JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 9 (1998) 409–414

Complement activation by cellulose: investigation of the effects of time, area, flow rate, shear rate and temperature on C3a generation *in vitro*, using a parallel plate flow cell

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The development and utilization of a parallel plate flow system to study the blood response to flat sheet biomaterials, is described. Unlike most other parallel plate flow systems, which have been used to study cellular interactions with biomaterials, the controlled flow test cell described below employs the test materials on both sides of the channel through which the blood flows. The flow cell is used to conduct an investigation into the *in vitro* generation of C3a by a regenerated cellulose membrane, Cuprophan. The effects of experimental variables such as temperature, blood flow rate, contact area and wall shear rate on C3a generation by Cuprophan were studied. The results show that C3a generation by Cuprophan is lower at 12 °C than at 22 °C, which is in turn lower than C3a generation at 37 °C. Furthermore, a decrease in contact area, and increase in wall shear rate and blood flow rate, can produce a decrease in C3a concentration.

1. Introduction

The increasing use of biomaterials in blood-contacting applications has created a desire for a more profound understanding of the blood response to artificial surfaces. It is believed that an improved understanding will not only improve patient treatment, but also contribute to the development of materials that are better suited for use in the clinic. Despite the dependence on artificial materials in a wide variety of clinical applications, comparatively little is understood regarding the basic interactions between blood and artificial surfaces, or their relationship to the clinical response. It is believed that this lack of fundamental information has hampered the development of improved materials for use in the clinic [1]. In addition, the lack of a generally accepted test procedure to assess the blood response to biomaterials has meant that materials have reached clinical utilisation without contributing basic information to advance related research [2].

The evaluation of blood responses to an artificial surface can be studied clinically, *in vivo*, *ex vivo*, or *in vitro* [3]. Clinical evaluation involves the fabrication of a device, which is then evaluated using human patients. *In vivo* procedures involve the implantation of the test material so that it is in direct contact with the host. *Ex vivo* procedures achieve blood contact extracorporeally and tests may be single pass, or involve recirculation of the blood. *In vitro* assessments are essentially laboratory investigations, where the blood is isolated from a donor prior to use. *In vitro* tests are the most widely used, as they are relatively simple and cheap. The choice in contacting medium, from whole blood to single protein solutions, and the ability to control experimental conditions impart a great degree of versatility to such procedures. The isolation of the blood from the host also permits the study of blood responses in isolation from the host response. *In vitro* tests can be assigned into two categories, static and dynamic procedures, according to the absence or presence of blood flow, respectively. Static tests involve the incubation of a finite amount of blood with the surface under investigation. Flow systems have mainly been used in the study of cellular interactions with materials. Unlike static tests, there is a continuous flow of contacting medium through the system. Although bead columns have been used in the past, parallel plate systems have been used more recently. In the latter, a rectangular cross-section

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channel is formed between two opposing plates with the blood or perfusate flowing in a single pass. Laminar flow conditions can be achieved and are easy to define and reproduce. Systems can be designed to produce a constant wall shear rate [4–6], or a graduated range of wall shear rates [7]. Most parallel plate systems support the test material on one plate only, the other plate being usually transparent to allow observation of the perfusate.

This paper presents the development and utilization of a parallel plate flow cell, to investigate the effects of time, temperature, flow rate, contact area and shear rate by a regenerated cellulose membrane. In this study, complement activation was to be investigated, and the ability to visualize blood cells was not required. Therefore, a flow chamber that employed the test material on both sides of the channel was preferred, increasing the contact area of the test materials and decreasing the contact area of non-test materials.

2. Materials and methods

In the past, Richardson *et al.* [4] developed a parallel plate flow cell to study platelet adhesion. One feature of this flow cell was the wide entry and exit ports, that allowed the introduction of medium across the full width of the chamber. Later, Weng *et al.* [8] designed a test cell with similar entry and exit ports; this cell used the test material on both sides of the chamber. The system had been reported to achieve laminar flow conditions as characterized by Hele-Shaw [9]. However, problems in sealing the chamber were encountered and further development was required to ease handling and improve reliability of the apparatus. The test cell used in this study was assembled as described below.

An exploded view of the test cell is shown in Fig. 1. Blood flows between two sheets of the test material which are supported on upper and lower poly(methyl methacrylate) (Perspex) plates. A silicone rubber O-ring sealing cord (not shown in Fig. 1) is embedded around the periphery of each plate. The blood flow channel is defined by a rectangular frame gasket (0.30 mm thick) made from two layers of Lexan sheeting (Stockline Plastics, UK). The Lexan layers, together with blood ports (fashioned from 14G hypodermic needles), are bonded together with an epoxy adhesive (Araldite®, Ciba-Geigy, UK). Blood enters and exits via the needles which are machined with an axial tapered section. The concavity of the tapered section is filled with a RTV silicone rubber resulting in a change in flow cross-section along the needle axis which promotes uniform flow distribution across the channel width. Semi-circular grooves machined in the Perspex plates mate with the blood port segments of the gasket. Assembly of the test cell is completed by clamping the plates in a jig.

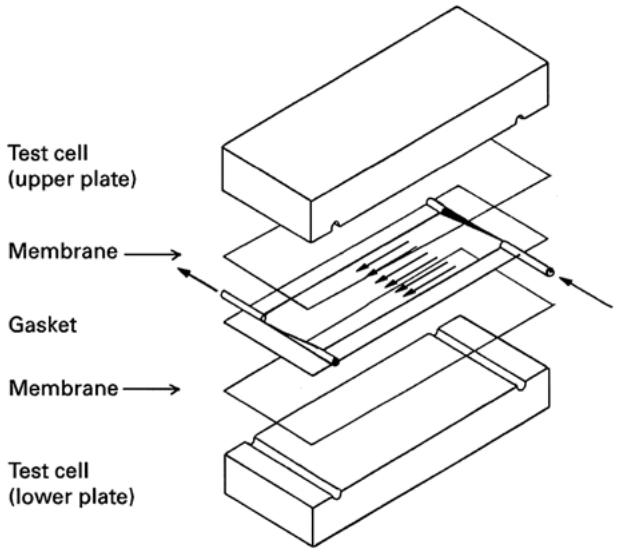

Figure 1 Schematic drawing of the controlled flow test cell.

The channel length was fixed by the distance between the grooves in the Perspex plates, and the width of the channel by the length of the tapered aperture in the modified needles. The aperture allowed the passage of blood through the channel between the plates. The channel dimensions defined the contact area, and the thickness of the gasket determined the depth of the channel, and the surface area to blood volume ratio. The latter was held constant throughout the study. Three test cells were constructed with the dimensions shown in Table I. The dimensions of the test cells A and B were chosen on the basis of the work performed by Weng *et al.* [8]. Complement activation was believed to be shear-independent [10], and so in order to verify this, Cell C was constructed. Cell C was designed to possess similar surface area, priming volume and residence time to cell B, but have a much higher wall shear rate for a given blood flow rate.

The membrane selected for this study was Cuprophan (Akzo Faser AG, Germany), a regenerated cellulose membrane. Cuprophan is a membrane that has been extensively reported to induce a high degree of complement activation. Thus, the membrane was selected as the most suitable material with which to conduct this investigation, as it would be the most likely to evoke responses that would allow discrimination between variables. The membrane was soaked overnight in saline prior to use. The test cells were then assembled and primed with saline. The priming of the system with saline allowed the blood to be introduced without meeting

TABLE I Dimensions of the test cells to assess the effects of surface area, flow rate and wall shear rate on C3a generation by Cuprophan. Contact area = $2Lw$; residence time (s), $t_R = 60(V/Q)$; volume = Lwh; wall shear rate (s^{-1}) = $(6Q)/(60\,h^2w)$.

	Cell A		Cell B	Cell C
Channel length, L (cm)	9.4		14.0	61.8
Channel width, w (cm)	2.0		3.0	0.7
Channel height, h (cm)	0.030		0.030	0.030
Contact area, A (cm^2)	37.6		84.0	86.5
Channel volume, V (cm^3)	0.564		1.26	1.30
Area: volume, A/V (cm^{-1})	67		67	67
Flow rate, Q (ml min^{-1})	0.5	1.2	1.2	1.2
Residence time, t_R(s)	68	28	63	65
Wall shear rate (s^{-1})	28	67	45	190

an air interface, and avoided the entrapment of air bubbles when the blood entered the cell. A control was required, and consisted of two needles, identical to those used in the construction of the gasket. Needles were siliconized by dipping into Repelcote (Sigma Chemicals, UK) and allowed to dry for a minimum of 24 h. They were then connected end-to-end with a piece of silicone tubing. The control was also primed with saline prior to the introduction of blood.

The nature of blood used in the study of blood–material interactions has the capacity to influence the response. It has been shown that the presence of antithrombotic agents can alter the blood response to a material [11], and that different anticoagulants exert different influences [12]. Therefore fresh, whole, human blood, without anticoagulant, from healthy donors was used. Blood was collected from the median cubital vein of volunteers, who had refrained from aspirin for 14 d prior to the test. Any air bubbles were removed and the initial (pre) sample collected. The syringe was then mounted on the syringe pump and flow started immediately. The outlet system contained a three-way stopcock, and the fluid displaced from the cell was collected in 5 ml disposable Plastipak syringes (Becton Dickinson, UK). Syringes at the outlet were changed every 3 min. The blood was then transferred from the syringe into a polystyrene tube, and pipetted into tubes containing disodium EDTA. The tubes were then placed on ice. The samples were then centrifuged for 15 min at 3000 g, at a temperature of 4 °C. Plasma was collected, snap frozen and stored at − 70 °C until assay. Each experiment was repeated twice ($n = 3$) . C3a measurements were determined with a commercially available radioimmunoassay kit (Amersham International, Little Chalfont, UK).

3. Results and discussion

3.1. Effect of temperature

The effect of temperature on the blood response was investigated. C3a generation by Cuprophan 280 HDF in cell B was measured at 12, 22 and 37 °C. A Perspex chamber with a heating unit was constructed and used to elevate the temperature. For the experiments at 12 °C, a radiator was connected to a pump that circulated chilled water. The test cell was then placed on top of the radiator. The data are presented in Fig. 2.

Analysis of the data reveals the following: when compared to the $t = 0$ (pre) value, at 37 °C, significantly higher amounts of C3a were generated by Cuprophan at 12 min. At 25 °C, C3a generation was significantly higher at 3, 9 and 12 min. At 12 °C, levels were significantly higher at 3, 9 and 12 min. C3a generation induced by Cuprophan compared to the respective control was significant at 0 and 12 min at 37 °C. At 22 °C, differences were significant at 6, 9 and 12 min. At 12 °C, a significant difference in C3a generation was observed at 6 min.

Comparison of the C3a generation between the three temperatures shows that generation at 37 °C was significantly higher at 6, 9 and 12 min. This is in agreement with results from Siemssen *et al.* [13] who also showed that C3a generation *in vitro* is much higher at 37 °C than at 20 °C. However, in our study, the amounts at $t = 0$ were also higher. This difference in pre values could indicate the introduction of considerable errors, owing to the extensive dilution of the plasma samples that was necessary during assay. Examination of the data also reveals that there is a larger spread in the results from the experiments at 37 °C when compared to the data obtained at 22 and 12 °C. This variability could be attributed to two factors: either it is an effect of elevating the temperature, so that the inherent biological variation is amplified as well as the response, or that the variability is an artefact introduced in the dilution step of the assay procedure. Examination of the amounts generated at 22 and 12 °C shows that C3a generation at 22 °C was greater than at 12 °C, although differences were only significant at 6 min. The reason for the slight elevation in C3a generation at 3 min is unclear. It is possible that this is an artefact of the priming procedure and the subsequent correction. The data generated at this time point should be interpreted taking this into consideration.

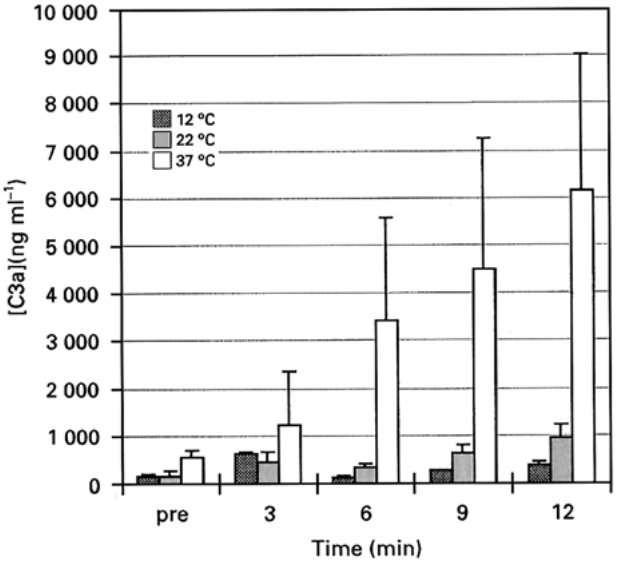

Figure 2 C3a generation by Cuprophan at (a) 37 °C, (b) 22 °C and (c) 12 °C.

Subsequent experiments investigating the effects of blood flow rate, contact area and shear rate were performed at 22 °C; a temperature of 37 °C was excluded, owing to the large variability of the readings generated. The response generated at 12 °C was not readily distinguishable from the response generated by the control. The response at the intermediate temperature (22 °C) induced responses that were within the sensitivity limits of the assay and could be distinguished from the control. Thus, 22 °C was selected as the experimental temperature.

3.2. Effect of flow rate

The influence of flow rate upon complement activation was investigated. The smallest test cell, Cell A, was selected for use in this study, to ensure that a reading could be obtained after 3 min at the lower

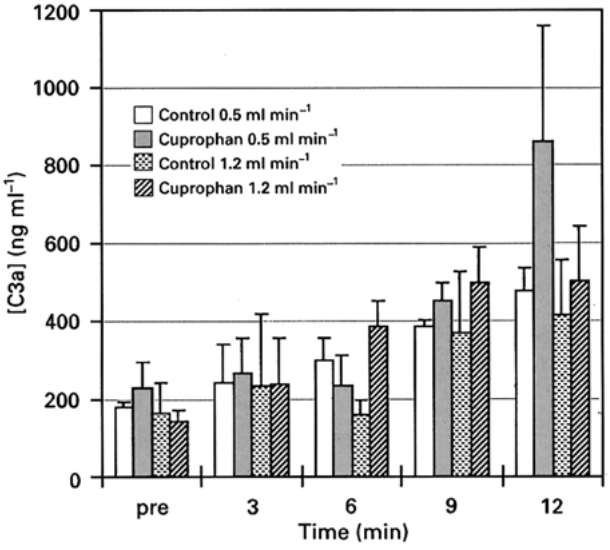

Figure 3 Effect of blood flow rate on C3a generation by Cuprophan.

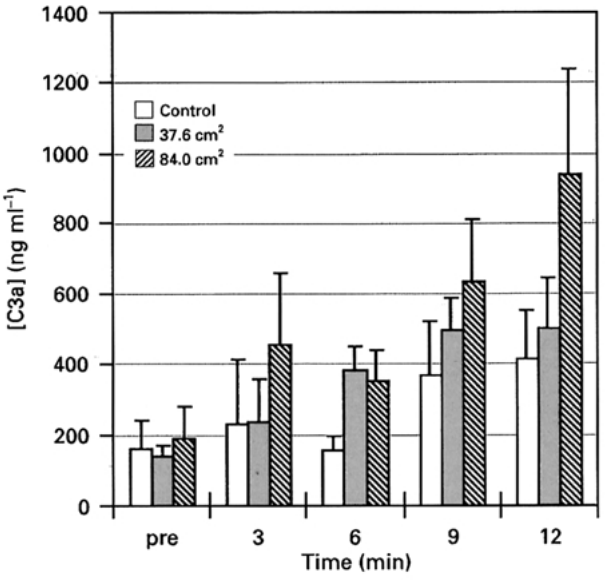

Figure 4 Effect of contact area on C3a generation by Cuprophan.

flow rate. The two flow rates employed were 0.5 and 1.2 ml min^{-1}. Controls were run at both flow rates. The results are presented in Fig. 3.

Analysis of variance on the data revealed that significant differences in C3a generation were obtained at 9 and 12 min when compared to the initial value for both controls. The 0.5 ml min^{-1} control also showed a significant difference at 6 min. When the blood was in contact with Cuprophan, significant differences in C3a generation were observed after 9 and 12 min when compared to the initial reading at both flow rates. A further significant difference was seen at 1.2 ml min^{-1} between the values obtained at 0 and 6 min.

Comparison between sets shows that significant differences between the controls were only present at 6 min. Differences were also significant between Cuprophan and the control, both at 1.2 ml min^{-1}, at 6 and 9 min. At 0.5 ml min^{-1}, significant differences between Cuprophan and the control were seen at 12 min only. Differences between the C3a generation by Cuprophan at two different flow rates were significant at 6 and 12 min.

These results suggest that the generation of C3a when in contact with a cellulose membrane is dependent on the flow rate of the blood along the surface of the membrane. A reduction in flow rate increases the generation of C3a. This could be due to the fact that a reduction in flow rate is accompanied by a decrease in wall shear rate and an increase in residence time. Because a finite amount of time is required for any reaction to occur, an increase in residence time can increase the extent of reaction [13].

3.3. Effect of contact area

The influence of contact area on C3a generation was also investigated. Cells A and B were employed, with blood flowing at the same flow rate. The results are shown in Fig. 4. Analysis of variance of the data with respect to time revealed that significant differences in C3a generation were present at 9 and 12 min for the control, 6, 9 and 12 min for cell A (37.6 cm^2) and 9 and 12 min for cell B (84.0 cm^2), when compared to the pre value. Comparison of the three groups shows that differences between the two areas and the control were significant at 6 min. Further significant differences were observed between cell B and the control at 9 and 12 min. The quantity of C3a generated by the larger contact area of Cuprophan was significantly higher than levels generated by the smaller area at 12 min. At this time, there was no significant difference between the smaller area and the control. However, further experiments are required to separate the effects of increased surface area and increased residence time. Each factor could cause an elevation in the extent of complement activation. A relationship between increasing surface area and increasing complement activation has been shown by Vienken and Baurmeister [14], Mahiout *et al.* [15], and Goldman *et al.* [16]. There have also been reports of a relationship between membrane surface area and C5a [17], βTG generation [15], granulocyte elastase release [18], and phagocyte oxidative activity [19]. The influence of factors other than contact area were not acknowledged in any of these reports.

3.4. Influence of wall shear rate

The influence of wall shear rate on complement activation, as monitored by C3a generation, was investigated. Cell B (45 s^{-1}) and Cell C (190 s^{-1}) were used with a blood flow rate of 1.2 ml min^{-1}. Both cells have similar contact area, area:volume ratio and residence time, but different wall shear rates. The wall shear rate in the control was 50 s^{-1}. The results are shown in Fig. 5.

Analysis of variance reveals that the C3a generated at the lower wall shear rate is significantly higher than the pre value at 9 and 12 min after the introduction of blood into the cell. Blood exposed to a wall shear rate of 190 s^{-1} generates significant amounts of C3a at 3,

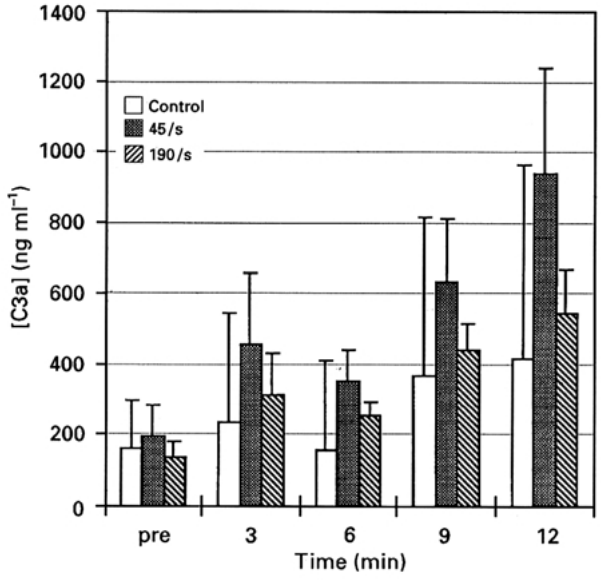

Figure 5 Effect of wall shear rate on C3a generation by Cuprophan.

9 and 12 min. The control also produced significantly higher amounts of C3a at 9 and 12 min. Comparison of the amounts generated between the three tests shows that the amounts generated by Cuprophan at the higher wall shear rate are significantly lower than the amounts generated at the lower wall shear rate at $t = 6$, $t = 9$ and $t = 12$ min. The slight difference in surface area (2.5 cm^2) between the two test cells is assumed to produce a negligible increase in C3a generation in Cell C (wall shear rate = 190 s^{-1}). The data indicate that reduction of the wall shear rate from 190 s^{-1} to 45 s^{-1} increases the activation of C3 by the membrane, as variables such as flow rate and area-to-volume ratio were held constant. Platelet adhesion to artificial surfaces has also been reported to be shear-dependent [4–6, 20].

If the system were shear-independent, complement activation would be reaction-limited and not diffusion-limited. However, C3a generation has been shown to be dependent on the wall shear rate, implying that within the range of wall shear rates studied, complement activation is diffusion-controlled. The Reynolds number of the flow between the parallel plates is low, and flow is therefore laminar. Reaction-controlled systems are rarely encountered in laminar blood flow conditions [21], particularly within the range of wall shear rates employed in this study [22]. C3a generation under the conditions employed in this study is likely to be either diffusion-controlled, or reaction and diffusion controlled [20].

The dependence of C3a generation on wall shear rate also affects the interpretation of the data examined earlier, with respect to alteration of the contact area and flow rate. An increase in flow rate increases the wall shear rate, which could contribute to the observed reduction in C3a generation. In relation to the study of the effect of contact area, the channel dimensions in cells A and B are not the same. The difference in width produces a higher wall shear rate in cell A than in cell B. This increase in wall shear rate could also partly contribute to the drop in C3a generation in Cell A, which can also be attributed to a reduction in contact area. In both cases, the observed differences are unlikely to be solely attributable to an alteration in one factor, but instead to be a summation of effects.

4. Conclusions

This report has outlined the construction of a parallel plate flow cell designed to investigate the blood response to biomaterials in flat sheet form, under controlled, defined conditions. It is also feasible that the procedure can be used to conduct investigations *ex vivo*. In this study, the procedure was used *in vitro* to evaluate the effects of temperature, blood flow rate, contact area and wall shear rate on C3a generation by Cuprophan. An increase in temperature and contact area increased C3a generation; an increase in blood flow rate and wall shear rate decreased C3a generation. This study highlights the need to control and define experimental conditions when conducting an investigation into the blood response.

Acknowledgements

N. Lamba was in receipt of a Medical Research Council studentship whilst conducting this investigation. The authors would also like to thank Dr J. Vienken, Akzo Nobel Membrana, Wuppertal, Germany, for donating the membrane used for this study.

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Received 12 June
and accepted 16 September 1997