Complement activation and protein adsorption by carbon nanotubes

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Abstract

As a first step to validate the use of carbon nanotubes as novel vaccine or drug delivery devices, their interaction with a part of the human immune system, complement, has been explored. Haemolytic assays were conducted to investigate the activation of the human serum complement system via the classical and alternative pathways. Western blot and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) techniques were used to elucidate the mechanism of activation of complement via the classical pathway, and to analyse the interaction of complement and other plasma proteins with carbon nanotubes. We report for the first time that carbon nanotubes activate human complement via both classical and alternative pathways. We conclude that complement activation by nanotubes is consistent with reported adjuvant effects, and might also in various circumstances promote damaging effects of excessive complement activation, such as inflammation and granuloma formation. C1q binds directly to carbon nanotubes. Protein binding to carbon nanotubes is highly selective, since out of the many different proteins in plasma, very few bind to the carbon nanotubes. Fibrinogen and apolipoproteins (AI, AIV and CIII) were the proteins that bound to carbon nanotubes in greatest quantity.

Keywords: Carbon nanotubes; Complement; Inflammation; Adjuvant; Apolipoproteins

1. Introduction

Carbon nanotubes (CNTs) can be described as rolled hexagonal carbon networks that are capped by half fullerene molecules. There are three main types of carbon tubes: single-walled (SWNTs), double-walled (DWNTs) and multi-walled (MWNTs). CNTs can be synthesised using the arc-discharge method (Ajayan et al., 1993) catalytic chemical vapour deposition (CVD) (Dai et al., 1996), and laser ablation (Guo et al., 1995). The dimensions of these tubular structures range from 0.4 to 2 nm in diameter for SWNTs, and from 2 to 100 nm for MWNTs. Both types have length typically ranging from 1 to 50 μm. The diameters of

DWNTs are typically from 1 to 3.5 nm and they are from several micrometers to tens of micrometers in length (Flahaut et al., 2003).

The possibility to combine the remarkable specificity and parallel processing of biomolecules with the hollowed cavity, size and electrical properties of carbon nanotubes has attracted considerable attention for several types of applications, ranging from the creation of new types of biosensors (Chen et al., 2003) to the fabrication of drug or vaccine delivery devices (Georgakilas et al., 2002, Pantarotto et al., 2003a, Pantarotto et al., 2003b, Pantarotto et al., 2004 and Wong Shi Kam et al., 2004).

It has been shown (Pantarotto et al., 2003b) that peptide–carbon nanotube complexes enhance the immune (antibody) response against the peptides with no detectable cross-reactivity to the carbon nanotubes (i.e. carbon nanotubes are not intrinsically immunogenic). Functionalised carbon nanotubes have been shown to cross cell membranes and to accumulate in the cytoplasm without being toxic for the cell (Pantarotto et al., 2004). The absence of immunogenicity of carbon nanotubes in comparison to common protein carriers, the translocation of carbon nanotubes across the cell membrane without being toxic and the ability to enhance an immune response when attached to an antigen, strengthens the possibility of using carbon nanotubes as therapeutic and vaccine delivery tools.

The mechanism by which a functionalised single-walled nanotube can be internalised into the human promyelocytic leukemia cell line HL60 and human T cells without being toxic has been studied (Wong Shi Kam et al., 2004). A fluoresceinated protein was shown to enter cells only when conjugated to a carbon nanotube (Wong Shi Kam et al., 2004). This work demonstrates that carbon nanotubes can be exploited as molecular transporters to carry cargoes into cells.

The biocompatibility and the possible hazardous effects that carbon nanotubes might induce in body fluids (e.g. human serum, plasma and blood) have to date not been reported. Such studies are required to help bring carbon nanotubes closer to the reality of pharmaceutical applications. A major problem in medical applications of nanoscale materials is whether the body's immune system can recognise carbon nanotube materials (Gewirth and Siegenthaler, 1996). Little is known about the interaction between nanomaterials such as carbon nanotubes and the immune system. In order to validate the use of carbon nanotubes as a building block

for the next generation of novel medical devices for diagnosis (e.g. ultra fast biosensors) and therapy (drug delivery) we have explored their interaction with the complement system.

The complement system is a group of about 35 soluble and cell-surface proteins in blood which interact to recognise, opsonise and clear or kill invading micro-organisms, altered host cells (e.g. apoptotic or necrotic cells) and other foreign materials (Sim and Tsiftsoglou, 2004). It can be activated by synthetic materials such as polystyrene (Andersson et al., 2002). Activation may occur by any of three pathways, termed the classical, lectin and alternative pathways. In the classical pathway, the protein C1q recognises activators mainly via charge and hydrophobic interactions, and binds to them. In the lectin pathway MBL binds to targets via interaction with neutral sugar residues (e.g. mannose). Similarly l-ficolin can initiate the lectin pathway, but its recognition specificity is uncertain. The activation of complement via the alternative pathway starts by the binding of C3b to the pathogen surface and the subsequent events of complement activation via this pathway are analogous to those of the classical pathway.

2. Materials and methods

2.1. Synthesis of carbon nanotube samples

For this study two types of carbon nanotubes were used: single-walled and double-walled. SWNTs were produced by two different methods. Arc discharge SWNTs were synthesised at the Inorganic Chemistry Laboratory, University of Oxford, UK (Journet et al., 1997). SWNT samples were purified by refluxing them in a concentrated HNO_3 solution (3 h, 110 °C) followed by repeated washing with deionised water and by a drying procedure (overnight at 110 °C). Subsequently, these samples were partially oxidised in air for 45 min in a furnace at 400 °C. Finally, the samples were annealed at 1400 °C under Ar flow for 2 h. Purified catalytic chemical vapour deposition SWNTs (CVD SWNTs) and high pressure carbon monoxide SWNTs (HIPco SWNTs) were purchased from Nanocyl S.A. company (Namur, Belgium) and Carbon Nanotechnology Inc. (Houston, TX), respectively. Purified catalytic vapor deposition double-walled nanotubes were made as described by Flahaut et al. (2003).

Plasma atomic emission spectroscopy studies were performed on the purified carbon nanotube samples to quantify the remaining traces of metal element impurities after the purification process. It was found that Arc discharge SWNTs contained 1.4% (w/w) Ni, HIPco SWNTs

contained 1% (w/w) Fe, CVD SWNTs contained 0.2% (w/w) Co, CVD DWNTs contained 1.9% (w/w) Mo. There were no other significant metalic impurities.

2.2. Handling of nanotubes in aqueous buffers

Nanotubes have a hydrophobic surface and do not disperse rapidly in aqueous buffers. To form rapidly a reasonably stable suspension nanotubes can be first wetted by use of a surfactant, such as 0.5% Triton X-100. Initial experiments were done with nanotubes wetted in 0.5% Triton X-100. It was then observed that serum and plasma would also act as a "wetting agent" and experiments were repeated with nanotubes, which had been suspended directly in serum or plasma. No difference in complement-activating capacity was found between nanotubes wetted in Triton X-100 and nanotubes wetted directly in serum or plasma.

To study protein binding to nanotubes, suspending the nanotubes in protein solution, incubating and washing by centrifugation or filtration is impractical, as the centrifuged nanotubes are difficult to resuspend. For filtration, they form a layer, which is not sufficiently porous for efficient washing. For this reason, nanotubes were dispersed in a relatively inert porous scaffold (Sepharose 4B beads, Amersham Biosciences, Bucks, UK) for protein binding experiments.

2.3. Preparation of antibody-sensitised erythrocytes, rabbit erythrocytes, serum, plasma and radioiodinated C1q

2.3.1. Preparation of antibody-sensitised erythrocytes

Antibody-sensitised sheep erythrocytes (EA) were prepared as follows. Erythrocytes from sheep blood in Alsevers solution (TCS Biosciences, Buckingham, UK) were washed (10 min, 3000 rpm room temperature (RT)) three times in phosphate-buffered saline (PBS (137 mM NaCl, 2.6 mM KC1, 8.2 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.4)) before resuspending the erythrocytes in dextrose gelatin veronal buffer ($DGVB^{2+}$; 2.5 mM sodium barbital, 71 mM NaCl, 0.15 M $CaCl_2$, 0.5 mM $MgCl_2$, 2.5%, w/v glucose, 0.1%, w/v gelatin, pH 7.4). Ten millilitres of sheep erythrocytes (adjusted to 10^9 cells/ml) were then incubated with 50 μl of rabbit anti-sheep haemolytic serum (C12HSA, Serotec, Kidlington, UK) for 1 h at 37 °C. EA were washed once in PBS, then three times in $DGVB^{2+}$, and the concentration adjusted to 10^9 cells/ml in the same buffer.

2.3.2. Preparation of rabbit erythrocytes

Rabbit erythrocytes from rabbit blood in Alsevers solution (TCS Biosciences) were washed three times in PBS and the concentration adjusted to 10^9 cells/ml in Mg–EGTA buffer (10 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*,*N*′-tetraacetic acid (EGTA)), 7 mM $MgCl_2$; 2.1 mM sodium barbital, 59 mM NaCl, 2.08% (w/v) glucose, 0.08% gelatin, pH 7.4.

2.3.3. Preparation of serum and plasma

Serum was made by clotting human citrated plasma (HD Supplies, High Wycombe, UK) by addition of 16 mM $CaCl_2$ with subsequent removal of the clot. This material was used for classical pathway and protein binding studies. Fresh human serum obtained from blood samples with no anti-coagulant was used for alternative pathway assays. Clotted blood was centrifuged at 3000 rpm for 30 min and the serum directly aliquoted and stored at −80 °C.

2.3.4. Iodination of C1q

C1q was isolated from pooled human serum using affinity chromatography on IgG Sepharose (Reid, 1981). C1q was iodinated as follows: CPG-10 (BDH Chemicals, Poole, UK) controlled pore glass beads (100 mg) was mixed with 1 ml of chloroform solution containing iodogen (Sigma, Poole, UK) (200 μg iodogen/ml) and incubated at room temperature for 5 min before the beads were dried under a stream of oxygen-free nitrogen. Ten milligrams of iodogen-coated CPG-10 glass beads (20 μg iodogen/10 mg beads) were mixed with 500 μl of PBS–0.5 mM EDTA, pH 7.4, 20 μl 1 M potassium phosphate buffer pH 7.4, 0.3 mCi of Na ^{125}I (Amersham Bioscience) and 50 μg of C1q in PBS–0.5 mM EDTA, pH 7.4 and incubated on ice for 5 min after which the mix was transferred to a de-salting column (PD-10, Amersham Bioscience) to remove unbound Na ^{125}I.

2.4. SDS-PAGE and Western blotting

2.4.1. SDS-PAGE

Unless otherwise indicated the samples that required electrophoretic analysis were incubated at 95 °C for 5 min in sample buffer (0.2 M Tris, 8 M urea, 2% SDS, 0.2 M EDTA, 40 mM dithiothreitol (DTT), adjusted to pH 8.2 with HCl) and loaded on a 4–12% Novex Bis–Tris sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gradient gel (Invitrogen, Paisley, UK), then separated by electrophoresis for 45 min at 200 V using MES buffer (Invitrogen) in a Novex X Cell II Mini-cell gel apparatus. Invitrogen multimark

prestained standards were used. Proteins bands were stained with simply Blue SafeStain (Invitrogen).

2.4.2. Western blotting technique

Following SDS-PAGE, unstained proteins were transferred to a PVDF membrane by semidry blotting in 0.039 M glycine, 0.048 M Tris, 0.0375% (w/v) SDS buffer. The membrane was blocked with 5% (w/v) skimmed milk powder (Marvel, Premier Brands Ltd., UK) in washing buffer (PBS, 0.2%, v/v Tween 20, pH 7.4). The blot was then incubated with polyclonal rabbit anti-human C1q antibody (MRC Immunochemistry Unit: 20 μg of purified IgG/ml) for 60 min at RT, washed three times for 10 min in washing buffer and incubated with goat–anti-rabbit IgG-horseradish peroxidase conjugate (0.5 μg/μl) (TAGO, Burlingame, USA), in blocking buffer for 1 h at RT. The membrane was developed using an ECL™ Western blotting kit (Amersham Biosciences).

2.5. Mass-spectometry analysis of proteins

SDS-PAGE gel bands were destained 3 times for 20 min in 100 μl 50 mM NH_4HCO_3 in 50% (v/v) acetonitrile. The supernatant was discarded and gel pieces were soaked in 100 μl of 80% (v/v) acetonitrile for 20 min. The acetonitrile was removed and gel pieces dried under vacuum for 30 min using a centrifugal evaporator (Speed-vac, Savant, USA). The proteins in the gel pieces were re-reduced by swelling them in 50 μl 10 mM dithiothreitol (DTT) in 100 mM NH_4HCO_3 and incubating (45 min, 56 °C). Gel pieces were chilled to room temperature and DTT solution removed by aspiration. Fifty microliters of 55 mM iodoacetamide in 100 mM NH_4HCO_3 was added and the gel pieces were incubated for 30 min at room temperature to alkylate free SH groups. Liquid was removed and gel pieces were washed with NH_4HCO_3/acetonitrile as previously. Gel pieces were completely dried under vacuum, re-hydrated in 50 μl 20 ng/μl trypsin in 100 mM NH_4HCO_3 (Sigma sequence grade) and left to incubate at 37 °C overnight. The supernatant was removed and stored in a 0.5 ml conical centrifuge tube. Fifty microliters of acetonitrile:triflouroacetic acid (TFA):water (50:1:49, v/v/v) was added to the gel pieces and the samples agitated for 20 min at room temperature. The supernatant was removed and pooled with supernatant from the previous step before being dried under vacuum as before. The sample was reconstituted in 10 μl 0.1% (v/v) TFA or 5 μl if from a faint gel band. One microliter of sample was mixed with 1 μl of saturated α-cyano matrix solution of which 0.5 μl was loaded onto the Ettan MALDI-ToF Pro mass spectrometer (Amersham Biosciences) target slide for PMF (peptide mass fingerprint)

analysis in reflectron mode. Proteins were identified by comparison of the spectra to a computer-generated database of tryptic peptides from known proteins using ProFound, the instrument's built-in search engine (Proteometrics LLC), which utilises the NCBInr protein database (National Center for Biotechnology Information, Bethesda, USA).

2.6. N-terminal sequence analysis

Proteins, which were not identified by mass spectrometry were subjected to N-terminal sequence analysis. Samples were reduced and run on SDS-PAGE (as in Section 2.4.1). The gel was electroblotted to Novex 0.2 μm PVDF membrane (Invitrogen) in a Novex blot module. The membrane was then stained with Coomassie Brilliant Blue. The bands of interest were excised from the PVDF membrane and washed extensively with 10% methanol in water prior to sequencing. Samples were then sequenced on an Applied Biosystems 494A 'Procise' protein sequencer (Applied Biosystems) using standard sequencing cycles.

2.7. Haemolytic complement assay (complement activation/consumption)

2.7.1. Complement activation/consumption via classical pathway

To investigate whether carbon nanotubes activate human serum complement via the classical pathway a complement consumption assay was done. Nanotubes (0.62–2.5 mg) were suspended in 500 μl of undiluted human serum and placed in the wells of a flat-bottomed 24-well plate (Greiner, Stonehouse, Gloucestershire, UK). Similarly, zymosan samples (Sigma) (0.62–2.5 mg) were suspended in 500 μl of undiluted serum and placed in wells of the same plate. Zymosan is a positive control as it activates the classical pathway by binding anti-yeast antibodies present in human sera. A negative control sample consisted in placing 500 μl of undiluted human serum in empty wells. Samples were incubated at 37 °C for 1 h followed by centrifugation (2500 rpm, 10 min).

To test the classical pathway (Whaley, 1985 and Whaley and North, 1997), the supernatants of each sample were serially diluted (two-fold serial dilution 1/2 to 1/1024 in $DGVB^{2+}$) and placed in a microtitre (96-well) plate. One hundred microliters of each dilution was incubated with 100 μl of antibody-sensitised EA (10^8 cells/ml in $DGVB^{2+}$) plus 100 μl $DGVB^{2+}$ in V-well microtitre plates (Dynex Technologies, Ashford, UK) for 1 h at 37 °C. After incubation, cells were spun down (2500 rpm, 10 min, RT), and haemoglobin was measured at 405 nm in the supernatant. Total haemolysis (100%) was measured by lysing EA with water.

Background spontaneous haemolysis (0%) was determined by incubating EA with buffer only. In this type of assay, incubation of human serum with an activator activates (consumes) complement and therefore depletes complement activity. The extent of depletion is determined by assaying remaining complement activity by determining complement dependent lysis of red blood cells. Data relative to the kinetics of the activation of complement system compared to zymosan were obtained by incubating 500 μl of undiluted serum with DWNTs (1.25 mg) or zymosan (1.25 mg) for 0, 5, 30 and 60 min at 37 °C. Each supernantant was assessed for the activation of complement via classical pathway following the procedure described above.

2.7.2. Complement activation/consumption via alternative pathway

To test the alternative pathway, 500 μl of fresh human serum was diluted 1:1 (v/v) with Mg–EGTA buffer and incubated for 1 h at 37 °C with zymosan or carbon nanotubes in wells of a 24-well plate as described above. Then quadruplicate 50 μl samples of the supernatant were again diluted 1:1 with the same buffer and placed in a microtitre plate. Rabbit red blood cells (200 μl of 10^9 cells/ml) were added to each well and incubated at 37 °C for 1 h followed by centrifugation (2500 rpm, 10 min). Fifty microliters of the supernantants were taken and mixed with 200 μl Mg–EGTA buffer. Haemoglobin was measured at 541 nm in the supernatant. Total haemolysis (100%) was measured by lysing the rabbit red cells with water. Background spontaneous hemolysis (0%) was determined by incubating rabbit red cells with Mg–EGTA buffer only. Kinetic studies of the activation of the complement system via alternative pathway were performed by incubating 500 μl of fresh serum diluted 1:1 (v/v) with Mg–EGTA buffer with 1.25 mg of DWNTs or 1.25 mg of zymosan for 0, 5, 30 and 60 min at 37 °C, followed by haemolytic assay as above.

2.8. Selective binding of serum and plasma proteins to carbon nanotubes

2.8.1. Selective binding of serum proteins to carbon nanotubes

Experiments using human serum and human plasma were carried out to determine whether protein binding to carbon nanotubes is selective. Two Sepharose 4B columns were set up: a larger Sepharose column (10 ml) (not containing carbon nanotubes), and a small Sepharose column (1 ml, containing 5 mg DWNT suspended among the Sepharose beads). These were equilibrated in 20 mM Hepes, 50 mM NaCl, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, pH 7.0.

Human serum (10 ml) was diluted with 10 ml running buffer and passed through the large column to remove proteins which bind to Sepharose (these include IgG, IgM and fibronectin) then through the small column, to bind proteins which interact with the nanotubes. After this, each column was thoroughly washed with the running buffer. From each column, the resin was resuspended to a 1:1 slurry in the running buffer. One hundred microlitres of each slurry was centrifuged at 13,000 rpm for 5 min. The supernantants were removed. Fifty microlitres of SDS-PAGE sample buffer was added to the resins. Samples were incubated at 95 °C for 5 min and loaded on an SDS-PAGE gradient gel as in Section 2.4.1.

2.8.2. Selective binding of plasma proteins to carbon nanotubes

For binding of plasma proteins, 10 ml of human plasma was diluted with 10 ml of running buffer (Alsever's solution: 294 mM trisodium citrate, 1.9% (w/v) glucose, 72 mM sodium chloride, 2.4 mM citric acid, pH 7.0) and then passed through the two columns, equilibrated in Alsever's solution. After washing, resins were analysed by SDS-PAGE as above.

Proteins bands on the SDS-PAGE gel were stained, then low and high intensity protein bands were excised from the gel and prepared for protein identification by tryptic peptide finger printing by mass spectometry and N-terminal sequence analysis as indicated in Sections Sections 2.5 and 2.6, respectively.

2.9. C1q binding to carbon nanotubes

To detect C1q bound to the DWNTs from serum, 100 μl of slurry suspension of the Sepharose–DWNT mixture (Section 2.8.1) and Sepharose (control from Section 2.8.1) were centrifuged at 13,000 rpm for 5 min. Supernantants were removed and 50 μl of SDS-PAGE sample buffer was added to each sample. Subsequently, these samples and a standard C1q sample (375 ng) were load in a 4–12% SDS-PAGE gradient gel and analysed by Western blot technique as described in Sections Sections 2.4.1 and 2.4.2.

To provide additional evidence that C1q binds directly to carbon nanotubes a binding experiment with pure C1q was performed. Sepharose columns were used for each binding experiment. One column containing only Sepharose (1 ml packed volume) served as the negative control. For the other Sepharose column, Sepharose (1 ml packed volume) was mixed with a suspension of DWNT (0.2–1.5 mg) in running buffer (20 mM Hepes, 50 mM

NaCl, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, pH 7.0) and the Sepharose–nanotube mixture was poured into a 0.5 cm diameter column.

^{125}I-C1q (124 262 cpm (1.15 μg)) in 500 μl of the above running buffer or in 500 μl of undiluted human serum was loaded onto each column. This material was left in contact with the column for 30 min at 4 °C followed by an extensive wash with the running buffer. Subsequently, the columns were placed in a Mini-Assay type 6-20 manual γ counter (Mini Instruments, Burnham-on-Crouch, Essex, UK) in order to measure the amount of C1q bound to the carbon nanotubes. The serum contains many different proteins, at a total concentration up to 70 mg/ml and so provides a very large excess of competing proteins to minimise non-specific binding of C1q.

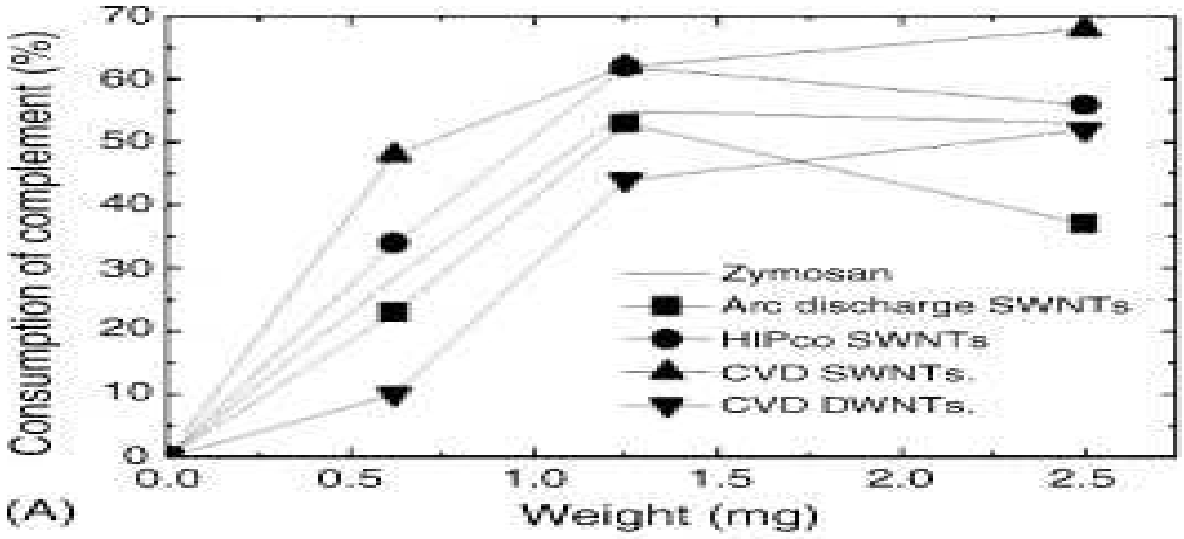
3. Results and discussion

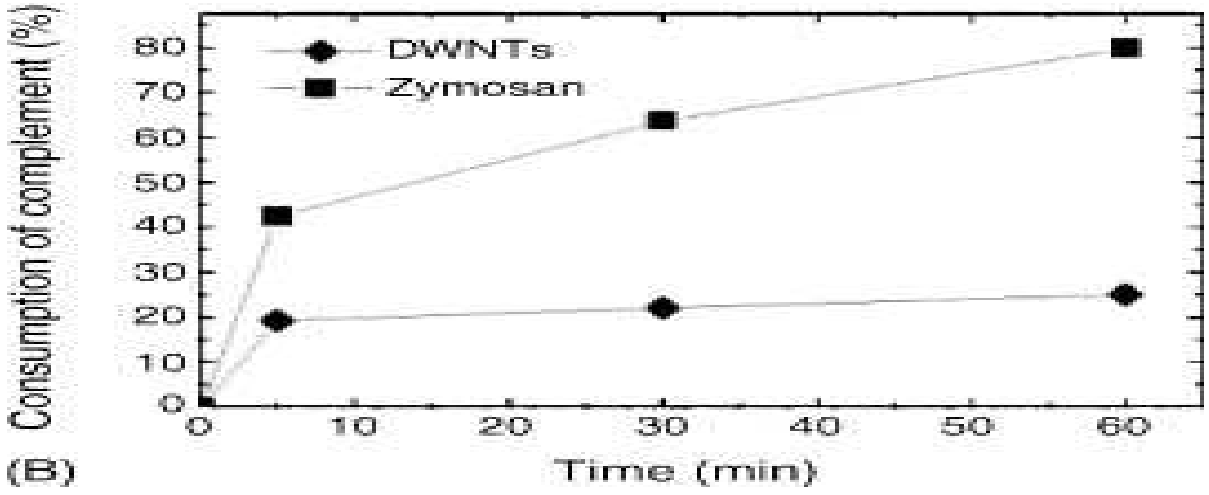
3.1. Consumption/activation of complement via the classical pathway and alternative pathway

The activation of the human serum complement system via the classical pathway by different types of carbon nanotubes, including SWNTs and DWNTs is shown in Fig. 1. Zymosan, a yeast cell wall extract which is a well-characterised and potent complement activator, was used as a positive control to test classical and the alternative pathway activation, while the incubation of a human serum sample at 37 °C was used as a negative control. Zymosan is generally used as an activator of the alternative pathway, but in classical pathway assay conditions anti-yeast antibodies in human serum bind to the zymosan, and activate the classical pathway. All the carbon nanotube samples tested activated complement to an extent comparable with zymosan. Activation is greater with 1.5 mg of zymosan or carbon nanotubes than with 0.62 mg, showing dose dependence. Because of the configuration of the experiment with materials settled in microtitre plate wells, the highest dose, 2.5 mg, presents a similar surface area to the 1.5 mg dose, and so results in similar complement consumption.

Fig. 1. (A) Percentage consumption of human serum complement activity via classical pathway due to the presence of different types of carbon nanotubes. Procedures were as described in Section 2.7.1. Zymosan samples (2.5, 1.25 and 0.62 mg) were used as the positive control for each amount of carbon nanotube samples incubated with undiluted human serum. A sample of undiluted human serum incubated at 37 °C served as the negative control experiment (zero complement consumption). Percentage of complement consumption was

calculated as $(C - C_i)/C \times 100\%$, where C represents the total complement activity (in CH50 units) of the negative control, C_i is the amount of activity remaining in the supernatant of the sample tested, therefore $(C - C_i)$ represents the amount of complement activity lost or consumed by the sample tested. The data points are the means of four determinations. (B) Time course of activation of complement system via classical pathway. Zymosan (2.5 mg) or DWNTs (1.25 mg) were incubated with 500 μl undiluted human serum for 0, 5, 30 and 60 min at 37 °C, and the remaining complement activity (CH50 units) measured by haemolytic assay. Complement consumption was calculated as for (A). Consumption of complement system by carbon nanotubes is rapid similar to zymosan (positive control).

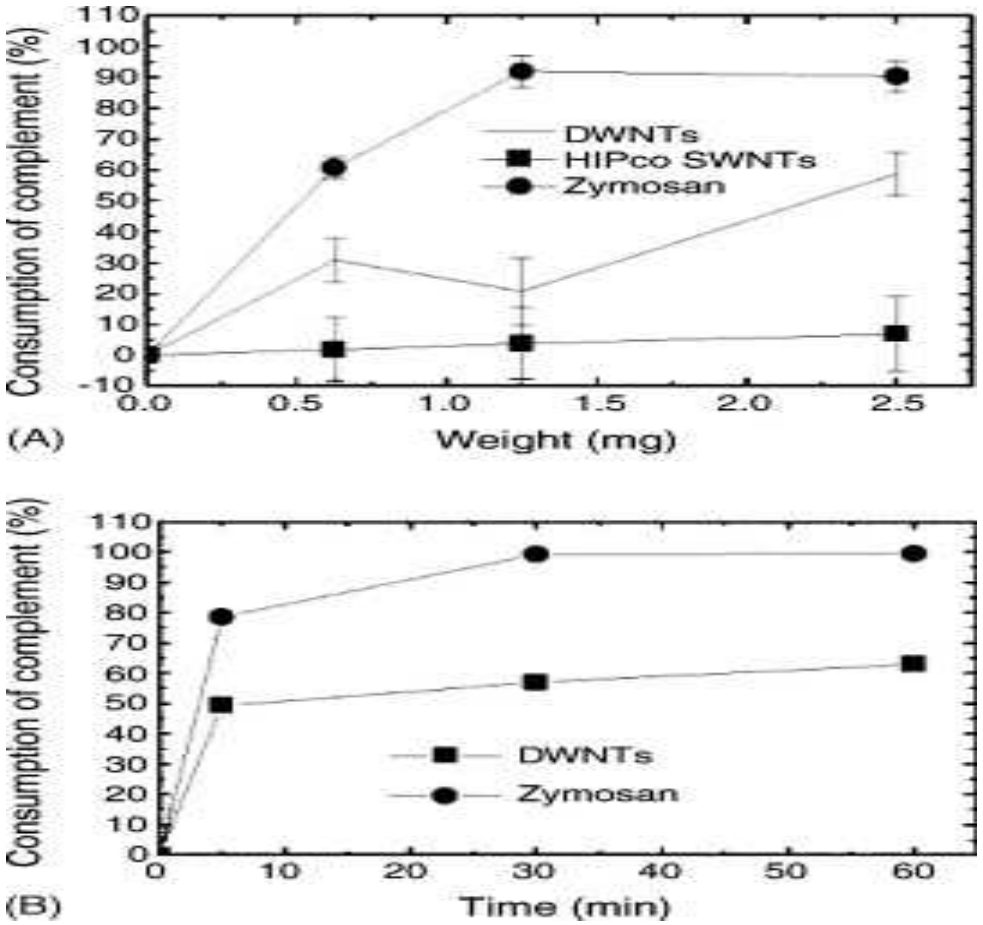




For testing activation of the human serum complement system via the alternative pathway, the incubation of serum with the potential activator, and the haemolytic assay were performed in the presence of only Mg^{2+}. The absence of Ca^{2+} in this assay blocks classical pathway activation. This is because the recognition complex of the classical pathway $C1qr_2s_2$ dissociates and is inactive in the absence of Ca^{2+}.

Complement consumption by DWNTs was dose-dependent and was about 50% of the consumption by a comparable weight of zymosan. Complement consumption by HIPco SWNTs was dose-dependent, but very low (Fig. 2). This is in contrast to classical pathway consumption, where HIPco SWNTs were more effective than DWNTs.

Fig. 2. (A) Percentage consumption of human serum complement activity via alternative pathway due to the presence of two types of carbon nanotubes. Procedures were as described in Section 2.7.2. Zymosan samples (2.5, 1.25 and 0.62 mg) were used as the positive control for each amount of carbon nanotube samples incubated with human serum diluted 1:1 in Mg–EGTA buffer. A sample of human serum incubated at 37 °C served as the negative control experiment (zero complement consumption). Percentage of complement consumption was calculated as $(C - C_i)/C \times 100\%$, where C represents the total complement activity of the negative control, C_i is the amount of activity remaining in the supernatant of the sample tested, therefore $(C - C_i)$ represents the amount of complement activity lost or consumed by the sample tested. Each data point represents the mean and standard deviation of four independent experiments. (B) Time course of activation of complement system via alternative pathway. Zymosan (1.25 mg) or DWNTs (1.25 mg) were incubated with 500 μl of human serum diluted 1:1 in Mg–EGTA buffer for 0, 5, 30 and 60 min at 37 °C and the remaining complement activity (CH50 units) measured by haemolytic assay. Complement consumption was calculated as for Fig. 1A. Consumption of complement system by carbon nanotubes is rapid similar to zymosan (positive control).



Kinetic analysis of activation of complement via both classical and alternative pathway compared to zymosan are shown in Fig. 1 and Fig. 2. These studies show that the consumption of complement by DWNTs via classical and alternative pathway is rapid, similar to zymosan. The similarity in kinetics indicates that the mechanisms of activation by zymosan and by nanotubes are likely to be comparable.

As a consequence of complement activation, fragments of various complement components are generated. C3b, C4b or the C3b breakdown products iC3b and C3d are likely to bind to nanotubes, and this may lead to adhesion onto the surface of a range of blood cells. They may also stimulate uptake into the cell. This may be advantageous in use of nanotubes in vaccination, as it may be improve the presentation of antigens. Drugs contained in nanotubes, however, could be prevented from reaching their targets sites and if cytotoxic could damage components of the cellular immune system.

The relatively high level of complement activation might lead to the generation of an inflammatory response and also might lead to the formation of granulomas at a later stage.

The creation of granulomas associated with the presence of carbon nanotubes has already been reported in studies of mouse lungs (Lam et al., 2004).

3.2. The selective binding of C1q and other plasma proteins to carbon nanotubes

To elucidate the mechanism of activation of complement via the classical pathway and to analyse the interaction of complement proteins with carbon nanotubes the binding of C1q to carbon nanotubes was studied.

Activation of the human serum complement system via the classical pathway takes place when C1q, the recognition subunit of the C1 complex, binds to complement activators. C1q is a 460 kDa protein composed of six heterotrimeric collagen-like triple helices that converge in their N-terminal half to form a stalk, then diverge to form individual stems, each terminating in a C-terminal heterotrimeric globular domain (Gaboriaud et al., 2003). C1 binds to target ligands via these globular domains, or heads, triggering activation of C1r and C1s, the proteases associated with C1q (Arlaud et al., 2002). In this study, we detected C1q protein bound to the carbon nanotube sample by using Western blotting (Fig. 3). Since C1q in serum circulates as the C1 complex ($C1qr_2s_2$), it appears that whole C1 is binding the nanotubes.

Fig. 3. C1q binding to DWNTs. Samples of proteins bound to DWNTs from human serum were analysed by SDS-PAGE and Western blotting as described in Sections Sections 2.8.1 and 2.9. DWNTs suspended in Sepharose were used as an affinity medium to select serum proteins which bind DWNTs. Serum diluted 1:1 was incubated with Sepharose–DWNTs equilibrated in the buffer (20 mM Hepes, 50 mM NaCl, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, pH, 7.0). After removing unbound protein by exhaustive washing with the same buffer, a suspension of the Sepharose DWNT mixture was analysed for bound proteins by SDS-PAGE. Total bound serum proteins are shown by Coomassie blue staining in Fig. 4, track 3; while the presence of bound C1q was shown by Western blotting (Fig. 3, track 2). Sepharose without DWNTs was used as a negative control for the whole procedure (Fig. 3, track 1). C1q A, B chains (which co-run) and C chains are visible in Fig. 3, track 3, while the lower intensity signal in track 2 reveals only the A/B chain band. Anti-C1q antibodies were used to detect C1q. Lane 1: control experiment (human serum proteins bound to Sepharose); lane 2: human serum proteins bound to DWNT Sepharose; lane 3: standard C1q (375 ng).

It was concluded that the binding of C1q to carbon nanotubes is highly selective, since out of thousands of different proteins in serum, very few proteins bind to the carbon nanotubes (Fig. 4). When DWNTs are exposed to human serum and plasma, only a few proteins bind in large quantity. These were identified by tryptic fingerprinting and mass spectrometry as fibrinogen and apolipoprotein A1. Several other bands are visible at lower intensity, these include apolipoproteins AIV and C-III. From serum (which lacks fibrinogen) apolipoprotein A1 is the dominant protein bound, with several other proteins including apolipoprotein AIV and C-III bound in low quantity.

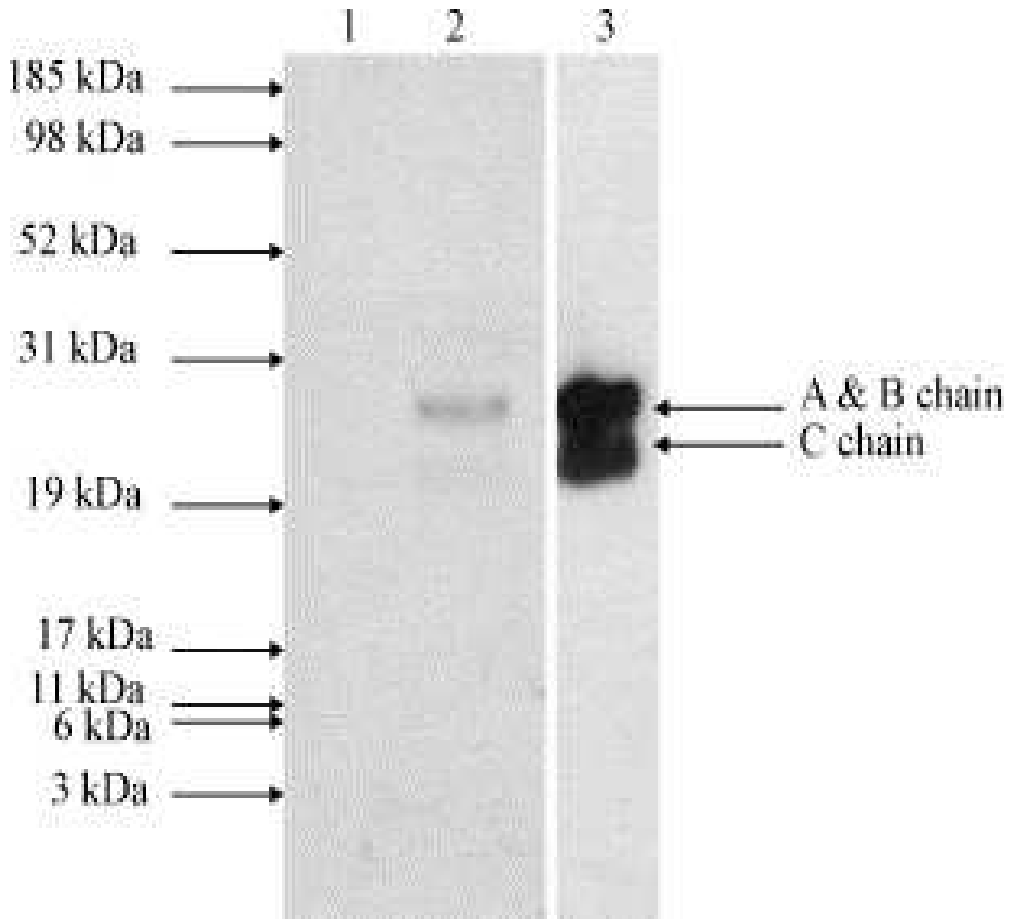
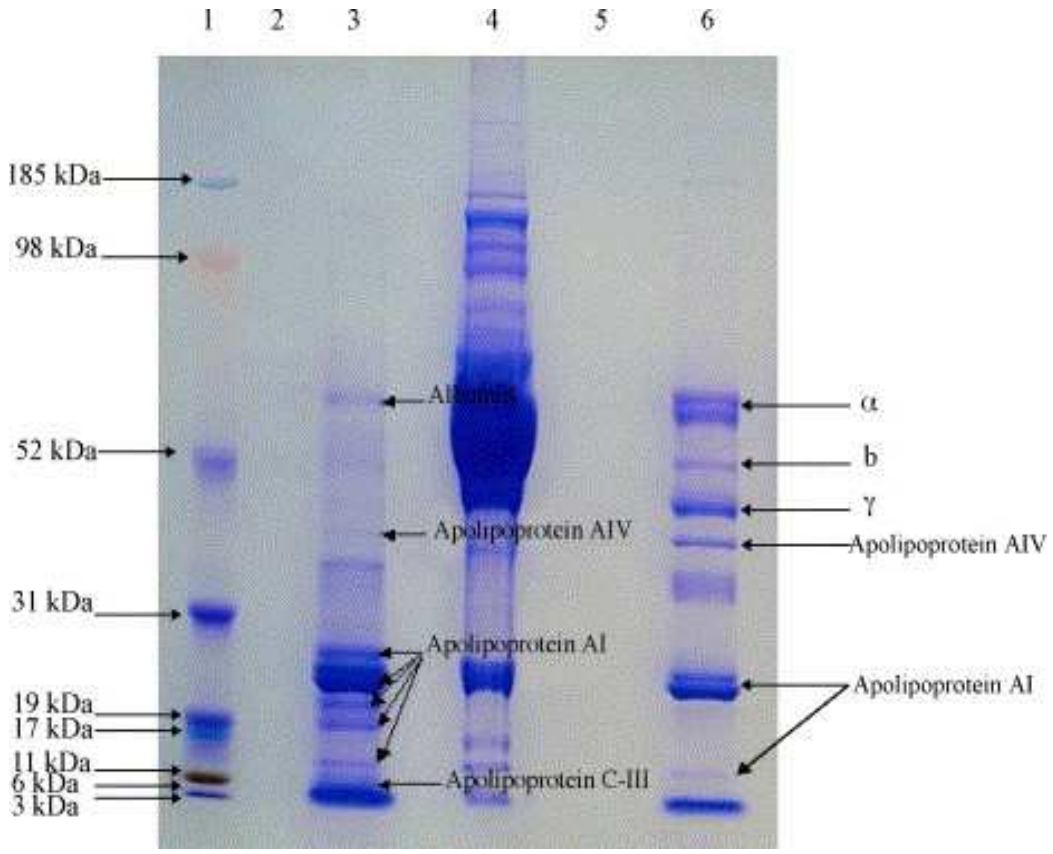


Fig. 4. Selective binding of human serum and plasma proteins to DWNTs. As described in Section 2.8 the binding of serum and plasma proteins to DWNTs was tested. Serum diluted 1:1 in 20 mM Hepes, 50 mM NaCl, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, pH, 7.0 or plasma diluted 1:1 in 294 mM trisodium citrate, 1.9% (w/v) glucose, 72 mM sodium chloride, 2.4 mM citric acid, pH 7.0, were run through affinity columns containing Sepharose (negative control) or Sepharose–DWNTs, each column equilibrated in the dilution buffer for plasma or serum as appropiate. After exhaustive washing in the same buffer, samples of the affinity resins were analysed by SDS-PAGE (reduced) (see Section 2.4). Lane 1: molecular weight marker; lane 2: control experiment (human serum bound to Sepharose); lane 3: human serum bound to DWNT Sepharose; lane 4: human serum (1.25 μl); lane 5: control experiment (human plasma bound to Sepharose); lane 6: human plasma bound to DWNT Sepharose; protein bands from the gel were identified by mass spectrometry tryptic digest fingerprinting or N-terminal sequence analysis as described in Sections Sections 2.5 and 2.6. Protein bands corresponding to ~35 and 37 kDa that are not identified in lane 3 and 6 were also analysed by mass spectometry and N-terminal sequence analysis but there was insufficient protein for their identification. Albumin, apolipoprotein AI, AIV and C-III were identified in serum samples. Albumin and apolipoprotein C-III were not identifiable by fingerprinting but were identified by N-terminal sequencing. Fibrinogen (a protein comprised of α, β and γ chains), apolipoprotein AI and AIV were identified in plasma samples. The spectrum of proteins bound (lanes 3 and 6) is very different from the spectrum of proteins in whole serum (lane 4), showing a high degree of selectivity in binding.



In Fig. 4, C1q is not identifiable as a band on the gel tracks showing plasma or serum proteins bound to carbon nanotubes, but it is detectable using specific antibody recognition (Fig. 3). In Fig. 4, tracks 4 and 6, C1q chains would co-run with the ~25 kDa bands of apolipoprotein AI, and so it would be difficult to identify C1q "under" this protein using the methods available to us. In general, classical pathway activation does not require a large quantity of C1q to bind: since complement activation has several amplification steps, small numbers of C1q molecules are sufficient for activation.

Complement activation by the carbon nanotubes may be due to direct binding of C1q (classical pathway) or C3b (alternative pathway) to the carbon nanotubes, or may be mediated by binding of C1q or C3b to other plasma/serum proteins adsorbed to the carbon nanotubes. The regular structure of the carbon nanotubes might create, for example, an array of bound proteins, which could be recognised by the complement system as "foreign". C1q binds to IgG and IgM, and to the pentraxins, and also fibronectin, but none of these is present (Fig. 4) as a major species binding to carbon nanotubes. There is an isolated report of C1q binding to fibrinogen (Entwistle and Furcht, 1988) but the complement consumption assays reported in

Fig. 1 and Fig. 2 were done in serum, not plasma, so fibrinogen is unlikely to be involved in complement activation.

Further tests were done using ^{125}I-labelled C1q to show that C1q binds directly to carbon nanotubes, which have not been exposed to other serum proteins (Table 1). Therefore, it seems likely that classical pathway activation arises from direct binding of C1q to carbon nanotubes. As shown in Table 1, there is some residual binding of C1q to Sepharose alone (no nanotubes) in the presence or absence of serum. Increasing the quantity of DWNT in the Sepharose increases C1q binding, in the presence and in the absence of serum.

Table 1.

Binding of ^{125}I-C1q to DWNT in presence and absence of human serum

Quantity of DWNT (mg)	Presence (+)/absence (−) of serum	% C1q bound
0	+	6.4[a]
0	−	6.2[b]
0.2	+	9.1
0.4	+	17.9
0.8	+	19.7
1.5	−	22

Binding of ^{125}I-labelled C1q to carbon nanotubes. DWNTs were immobilised in Sepharose. Sepharose alone was used as a negative control. Carbon nanotubes were exposed to 124,262 cpm (1.15 μg) of ^{125}I-C1q. Binding of ^{125}I-C1q was tested in the presence or absence of 500 μl of undiluted human serum.

[a] % of labelled C1q bound to Sepharose in absence of nanotubes but in the presence of serum.

[b] % of labelled C1q bound to Sepharose in absence of nanotubes and in the absence of serum.

For the alternative pathway also, C3b binding might be direct or indirect. SWNT barely activate the alternative pathway, while the DWNT activate well (Fig. 2). Both DWNT and SWNT however show very similar overall binding of plasma and serum proteins (as in Fig. 4: CSM unpublished results), so the major carbon nanotube binding proteins identified in Fig. 4 are unlikely to be involved in indirect binding of C3b. Carbon nanotubes could potentially

cause complement activation (consumption) by sequestering factor H, causing increased C3 turnover in serum. However, it is clear from Fig. 4 that carbon nanotubes do not bind factor H (a 155 kDa single chain glycoprotein).

Fibrinogen has been previously reported not to be significantly absorbed on SWNTs (Shim et al., 2002). Shim et al. (2002), attributed this phenomenon to the much larger size of fibrinogen (~340 kDa and about 45 nm long (Brown et al., 2000)) relative to the diameter of SWNTs (0.4–2 nm). We however find that fibrinogen in human plasma binds to DWNTs, which have diameter 1–3 nm. Since only a small segment of the protein is likely to interact directly with the carbon nanotubes, the overall size of the protein may be irrelevant in this context. Absorption of fibrinogen to "biocompatible" polymers such as polyester terephthalate (PET) has been shown to promote inflammatory responses, including phagocyte recruitment (Tang and Eaton, 1993).

4. Conclusions

We have demonstrated for the first time that two types of carbon nanotubes, SWNTs and DWNTs activate the human serum complement system via the classical pathway. DWNT also activate the alternative pathway, but SWNT hardly activate this pathway. The level of activation in both pathways was compared to that produced by an equal weight of zymosan, a well characterised and potent complement activator (Fig. 1 and Fig. 2).

Activation of complement by carbon nanotubes via classical pathway will lead to generation of inflammatory peptides C3a, C4a and C5a. C5a is a neutrophil chemotactic factor. The carbon nanotubes will also become coated with the opsonins C3b and iC3b. When these nanomaterials are introduced into a mammalian host, complement activation will result in accumulation of neutrophils and in adherence of phagocytic cells around them. Morevor, since these nanowires are too large to be phagocytosed, there may be discharge of degradative enzymes by neutrophils, causing tissue damage. There will probably be granuloma formation originating mainly from macrophage adherence (Diaz et al., 2000).

Complement activation products such as C3b, C4b, iC3b and C3d also act as adjuvants (i.e. they increase the immune response to foreign materials). This means that complement activators, including carbon nanotubes, which become coated with iC3b and C3d, may act as adjuvants. These findings are consistent with and may provide an explanation for the finding

of Pantarotto et al. (2003b) who showed enhance anti-peptide antibody response by immunising with peptides coupled to nanotubes. In that study (Pantarotto et al., 2003b) however, the nanotubes were functionalised (chemically modified). The mechanism of adjuvant activity relies on complement and complement receptor interactions such as interaction of C3d, bound to the complement activator, with its receptor CR2 on antigen presenting cells, including B lymphocytes. This receptor–ligand interaction can stimulate antigen presentation, B lymphocyte proliferation and immunoglobulin secretion (Fearon and Carroll, 2000).

The activation of human complement induced by carbon nanotubes might be diminished or eliminated by alteration of surface chemistry. Variations in surface charge, for example, might promote binding of Factor H, a down-regulator of complement activation (Sim et al., 1993). Studies are in progress to determine the effects of chemical modification of nanotubes on complement activation.

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