In vitro complement activation after contact with pyrolytic carbon-coated and uncoated polyethylene terephthalate

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This study was undertaken to evaluate whether the pyrolytic carbon coating of polyethylene terephthalate induces complement activation. Complement activation induced by pyrolytic carbon-coated polyethylene terephthalate (PET + PC) in comparison with uncoated polyethylene terephthalate (PET) was assessed on whole blood collected with heparin. The activation of the classic pathway was evaluated by C4d fragment enzyme immunoassay. The activation of the alternative pathway was evaluated with Bb fragment enzyme immunoassay. The results show that uncoated PET activates the alternative pathway, but not the classic one. PET + PC does not induce complement activation, not even through the alternative pathway. Pyrolytic carbon coating therefore contributes to improving blood compatibility.

1. Introduction

Complement activation is one of the causes of the failure of blood-contacting devices [1, 2]. Complement activation leads to the formation of peptides with biological activity, which activate platelets and induce granulocytes and monocytes to adhere to the endothelium, with subsequent migration into tissues and release of their enzymes [3–7]. Complement activation therefore contributes to the inflammatory response, the onset of immune reaction and the formation of thrombi.

The importance of complement in the pathogenesis of medical device failure is such that the evaluation of complement activation is recommended by standard EN 30993-4 [8]. Usually biomaterials activate the complement through the alternative pathway, even though the involvement of the classic pathway cannot be excluded [9]. Therefore, the standard recommends evaluation of complement activation by assaying both the classic and the alternative pathway. One of the markers of the classic pathway is the fragment C4d, which is split away from C4b. The latter derives from C4 and participates in the formation of C3 convertase. Bb fragment, which derives from the conversion of factor B zymogen into the active enzyme, is a marker of the alternative pathway. Factor B zymogen forms a complex with C3(H2O),B or with complex C3b,B and is split into Ba and Bb fragments by factor D. The resulting bimolecular complex (C3b, Bb) is the C3 convertase, which splits C3 in C3a and C3b. Complement activation induced by materials used in haemodialysis circuits [10, 11], extracorporeal circulation [12] and apheresis [13] has been particularly investigated, as well as complement activation induced by vascular catheters, as a factor able to affect the onset of infectious complications [14]. Few studies have been performed on complement activation induced by materials used for vascular prostheses. Some data reported in the literature show that uncoated PET activates complement [15–17]. Such activation, favouring platelet adhesion and pseudointima iperplasia, can contribute to vascular implant failure. Research has also been done on complement activation induced by different coatings of PET. The coating with bovine collagen does not reduce complement activation compared to uncoated PET [18, 19]. A promising coating for PET is pyrolytic carbon. The surface properties of this material have been thoroughly investigated, as well as its effects on platelets and plasmatic phase of coagulation, but its action on complement has been poorly investigated.

The aim of this study was to compare complement activation, through the classic or alternative pathway, induced by pyrolytic carbon-coated PET with that induced by uncoated PET.

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2. Materials and methods

2.1. Materials

The following materials, produced by Sorin Biomedica (Saluggia, Italy), were tested: uncoated polyethylene terephthalate (Albograft) (PET), and polyethylene terephthalate coated with pyrolytic carbon (Carbograft) (PET + PC). Both materials were sterilized with ethylene oxide and stored at room temperature. The specimens were assayed as 9.0 cm^2 squares.

2.2. Endotoxin preparation

A sterile solution of 1 mg bacterial endotoxin (LPS) (lipopolysaccharide of *Escherichia coli* 055:B5 by Sigma, assayed for cell cultures) in 1 ml AIM-V (Gibco) [20] was used as positive control, i.e. inducer of complement activation, to be compared with test materials.

2.3. Blood collection and treatment

Blood samples were collected from healthy donors in tubes containing lithium heparinate and stored on ice until tested. The contact between blood and materials was performed within an hour from the collection.

2.4. Contact between whole blood and materials

Each sample of whole blood was aliquoted as follows:

(i) 3 ml were put in contact with a 9.0 cm^2 PET square;

(ii) 3 ml were put in contact with a 9.0 cm^2 PET + PC square;

(iii) 3 ml were put in a tube with 0.03 ml AIM-V;

(iv) 3 ml were incubated with 0.03 ml LPS (at the concentration of 1 mg ml^{-1}) to obtain a final concentration of 10 μg ml^{-1}.

All samples were incubated for 30 min at 37 °C, under static conditions. Immediately after incubation, the materials were separated and dysodic EDTA (Carlo Erba) was added to whole blood (final concentration: 5 mM) [21]. After centrifugation of blood for 15 min at 2000 ***g*** at + 4 °C, the plasma was separated and stored at − 70 °C.

2.5. Negative control

In C4d and Bb assays, a negative control was also tested. This was obtained from blood collected in tubes containing lithium heparinate and dysodic EDTA. The sample was centrifuged at 2000 ***g*** for 15 min at + 4 °C, then the plasma was incubated for 30 min at 56 °C to inactivate complement, and finally stored at − 70 °C.

2.6. Positive control

In Bb assay, a positive control consisting in Zymosan-activated plasma was also tested. Zymosan (Sigma) was reconstituted with distilled water, boiled for 30 min and centrifuged at 2000 ***g*** for 20 min at + 4 °C. The pellet was added to plasma (final concentration 20 mg ml^{-1}) and incubated for 60 min at 25 °C. After incubation, the plasma was centrifuged at 2000 ***g*** for 20 min at + 4 °C and the pellet was discarded. The supernatant was immediately frozen at − 70 °C.

2.7. Determination of complement fractions

The complement fractions C4d and Bb were determined by enzyme immunoassay (Quidel, San Diego, CA). The assays were carried out according to the instructions of the manufacturer. The samples and the controls were stored on ice after thawing and were tested within 2 h. Both standards, controls and samples were tested in duplicate. Controls and samples were diluted 1:70 for C4d and 1:10 for Bb using the Specimen Diluent of the kits. The absorbances were read at 405 nm and the best fit line was derived using linear regression analysis. Only the fit lines whose correlation coefficient was > 0.95 were accepted.

2.8. Statistical analysis

The test results are expressed as arithmetic mean plus or minus standard error of ten separate experiments. The effects of the materials were evaluated by the analysis of variance (ANOVA), using the StatView 4.5 software for Macintosh (Abacus Concepts, Inc.). The Bonferroni–Dunn's multiple comparison test was applied to detect specific differences between groups. The p value $\leqslant 0.0083$ was considered as statistically significant.

3. Results

3.1. C4d fragment

The negative control, i.e. plasma inactivated at 56 °C for 30 min, yielded a value of 3.08 μg ml^{-1}. In the samples of blood not in contact with the materials, C4d concentration was similar to the negative control. Incubation with LPS determined an insignificant increase of C4d. Also PET and PET + PC determined insignificant variations in C4d. No significant difference between PET and PET + PC in respect to the generation of C4d were observed (Table I).

3.2. Bb fragment

The negative control, i.e. plasma inactivated at 56 °C for 30 min, yielded a value of 0.35 μg ml^{-1}. Plasma activated with Zymosan yielded a value higher than 2.52 μg ml^{-1}. In the samples of blood not in contact with the materials, Bb concentration was lower than the activated plasma. Incubation with LPS determined a significant increase. Also the contact with PET determined a significant Bb generation with respect to the control without materials. The contact with PET + PC did not determine any significant variation in Bb fragment concentration. A significant difference between PET and PET + PC with respect to the formation of Bb fragment was observed (Table II).

TABLE I Mean, standard error and significance of complement fraction C4d after contact with PET and PET + PC

Material	C4d, mean ± S.E. ($\mu g\,ml^{-1}$)	p
Control	1.974 ± 0.173	–
LPS	2.674 ± 0.180	n.s.[a]
PET	2.338 ± 0.215	n.s.
PET + PC	1.764 ± 0.176	n.s.

[a] n.s. = not significant.

TABLE II Mean, standard error and significance of the Bb fraction of the complement after contact with PET and PET + PC

Material	Bb, mean ± S.E. ($\mu g\,ml^{-1}$)	p
Control	1.148 ± 0.095	–
LPS	1.660 ± 0.122	0.0018 (versus control)
PET	1.683 ± 0.069	0.0012 (versus control) 0.0013 (versus PET + PC)
PET + PC	1.153 ± 0.132	n.s.[a] (versus control)

[a] n.s. = not significant.

4. Discussion

Some previous studies on complement activation were performed after contact of biomaterials with plasma or serum [22, 23]. We preferred to put the test materials in contact with whole blood, in order to mimic the complement activation which occurs *in vivo*. The same type of contact was also used by other authors, who demonstrated the capability of PVA hydrogels to activate the complement with a significant increase in Bb and sC5b-9 fragments [24].

In evaluating material-induced complement activation, much attention must be paid in sample manipulation. Artefacts derived from sample processing, which can determine complement activation, must be avoided. The risk of activation during sample management was lowered by (i) storage of blood on ice, (ii) performance of the contact within 1 h from collection, (iii) inactivation of complement with 5 mM EDTA immediately after the contact [21], and (iv) immediate centrifugation of blood at low temperature.

The activation induced by PET and PET + PC was always compared with a negative control consisting in a sample of blood processed in the same way but without the materials. In this negative control, complement was not inactivated by incubation at 56 °C (not inactivated control). As positive control in each experiment, a sample incubated with LPS was always tested. In such a sample, significantly higher levels of Bb were observed. In the not inactivated control, C4d and Bb levels were similar to those measured in a plasma sample where the complement was inactivated by the 30 min incubation at 56 °C (inactivated control). In the not inactivated control, Bb levels were much lower than those found for a plasma sample in which the alternative pathway was activated with Zymosan [25]. In addition, the accuracy of the assays was evaluated by using controls at the known value supplied with the kit.

Our results show that the complement activation induced by uncoated PET occurs through the alternative pathway, whereas the classic pathway is not activated by this material. These results agree with those of other authors [15, 17] who assayed other complement fragments to demonstrate the activation of the alternative pathway induced by PET.

Our results also show that the coating with pyrolytic carbon reduces complement activation as compared to uncoated PET. After the contact with PET + PC, only a slight and insignificant increase in Bb fragment can be observed, with a significant difference in respect to uncoated PET, and a slight increase in C4d, lower than that observed for uncoated PET. It can be assumed that on PET, which is an activating surface, C3b is protected from inactivation by factors H and I. On pyrolytic carbon, which is a non-activating surface, C3b interacts with factor H with a greater affinity, resulting in inactivation of C3b by factor I (Fig. 1). These data show that the blood compatibility of Dacron can be improved by the pyrolytic carbon coating. Other studies have demonstrated the better blood compatibility of the coating with pyrolytic carbon compared to uncoated PET [26, 27]. In particular, it was demonstrated that pyrolytic carbon determines platelet adhesion and activation lower than those induced by uncoated PET. Pyrolytic

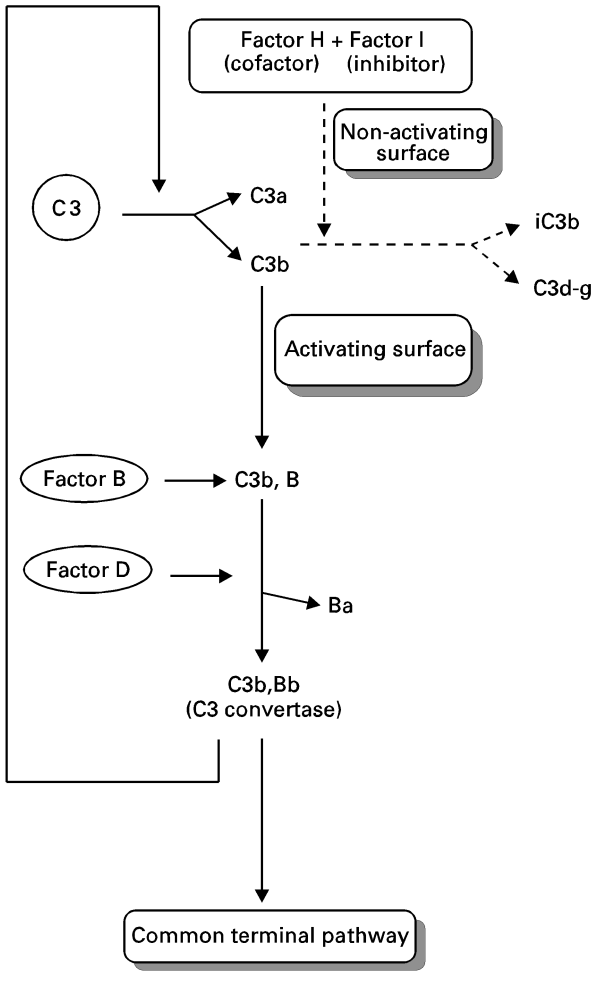

Figure 1 Activation of the alternative pathway of complement induced by PET. The coating with pyrolytic carbon reduces the ability of PET to activate the complement alternative pathway.

carbon blood compatibility is also enhanced by the fact that such material favours the growth of endothelium more than uncoated Dacron, and it does not modify the endothelium adhesion molecules [28–30].

5. Conclusion

Uncoated PET activates the complement through the alternative pathway, as demonstrated by the significant increase in Bb fragment. The classic pathway is not affected, as can be seen from the assay of C4d peptide.

Pyrolytic carbon coating significantly limits the complement activation. These results demonstrate the better blood compatibility of PET + PC in respect to uncoated PET.

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