothelial adhesion [24]. Hirafuji and Shinoda [24] also found influences of PMN on platelet–endothelial adhesion.

These multiple interactions between PMN-biomaterials and platelets suggest that the PMNs must be considered to be important with regard to the haemocompatibility of biomaterials, especially for the assessment of their thrombogenicity – the central issue for the suitability of biomaterials for vascular prostheses.

Analysis of the rheological properties of the used PMN suspensions attests that in our model laminar flow conditions are guaranteed ("Newtonian fluid") for the used wall shear rates employed. A typical behaviour comparable to the non-Newtonian flow properties of whole human blood [25] was not established in our model. The latter possesses, however, the advantage of a reproducible and well-defined shear stress in the test system. The minimal effective differences between laminar flow and the normal, physiological flow may be nonsignificant with regard to the adhesion process of the PMN, because flow-conditioned cell–cell interactions had a limited influence [25] on the adhesion processes.

The flow cytometry-based vitality analysis showed that at the beginning of the experiment a maximum of 12% of the PMN were irreversibly damaged by the isolation procedure [6]. The analysis of the cells at the end of the experiment suggest that the dead cells sedimented in the stock vessels so that only 2% of the circulating, non-adherent cells have to be regarded as dead. Nevertheless, a definite statement concerning the state of functionality and pre-activation of the used PMNs by the isolation and preparation procedure cannot be made, although the vitality test suggested that the isolation protocol [6, 17] is reliable for the adhesion experiments.

Analysis of the morphometrical data demonstrated that on the surface of the different material films different amounts of PMNs adhere. The ranking of materials with the highest adhesion on the PE-film and the smallest on PU-film was independent of the flow conditions applied. In comparison to the static model we found the same ranking in adherence on the materials surface as described by Klein [11].

The expanded polytetrafluoroethylene prostheses, which have been used since 1975 [26], consist of a fine-meshed PTFE tube in a single layer. The layer consists of solid PTFE knots with fibrils radiating longitudinally, forming a felt-like network. The quantification of adherent PMN on ePTFE surfaces indicates that on these fibrillar structured surfaces

adhesion is the highest compared to the other materials in our experiments.

When compared to our previous analysis of FEP-teflon-films under static conditions [11] and under venous and arterial flow conditions [17,27] a large difference exists in the adhesion properties between the films (PP, PU, PE, PVC) used in this study and fine-meshed teflon material (ePTFE) from a conventionally used vascular prosthetic device. The static and flow analyses of FEP-teflon showed that PMN adherent to this surface were comparable to PU [11,27]. These results suggest that, independent of the chemical structure, other material properties or experimental conditions might induce this high PMN adhesion, a state which could contribute to the early graft thrombogenicity of the ePTFE grafts [28].

Study of the continuous video microscopy indicates that close to the wall microturbulent flow is present. This flow consists of different components, particularly high speed flow areas and on the other hand multiple areas with parastatic flow conditions. We found that in these areas PMNs developed larger aggregates. We suggest that under venous flow conditions these semi- or parastatic areas will be expanded although a quantification of this is not available. However, this concept is corroborated by some *in vivo* results with ePTFE vascular grafts from Lundell *et al.* [29]. The examination of ePTFE grafts in sheep showed that in unrestricted carotid vascular grafts an occlusion by a thrombus could not be established, whereas in 85% restricted grafts a thrombogenic occlusion was described.

We propose an analogous interpretation for the higher amount of adherent PMN on the polymer films under venous flow conditions. The physiological analysis of blood flow by Goldsmith and Turitto [25] indicated that in the area of the nearest boundary layer the relative flow velocity is very low and often stagnation of flow could be found. With a decrease of flow the thickness of the peripheral "large cell-depleted layer" should be reduced with a consecutive marginalization of the large cells, i.e. the PMN, and a prolongation of the contact time between the material surface and the PMN.

The morphology of the PMN which adhere under static conditions differs from that of cells exposed to shear stress [30]. Under static conditions a moderate spreading of PMN has been described. The morphological changes of the PMN must be interpreted as a function of the physical properties of the biomaterial surfaces, i.e. the contact angle, the surface free energies of the substrata, as well as the ionic composition of the biomaterial surface, including the amount and localization of hydroxyl groups [3]. A direct morphological classification of PMN activation could not be clearly demonstrated. It is possible that morphology may be an indicator for a level of common metabolic activity of the PMN [30].

The biochemical analyses indicate that in the system used only a weak effect on LTB_4 release induced by the different materials could be determined. A significant effect caused by flow conditions was not observed. The weak effects on the release of arachidonic metabolites may be caused by the stimulation of the PMN through the polymer contact areas in the flow circuit. The proportion between the unspecific, the flow circuit, and specific, tested biomaterial, contact area can be expressed as 1.93 : 1. It is possible that this could affect the leukotriene output in a significant way. Of further importance is the observation that LTB_4 release was not time dependent. This can be interpreted as a constant stimulation effect, induced by the combination of fluid shear stress and the individual materials, without a time-dependent stimulation of the arachidonic acid pathways.

On a broader note, biocompatibility testing has undergone serious changes of emphasis and is now generally accepted as involving two principle areas. The first is the principle of "biosafety", which involves the exclusion of deleterious effects of potential biomaterials on the organism. The second area, commonly known as "biofunctionality", is an aspect which deals with the ability to perform with an appropriate host response in a specific application [31–33].

On the basis of the ISO standards we have established a new *in vitro* flow model for biomaterial evaluation based on a parallel plate flow chamber designed by Klein [11]. The decisive advantages of this model are the multiple, reproducible modifications of the experimental environment, the simultaneous registration of the morphological changes of flowing and adherent cells within the flow chamber, as well as the continuous availability of flow media specimens (supernants) for further biochemical analysis without disturbing the flow properties in the chamber. Furthermore, at the end of the experiment multiple additional techniques can be performed, such as scanning and transmission electron microscopy, as well as many other investigative techniques. The dynamic system has the advantage that through additional modification of the flow conditions, physiologic and pathophysiologic principles can be simulated and studied to complete the static *in vitro* tests. Further experiments, both static as well as dynamic, must be performed for multiple, relevant parameters of haemocompatibility, for potential biomaterials as well as those already used in vascular prostheses. These experiments include the quantification of protein absorption, evaluation of the activation of the coagulation system, the quantification of platelet adhesion, as well as the testing of cell-to-biomaterial and cell-to-cell interactions, including stimulation of immunocompetent cells, such as macrophages and lymphocyte subpopulations. All this is needed in order to complete the picture of biofunctionality of a biomaterial with respect to blood contact.

The results presented prove that the parallel plate flow chamber appears to be a useful tool to examine the interactions between suspended blood cells, such as PMNs, and biomaterial surfaces to predict the potential interactions of the blood–biomaterial interface. Contrary to the opinion of Slack and Turitto [34], that parallel plate flow chambers do not represent a reliable system for studying the effects of shear rate or shear stress on suspended blood components, we suggest that at least this system offers all the

opportunities to study these effects. The options of this flow system, including visualization and imaging of circulating blood cells in the fluid stream, during the adhesion process, such as rolling [35] as well as after the adhesion on the biomaterial surface, demonstrate the special suitability of this device to detect the morphological changes in a quantitative manner with simultaneous biochemical analysis of biochemical mediators and specific metabolic products in the flow media.

The demonstrated model offers, besides multiple modulation options of the microenvironment, i.e. "pathologic" shear stress, modified system pressures, modified pH values, etc., further possibilities for a continuous analysis of morphological changes in different coculture models using human endothelial cells together with platelets, and white as well as red blood cells.

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