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Using Elisa to evaluate complement activation by reference biomaterials

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The interaction between blood and an artificial surface results in the activation of complement and can lead to extensive inflammatory reactions. *In vitro* work was undertaken to validate a system of assays to evaluate the complement activation of a candidate biomaterial by comparing its capacity of activation with that of reference biomaterials. The biomaterials tested were cellulose acetate, cellulose dialysis tubing, low density polyethylene, polydimethylsiloxane and AN69. Complement activation was assessed using ELISA for iC3b, C3a and Factor Bb. Results showed that (1) iC3b measurements may underestimate complement activation, (2) the presence of Bb in plasma or serum was a reliable indicator of complement activation, (3) measurements of C3a are of interest but are difficult, (4) adsorption or/and binding of complement proteins occured on the surface. Evaluation of complement activation for a biomaterial should include the assessment of the fluid phase (in serum or plasma) as well as the bound phase (on the surface of the biomaterial) of complement.

1. Introduction

Complement plays an important role in the body's defence mechanism. When a biomaterial comes into contact with blood, complement activation is one of the reactions of the inflammatory response. It may also contribute to the activation of platelets and coagulation when a foreign surface is exposed to blood [1]. Complement activation has many clinical consequences; e.g. the leukopenia and hypoxia associated with hemodialysis [2, 3]. Some complement products have specific receptors for neutrophils, monocytes, macrophages and mast cells. Their interaction with the respective receptors induces cellular responses that may enhance, if not induce, the inflammatory response [4].

Complement activation has been recognized as an important component of the biocompatibility of materials and the capacity of a biomaterial to activate complement is an important index of biocompatibility. This work was undertaken to validate an ELISA (Enzyme Linked Immunosorbent Assay) for complement activation induced by biomaterials. *In vitro* evaluation was performed with human serum or plasma. ELISA kits are commercially available to measure the complement proteins iC3b, C4d, Factor Bb, SC5b-9 and more recently C3a. A screening test to evaluate complement activation of a candidate biomaterial using these kits involved comparing its capacity to activate complement with that of reference biomaterials. Complement activation of the reference biomaterials was investigated and the complement proteins being assayed were iC3b, Factor Bb and C3a.

2. The complement system

Complement represents a non-cellular and non-specific defence system against invading foreign elements. The complement system consists of at least twenty plasma proteins, functioning either as enzymes or binding proteins. The system is organized in two activation pathways that lead to a cascade of cleavage reactions: the classical and the alternative pathways. In either pathway, an initial enzyme catalyses the formation of the C3 convertase (enzyme cleaving C3 into C3a and C3b) which further generates the C5 convertase leading to the assembly of the terminal complex. At the end of the cascade, the membrane attack complex (MAC) sequence is common for both pathways and corresponds to the cytolytic pathway: the MAC is responsible for cell damages and lysis.

The classical pathway is triggered by antigen-antibody complexes and is not frequently activated by biomaterials [5]. The alternative pathway is triggered by many foreign substances. In the fluid phase, activation occurs spontaneously at a slow rate and is amplified by the presence of a foreign surface. A spontaneous hydrolysis of the internal thioester of C3 takes place and generates a C3(H_2O) molecule that can bind and activate Factor B and this C3(H_2O), Bb fluid convertase cleaves C3 into C3a and C3b. Amplification follows if the metastable C3b binds to a

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surface. Attachment of C3b to an activating surface favours the binding of Factor B to C3b, and thus the alternative convertase, C3b, Bb, is formed and cleaves more C3 molecules into C3a and C3b. If C3b binds to a non-activating surface, it is assumed that binding to the regulatory proteins factor H and factor I is favoured: C3b is inactivated by factor I and becomes iC3b [6, 7]. iC3b is further degraded to C3c and C3d. When a sufficient amount of C3b has been generated, the C5 convertase (C3b, Bb, C3b complex) is formed and begins cleaving C5 into C5a and C5b. The terminal complement complex C5b-9 is generated (assembly of C5b with C6, C7, C8, and C9), it exists in two forms: the membrane attack complex mC5b-9, that penetrates cell membrane and can lead to cell lysis and the non-lytic complex, SC5b-9, in the fluid phase, associated with vitronectin.

C3a, C4a and C5a are anaphylatoxins and their presence in blood have biological effects such as chemotaxis, increase in vascular permeability, vasodilatation (action of C3a and C4a) and neutrophil activation (action of C5a) [8].

3. Materials and methods

3.1. Biomaterials

Four reference biomaterials were used. Cellulose dialysis tubing (CDT) was offered by Dr Josefowicz (material from the IUPAC Working Party on blood materials interactions), cellulose acetate (CA) was obtained from Warehouse Plastics (Toronto). Low density polyethylene (LDPE) and polydimethylsiloxane (PDMS) sheets were NIH primary reference materials obtained from Abiomed and Thoratec Laboratories Corporation, respectively. Dialysis membrane AN69 (Hospal), a gift from Dr J. Y. Breillatt (Baxter), was tested in some of the later experiments. CDT was used as a strong positive control, CA was a weak positive control, LDPE and PDMS were the negative controls.

3.2. Pooled plasma and pooled serum

For pooled plasma, blood from healthy donors was drawn into Vacutainer sterile tubes containing sodium heparin (100 USP) and centrifuged for 15 min at 4 °C. The plasma fractions were pooled and stored at − 70 °C until use. For pooled serum, blood was drawn into Vacutainer sterile tubes, allowed to coagulate at room temperature for 30 min and centrifuged at 4 °C for 10 min. The serum fractions were pooled and kept at − 70 °C until use.

3.3. Incubation

Discs were cut from the biomaterials and washed thoroughly: three washes of 30 min each in distilled water and then identically in 0.9% NaCl solution. Note that CDT discs had been previously washed following instructions for at least 4 h in dH_2O. CA and AN69 discs were also previously washed in an ultrasonic bath for 20 min and rinsed with dH_2O for 30 min. The samples were incubated with 200 µl of plasma – either from pooled or single drawings – or serum in a 24-well plate (Nunclon™ from Nunc, surface-treated wells for cell attachment) at 37 °C for 15 min to 4 h. Pieces of Silastic® (16 mm outside diameter) were used to anchor the discs onto the bottom of the wells in like fashion to Ziats *et al.* [9]. After exposure, 20 µl of 200 mM EDTA was added to the wells and the samples were aliquoted and frozen at − 70 °C until further analysis. Controls were run with plasma/serum alone (in an empty well) or plasma/serum incubated with Silastic® tubing.

3.4. Analysis of complement activation products in the fluid-phase by ELISA

Determinations of concentrations of iC3b fragment, Bb fragment, C3a-desArg were performed by enzyme immunoassays (Quidel Corporation). The principle of the procedure for all the complement components assayed was a three-step procedure utilizing: (1) a microassay plate coated with a mouse monoclonal antibody which binds specifically to the complement component, (2) a horseradish peroxidase conjugated antibody to antigens of the component, (3) a chromogenic substrate. Data were analysed with two-way analysis of variance test; $p < 0.05$ was considered as significant.

3.5. Analysis of complement activation products on the surface by SDS-PAGE

Complement components bound onto the surface were evaluated by sodium dodecyl sulfate-polyacryloamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis. Discs of biomaterials were incubated with serum for 1.5 h at 37 °C following the procedure described above. After removal of serum, discs were washed four times in phosphate buffer saline. The discs were then cut in pieces, transfered into Eppendorf tubes and incubated at 90 °C for 10 min in a 2% (w/v) SDS and 14.4 mM mercaptethanol solution. The whole mixture was ultra-centrifuged for 1 min and aliquoted; samples were kept at − 70 °C. Samples were analysed by SDS-PAGE according to the method of Laemmli [10]. Western blot was performed using polyclonal anti-C3c antibodies or a mixture of polyclonal anti-C3c and anti-C3d antibodies (DAKO) followed by biotynaled-conjugated immunoglobulins [11]. A streptavidin HRP reagent conjugate and ECL Western blotting detection reagents (Amersham) were used for revelation. For this experiment, discs of Silastic® tubing were also tested.

4. Results

All the biomaterials were found to be activators of complement but to different degrees. In all cases, the ranking of the biomaterials, was, as one would expect: CDT ≥ CA > PDMS ≥ LDPE.

For all the complement components tested, controls ran with empty well and empty well with Silastic® tubing showed that the piece of Silastic® tubing was not inducing complement activation to a level that

TABLE I Complement activation of the controls with Silastic®. Serum or plasma was incubated in an empty well or in an empty well containing Silastic® tubing, for different times at 37 °C. Complement activation was assessed using ELISA for iC3b and Bb fragments. Values for CDT (high activator) and LDPE (low activator) have been added for comparison

	Plasma iC3b			Plasma Bb Fragment		Serum Bb fragment
Surface	15 min	30 min	60 min	120 min	180 min	90 min
Empty well	10.13 ± 1.37	12.27 ± 1.06	0.899 ± 0.08	1.07 ± 0.05	2.12 ± 0.48[a]	7.04 ± 2.11
Empty well with Silastic	9.97 ± 1.5	9.3 ± 2.05	1.063 ± 0.13	1.277 ± 0.23	1.53 ± 0.11	5.44 ± 1.68
LDPE	12.91 ± 1.29	14.47 ± 3.11	1.025 ± 0.13	1.109 ± 0.14	1.45 ± 0.198	2.58 ± 0.91
CDT	20.54 ± 2.47	24.68 ± 3.08	1.279 ± 0.14	1.753 ± 0.24	2.28 ± 0.23	6.90 ± 1.13

[a] $P < 0.02$

would interfere with the complement activation mediated by the biomaterial tested (data in Table I). However, neither no disc values could be used as controls since the values obtained at long incubation times (with plasma) or with serum were quite high compared to other samples. This is not surprising since the polystyrene had been treated for cell attachment and this lead to enhanced activation.

4.1. iC3b fragments

Starting at 15 min, iC3b fragments were detectable in the plasma samples, but no significant differences appeared among the biomaterials before 1 h. After 30 min, a significant increase of iC3b was observed for CDT and CA (Fig. 1). At 1 h, CDT as well CA were significantly different from PDMS and LDPE.

4.2. Bb results

Complement activation was measured at first in plasma samples. Bb production at an almost constant rate was observed for all the biomaterials up to 2.5 h, the rate for LDPE being the smallest (Fig. 2). After 2.5 h, a plateau was reached for CA and PDMS, while

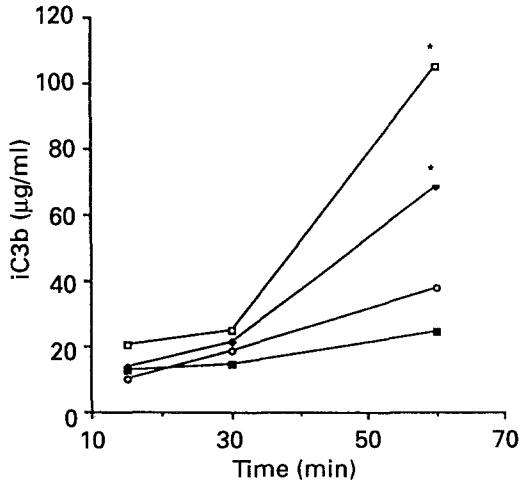

Figure 1 Quantification of iC3b fragments in human heparinized plasma incubated with biomaterials. Discs of biomaterials, anchored to the bottom of the wells of a 24-well plate with a piece of Silastic® tubing, were incubated at 37 °C with human plasma for different times. Plasma was assayed for iC3b using an ELISA. Resting sample: [iC3b] = 7.66 ± 0.79 µg/ml (□ CDT; ◆ CA; ○ PDMS; ■ LDPE) *significantly different ($p < 0.05$) from PDMS and LDPE, # statistically different ($p < 0.05$) from LDPE.

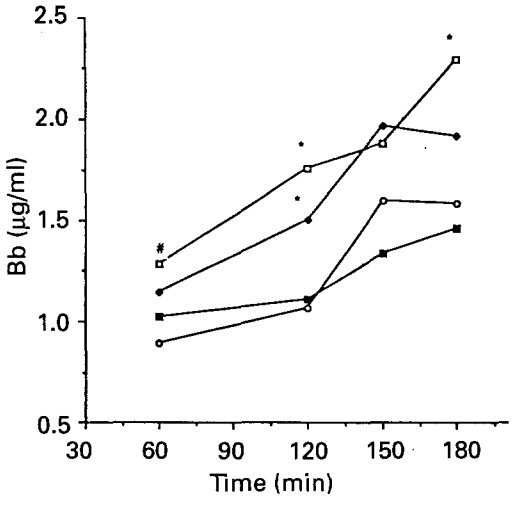

Figure 2 Quantification of Bb fragments in pooled human heparinized plasma incubated with biomaterials. Levels of Bb were determined using an ELISA. Resting sample: [Bb] = 1.13 ± 0.03 µg/ml (□ CDT; ◆ CA; ○ PDMS; ■ LDPE) *significantly different ($p < 0.05$) from PDMS and LDPE, # statistically different ($p < 0.05$) from LDPE.

[Bb] for LDPE and CDT continued its increase. Starting at 1 h, no significant differences were observed among the biomaterials. At 2 h, PDMS and LDPE were found to be significantly different from CDT and CA. At 2.5 h, LDPE was significantly different from CDT and CA. At 3 h, CDT was significantly different from PDMS and LDPE.

Results obtained with pooled serum led to the same kinetics (Fig. 3): LDPE and CDT activated complement with an almost linear rate from 1 to 4 h, while a plateau was reached for PDMS at 2 h ([Bb] ~ 7 µg/ml) and for CA at 2.5 h ([Bb] ~ 13.5 µg/ml). At 1.5 h, significant differences were found between LDPE and CDT and CA, respectively. CDT was also found significantly different from PDMS. At longer incubation time, LDPE was always found to be significantly different from CDT and CA. AN69 (data in Table II) was found to be significantly different ($p < 0.003$) from CDT and CA at 2 h.

4.3. C3a results

C3a generation began within the first 15 to 30 min. Two types of kinetics could be observed: for CA and PDMS, after 30 min, formation of C3a started to level off while for LDPE and CDT, a constant increase

TABLE II Complement activation of AN69. Serum was incubated with AN69 for different times at 37 °C. Complement activation was assessed using ELISA for Bb fragments and C3a. Values for CDT (high activator) and LDPE (low activator) have been added for comparison

Surface	Serum C3a 15 min	30 min	60 min	Serum Bb fragment 90 min	120 min
AN69	1.36 ± 0.14[a]	2.48 ± 0.81[a]	3.05 ± 1.15[b]	3.82 ± 1.38[b]	3.27 ± 0.75[a]
LDPE	1.99 ± 0.78	2.10 ± 0.03	2.66 ± 0.05	2.70 ± 0.91	4.23 ± 2.55
CDT	4.89 ± 0.67	5.49 ± 0.93	4.80 ± 0.88	7.15 ± 0.86	7.68 ± 2.65

AN69 significantly different from CDT and CA: [b]$p < 0.05$, [a]$p < 0.01$.

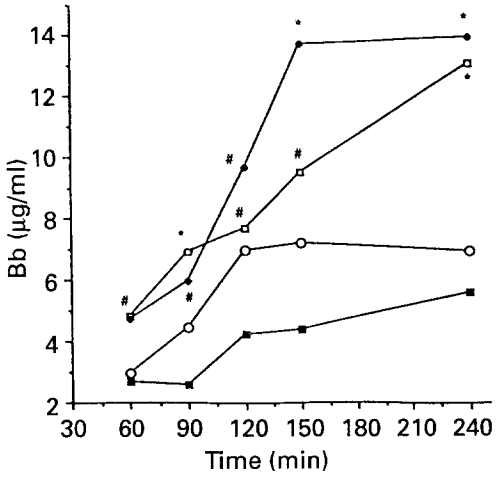

Figure 3 Quantification of Bb fragments in pooled human serum incubated with biomaterials. Serum was assayed for Bb using an ELISA. Resting sample: [Bb] = 1.58 ± 0.6 µg/ml (□ CDT; ◆ CA; ○ PDMS; ■ LDPE) *significantly different ($p < 0.05$) from PDMS and LDPE, # statistically different ($p < 0.05$) from LDPE.

from 15 to 90 min could be observed (Fig. 4). During the first minutes of incubation, activation of complement occured to high extent for CA and CDT, differences could be observed between the positive and negative controls at 15 min. At 30 min, only LDPE was significantly different from the two positive controls. At 90 min, a significant difference existed between LDPE and CDT. As far as AN69 is concerned, it was found to be significantly different from CDT,

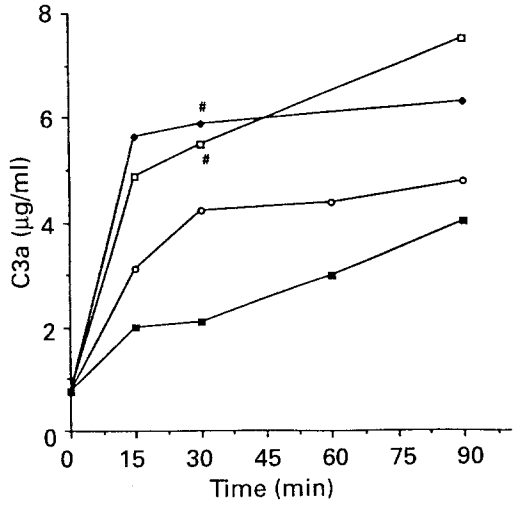

Figure 4 Quantification of C3a in pooled human serum incubated with biomaterials. Levels of C3a were determined using an ELISA. Resting sample: [C3a] = 0.755 ± 0.6 µg/ml (□ CDT; ◆ CA; ○ PDMS; ■ LDPE) *significantly different ($p < 0.05$) from PDMS and LDPE, # statistically different ($p < 0.05$) from LDPE.

CA and PDMS ($p < 0.02$) at 15 and 30 min (values were obtained only for these two incubation times). C3a generation for AN69 (in Table II) was even lower than that for LDPE.

4.4. SDS-PAGE followed by Western blot analysis

SDS-PAGE followed by Western blot analysis showed bound C3 fragments on the surface of every biomaterial. The predominant polypeptides on all surfaces had molecular weights of ~ 75 kDa, ~ 70 kDa and ~ 40 kDa, representing the β-chain of C3/C3b and fragments of iC3b, respectively (Figs 5 and 6). Bands at a molecular weight ~ 120 kDa

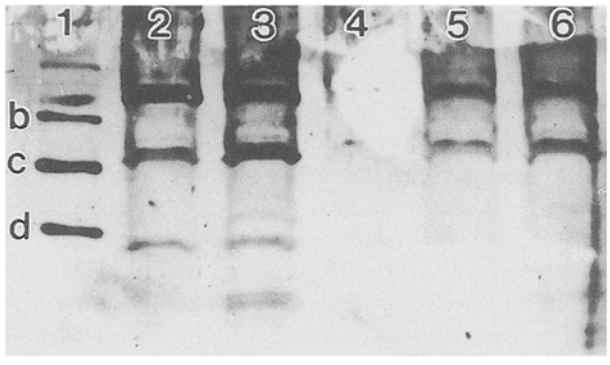

Figure 5 SDS-PAGE followed by Western Blot analysis of C3 fragments present on the surface of biomaterials incubated for 1.5 h with serum. Detecting antibodies were polyclonal anti-C3c. Lane 1: molecular weight standards: b = 58100 kDa, c = 39800 kDa, d = 29000 kDa; Lane 2: CDT; Lane 3: CA; Lane 4: LDPE; Lane 5: PDMS; Lane 6: Silastic®.

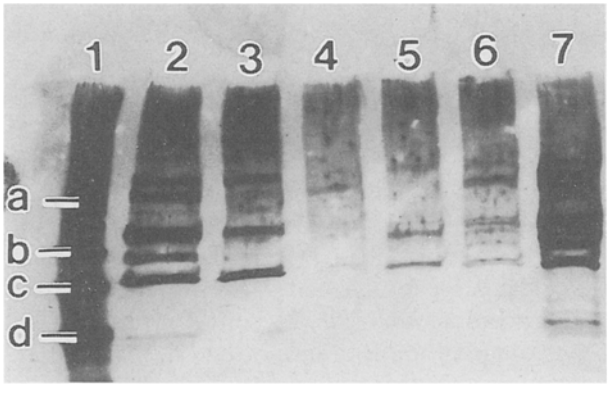

Figure 6 Immunoblot of C3 fragments present on the surface of biomaterials incubated for 1.5 h with serum. Detecting antibodies were a mixture of polyclonal anti-C3c and anti-C3d. Lane 1: molecular weight standards: a = 97400 kDa, b = 58100 kDa, c = 39800 kDa, d = 29000 kDa; Lane 2: CDT; Lane3: CA; Lane 4: LDPE; Lane 5: PDMS; Lane 6: Silastic®; Lane 7: AN 69.

(α-chain of C3/C3b) were also visible (Fig. 5). For LDPE (Fig. 6, lane 4), just the 75 kDa band seemed to be present (another band could sometimes be observed but its intensity was no greater than the background). On the blot incubated with anti-C3c and anti-C3d (Fig. 5), a weak band near 29 kDa (corresponding to C3d M W) was also visible for CDT, CA and AN69. The immunoblot incubated with polyclonal anti-C3c antibodies (Fig. 5) showed some evidence of C3c adsorbed to the surfaces of CDT and CA: lanes at 29 kDa, 43 kDa and 75 kDa. Generally, the intensity of the bands for PDMS, LDPE and Silastic® tubing was reduced compared to CDT, CA and AN69. The surface of AN69 appeared to have the most adsorbed protein.

5. Discussion

Classification of the tested biomaterials, using either iC3b, C3a or Bb values, conformed what was expected, and was similar to the ranking of the IUPAC working party on "interactions of polymer with living systems" [12].

Based on the known mechanism of complement activation in the presence of a surface, the values obtained from iC3b measurements were questionable: some iC3b from the conversion of the C3b bound to the surface may have remained bound and thus would not be assessed through plasma analysis. In fact, on the immunoblot, the two α-chain fragments 70 kDa and 40 kDa indicate the presence of iC3b on most of the surfaces. Concentrations of iC3b in plasma may then underestimate the extent of complement activation due to the remaining iC3b on the surface. This hypothesis was further verified with plasma analysis for Bb content. At 1 h, Bb values showed that Silastic® tubing in a well was activating complement to a low degree (compare to the values obtained with plasma alone in a well), while the iC3b test failed to show any supplementary activation. Activation of complement induced by the piece of Silastic® did not affect our findings, since statistical analysis pointed out that the difference of Bb generation in an empty well and in a well containing Silastic® tubing was not significant. As reported in the results (Table I), the Silastic® tubing used to anchor the samples did not appear to activate complement to a degree that influences activation by the tested discs.

Activation of complement is believed to occur essentially through the alternative pathway [13, 14], assessing activation via Factor Bb was then a justified choice because no adsorption of Bb has been reported. Two types of kinetics were observed among our samples: a linear curve for CDT and LDPE and a more sigmoidal curve for CA and PDMS, where a plateau was reached around 2 h. Reasons for this different behaviour may be due to surface and chemical properties, but need further exploration. The time where the full capacity of complement activation could be detected was thus different depending on the biomaterials. However, significant differences of Bb concentrations among the positive and negative controls started to appear around 1.5 h. The characteristics of a biomaterial could then be evaluated before a plateau is reached. An incubation time between 1.5 h and 3 h can thus be used to evaluate the complement activation of a candidate biomaterial comparing its Bb production with that obtained with our controls.

The ELISA for C3a was made commercially available only recently; C3a measurements appear to give direct assessment of C3 activation and it is also a more common measurement of complement activation than Bb. However its use as an *in vitro* evaluation tool has been quite difficult. Spontaneous cleavage of C3 into C3a and C3b occurs *in vitro* and very high background level in serum samples (around 7 μg/ml) are possible. While unthawing serum samples, complement activation may occur and this represents a major limitating factor in an anaphylatoxin assay as already reported by Morgan [15]. A main problem with our C3a experiments was also the fact that a significant variability in [C3a] existed between discs of the same biomaterials. Further experiments need to be performed to obtain a more suitable system for C3a measurements. A solution might be to use plasma instead of serum samples. Some recommend the use of plasma since it contains lower background levels [9, 16], while others prefer serum to evaluate complement activation by biomaterials [17, 18]. Serum, instead of heparanized plasma, better mimics the non-anticoagulant situation, since heparin has inhibitory effects on both classical and alternative pathways of complement [19]. Using serum may enable us to come closer to the natural mechanism of interaction between complement and biomaterial.

For C3a assays, interpretation of the concentrations obtained in the samples have to be done carefully: low concentrations of C3a may be due to adsorption of C3a on the surface. A test for C3a adsorption would then be required: analysis of the surface could be performed with a Western blot or a comparison between the kinetic patterns for C3a and Bb production could be done. Adsorption of C3a could be indicated for example by a plateau in [C3a] but none in [Bb].

For C3a formation, only within the first 30 min could significant differences be observed between the biomaterials. Early time points seemed then to be the appropriate incubation times to evaluate the capacity of activating complement of a biomaterial. This early activation measurements for C3a would also be a good indicator for testing hemodialysis membranes. Reports [2, 20] have shown that the first 15–30 min of dialysis might be the critical time where a high amount of C3a generated will induce an extensive leukopenia: this is the case for CDT, while for polyacrylonitrile membrane (AN69) minimal C3a formation had been observed – in fact most of it is adsorbed on its surface – and biological effects such as leukopenia seemed to be absent [21]. Our *in vitro* results were in accordance with these observations, AN69 was found to be significantly different from CDT and CA. Then, even if difficulties were encountered with serum samples for the C3a assay, our system seemed to be quite adequate to screen hemodialysis membranes.

Interesting results were obtained by Western blot analysis for C3 fragments. This iC3b fragment is a

receptor for neutrophils, monocytes and NK cells [1]; adherence of those cells may lead to phagocytosis or cytotoxic release reactions. iC3b fragments were found on most of the surfaces, except LDPE. The amount of iC3b on PDMS and Silastic® tubing was lower than on CDT and CA, but iC3b presence might still participate in the adhesion of inflammatory cells, thus leading to a less biocompatible material. C3d fragments were also found on the surface of CDT, CA and AN69. C3d is a receptor for B-lymphocytes and platelets. LDPE was the only material where no 40 kDa band could be observed, indicating that only C3b was present on the surface (revealed by the presence of the 75 kDa band). For LDPE, all the iC3b fragments, issued from inactivation by Factor I of the C3b bound to the surface, are directly released into plasma instead of staying bound to the surface as for the other materials studied here. The membrane AN69 is known to adsorb C3a but it also seemed to bind or adsorb a great amount of other C3 fragments such as iC3b and C3d.

6.Conclusions

Our incubation system at 37 °C proved to be a useful means to evaluate complement activation. Similar results in the capacity of complement activation of our biomaterials were found with the different complement proteins assayed. Quantification of Bb using an ELISA appears to be the most reliable assay to assess complement activation through the alternative pathway. It is an indirect measure of C3 activation but the advantages are that no adsorption of Bb seems to occur and that the background level is really low. The test for Bb is part of a screening test to assess the compatibility of biomaterials in contact with blood. C3a formation reflects activation of both pathways and despite the difficulties encountered, the C3a assay may be used to verify that complement activation occured only via the alternative pathway ensuring that low Bb generation was due to a weak activator of complement. Surface analysis should be performed while evaluating the complement activation of a biomaterial since the importance of the bound-phase of complement has not yet been explored; complement protein adsorption could also be an issue.

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