IN VITRO STUDY OF CHEMICALLY MODIFIED CARBON FIBRES

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Abstract

The objects of this study were three types of low-carbonised carbon fibres. The fibres differed in oxygen contents and in surface state resulting from chemical bonding. The behaviour of macrophages line J774 and peritoneal mouse macrophages in the presence of these fibres was studied. Viability, ability to proliferation and releasing level of IL-6 and NO were analysed. It has been shown that all examined fibres induce the formation of considerable high level of IL-6. Moreover, none of the analysed materials induced of NO forming. Macrophage viability varied depending on oxygen content on the carbon surface. Reaction of cells with modified surface carbon fibres brings about significantly different cells response in vitro.

Keywords: *Carbon fibres, surface modification, macrophages, cellular response.*

Introduction

Carbon materials prepared by carbonisation of organic precursors are of interest because of possible applications in medicine. Depending on the nature, crystallinity degree and surface properties these materials can be successfully applied in various areas of medicine [1, 2]. Up till now pyrolitic carbon which is believed to be highly biocompatible and biostable is the material most frequently used in medicine. The subject of our studies are amorphous carbon fibres containing stable oxygen-hydroxyl-carbon bonds on their surface. Such carbon materials - in contrast to the pyrolitic carbon - are active in biological systems. As it has been found in our previous studies these materials stimulate the tissue growth and can be used for the treatment of connective tissue defects [3]. Our previous studies have shown that the intensity of phagocytosis by macrophages depends on the carbon fibres surface composition [4]). The goal of the present studies is the analysis of the macrophage's behaviour in the presence of three types of low carbonised carbon fibres of modified surface.

Macrophages are multipotential cells which act in both, inductive and efector phases of the adaptive immunological response as well as in innate immunity [5-7]. Since they exist in many tissues as the so-called resting macrophages and they appear in the local inflammation states via migration , the studies of their behaviour seem to be important in the development of the potential biomaterials. In contact with a material of an appropriate size macrophage begins phagocitize it and a efficiency of phagocytozis depends on the numerous of the receptors interactions. These interactions initiate a whole chain of phagocytoze excitation reactions. In the case when the object in to large for phagocytoze, macrophage attach to surface via their non specific cell surface receptors and release many biologically very strong mediators.

Macrophages are sensitive not only to chemical composition of the surface of the biomaterial but also to its shape and size [8-11]. The fibres studied in the present work differed in the chemical composition, but they did not differ neither in diameters nor in the degree of surface roughness. The ability of the macrophages to proliferation and their viability on these materials as well as their excitation level has been determined by the measurement of IL6 and NO released.

Experimental

Characterisation of the material

Carbon fibres studied in the present work have been prepared by carbonisation of polyacrylonitrile at 1000°C. Three types of samples differing in the surface composition have been prepared: C1, C2 and C3. The C1 fibres have not been modified after the carbonisation process, the C2 ones have been oxidised in the boiling nitric acid for 30 minutes, whereas the C3 fibres have been oxidised for 90 minutes.

SEM and EDS studies of carbon fibres

Three types of carbon materials designated for cellular investigations have been subjected to microscopic as well as X-ray microprobe analyses. Samples of the fibres have been investigated using a Philips XL30 microscope connected to a Link ISIS-EDS attachment. FIG. 1 shows the microscope images of the samples whereas FIG. 2 presents the EDS curves.

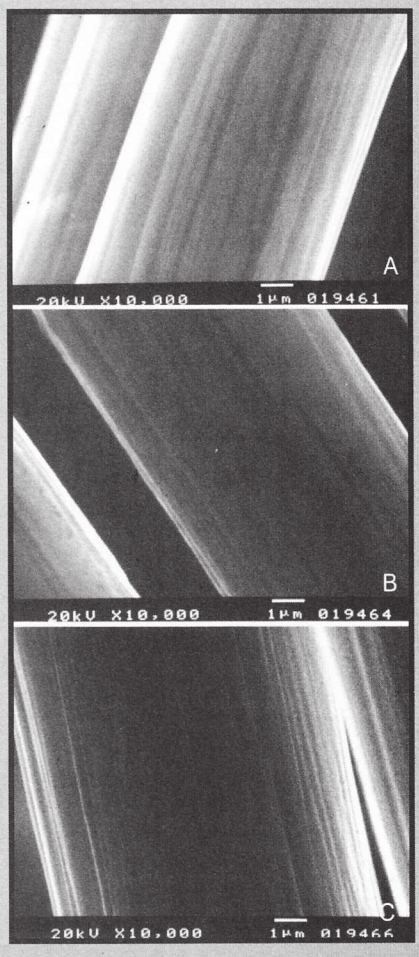


FIG. 1. Scanning electron microphotographs of carbon fibres:
A - C1; B - C2; C - C3.

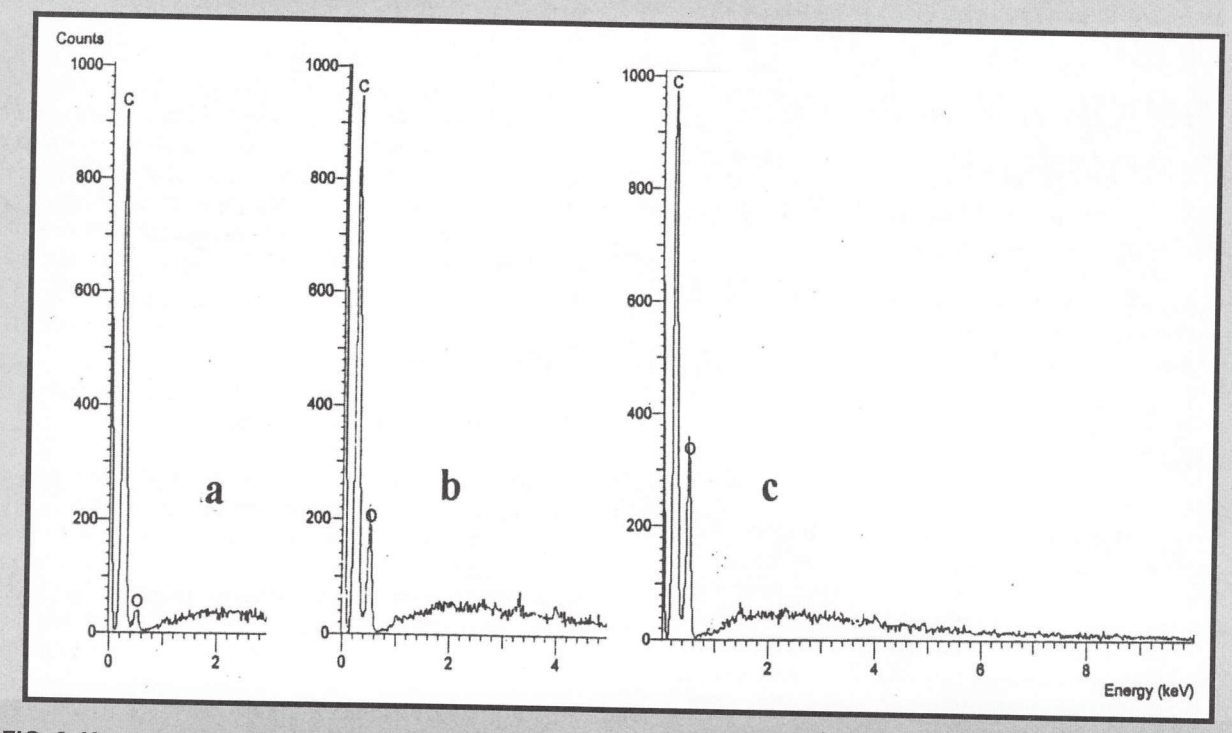


FIG. 2. X-ray microanalyses of carbon fibres (EDS): a)-C1, b)-C2, c)-C3.

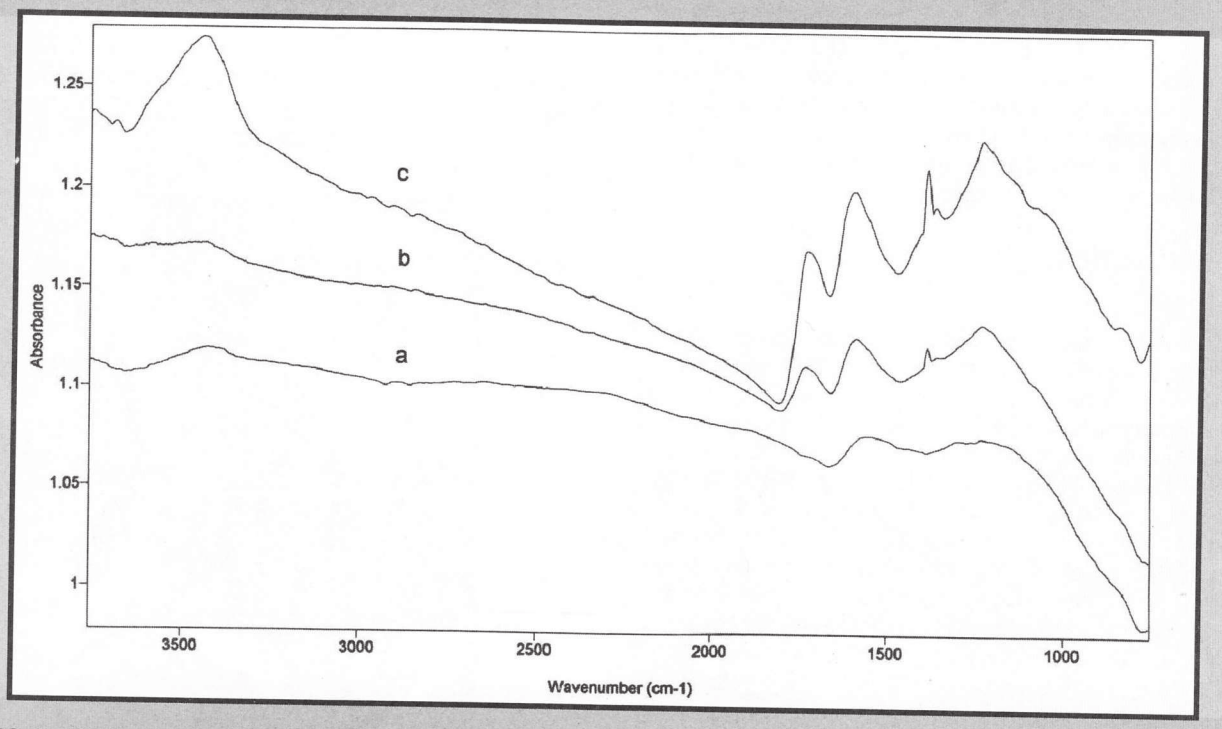


FIG. 3. FTIR spectra of carbon fibres: a)-C1, b)-C2, c)-C3.

FTIR studies of the carbon fibres

Spectra of the samples of carbon fibres in the range of 4000-500 cm^{-1} have been measured using a Digilab Bio Rad FTS 60 spectrometer. The spectra are shown in FIG. 3.

Cells - characterisation

For the studies the macrophages J774 [12, 13] of the culture grown at the Department of Immunology of Collegium Medicum of the Jagiellonian University were used. Peritoneal mouse macrophages were obtained from 6-8 week-old CBA/J male animals. Macrophages were induced by intraperitoneal injection of 2 ml thioglicolate medium (Difco). Cells were collected 5 days later by washing out of the peritoneal cavity with 5 ml of DPBS containing 5U heparin/ml. Cells were centrifuged and red cells were lysed by osmotic shock. Although the suspensions of these cells contained up to 5% of other cell types, they are termed "macrophages" throughout this paper.

Cell culture on carbon materials

Samples of 20 mg of carbon materials were washed twice with distilled water and dried at 160°C for 2 hours. Then the samples were transferred onto a flat-bottomed 12-well plate and treated with the suspension of the cells in

RPMI with 5% of FCS at an concentration of 10^6/ml. in the amount of 2 ml per well. The cells were grown for 1, 2 or 3 days in the atmosphere of 5% of nitrogen and 95% of air at 37°C. Then the number of viable cells in the culture was determined. After a 1day growth the supernatant was collected from the cells in order to determine IL-6 and NO.

Determination of the number of the viable cells after the growth

Cell respiration, as an indicator of cell viability, was assessed by the mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma) to formazon [13]. After removal of 1 ml of the liquid from above the culture, 100 ul of the MTT solution (5mg/ml) was added to the suspension of cells and incubated for 3.5 hours. Then it was frozen at -80°C and defrozen twice, treated with 1.9 ml of the solution isopropanol-HCl, centrifuged and the absorption of the solution at λ=570 nm was measured. The curves representing the dependence of the absorption on the number of cells were prepared for the suspension of the fresh J774 cells and peritoneal macrophages. The results are shown in FIGS. 4 and 5.

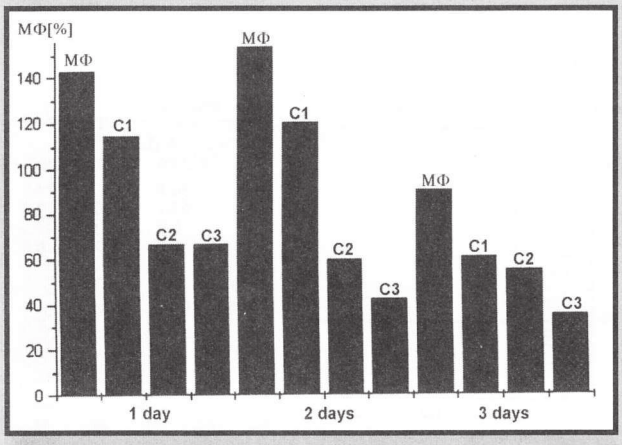


FIG. 4. Vitality of J 774 cells after carbon fibres (C1, C2, C3) contact.
Each result is mean value of 4 experiments in duplicates.

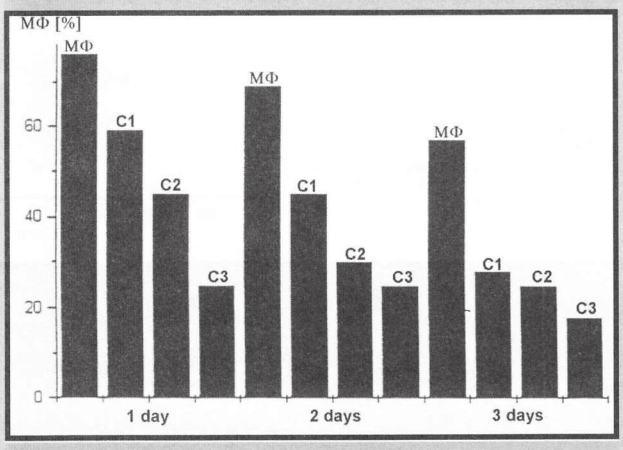


FIG. 5. Vitality of peritoneal macrophages after carbon fibres (C1, C2,C3) contact.
Each result is mean value of 4 experiments in duplicates.

Flow cytometry

J 774 cells were harvested from the 3 day culture by using 0,02% EDTA, washed and resuspended in staining buffer (PBS with 2% FCS and 0,01% sodium aside) to get a final concentration of 1×10^{-6}/ ml. Evaluation of percentage of viable cells was defined by propidium iodide exclusion. Duplicate preparations of each sample were analysed. FACS analysis was performed on Ortho Cytoronabsolute, for analysis of 1×10^4 cells sample. For data acquisition, a Immunocount II software was used. A typical set of results for the 3 day culture is collected in TABLE 1.

Determination of IL6

IL6 in the supernatants was determined for the 24 hour culture using the ELISA test (TABLE 2). **Nitrite (NO) determination**

Nitric oxide (NO), quantified by the accumulation of nitrite (as a stable end product), was determined by a microplate assay [14] Briefly, 100 ml samples were removed from the supernatants and incubated with an equal volume of Griess reagent at room temperature for 10 min. The absorbance at 550 nm was determined with a microplate reader .Nitrite concentration was calculated from a sodium nitrite standard curve

Results

SEM and EDS studies

Microscope analysis showed high similarity of the three types of carbon fibres studied. Surfaces of the fibres look the same, they are composed of oblong strips along the fiber axis which are typical of all the carbon fibres prepared from polyacrylonitrile. X-ray microprobe analysis revelead merely oxygen and carbon in the surface layers of all the three types of fibres studied. The amount of oxygen was different and growed as the oxidation time increased.

FTIR studies

IR spectra of the three types of carbon fibres studied are different. In the spectrum of the C1 sample irregular bands of low intensity are visible. They prove low amount of oxygen bonded with carbon. The spectra of C2 and C3 samples contain well resolved bands. The band located at 1725 cm^{-1} is due to the carbonyl group (C=O) vibrations, the band at 1590 cm^{-1} originates from the radical ions (C::O) [15], whereas the broad band with the maximum around 1240 cm^{-1} can be assigned to the single carbon-oxygen bonds in C-O-C or C-O-H groups. The band at 3440 cm^{-1} can be associated with the presence of the hydroxyl groups and it is visible in the spectrum of the sample C3.

Cellular studies

Results of the macrophage viability studies indicate that the J774 cells increase in number for the first 2 days and on the third day they begin to die (FIG. 4). During the first day the cells become more numerous in the presence of the C1 fibres, whereas the other two types of fibres cause the death of the equal number of cells. The death during the second and the third days is influenced to the highest degree by the C3 material and to the lowest degree by the C1 material. Peritoneal macrophages which do not divide in the culture behave similarly to the J774 macrophages depending on the carbon material they were grown with (FIG. 5). Already during the first day 75% of the cells grown in the presence of C3 material die. For the C1 and C3 materials

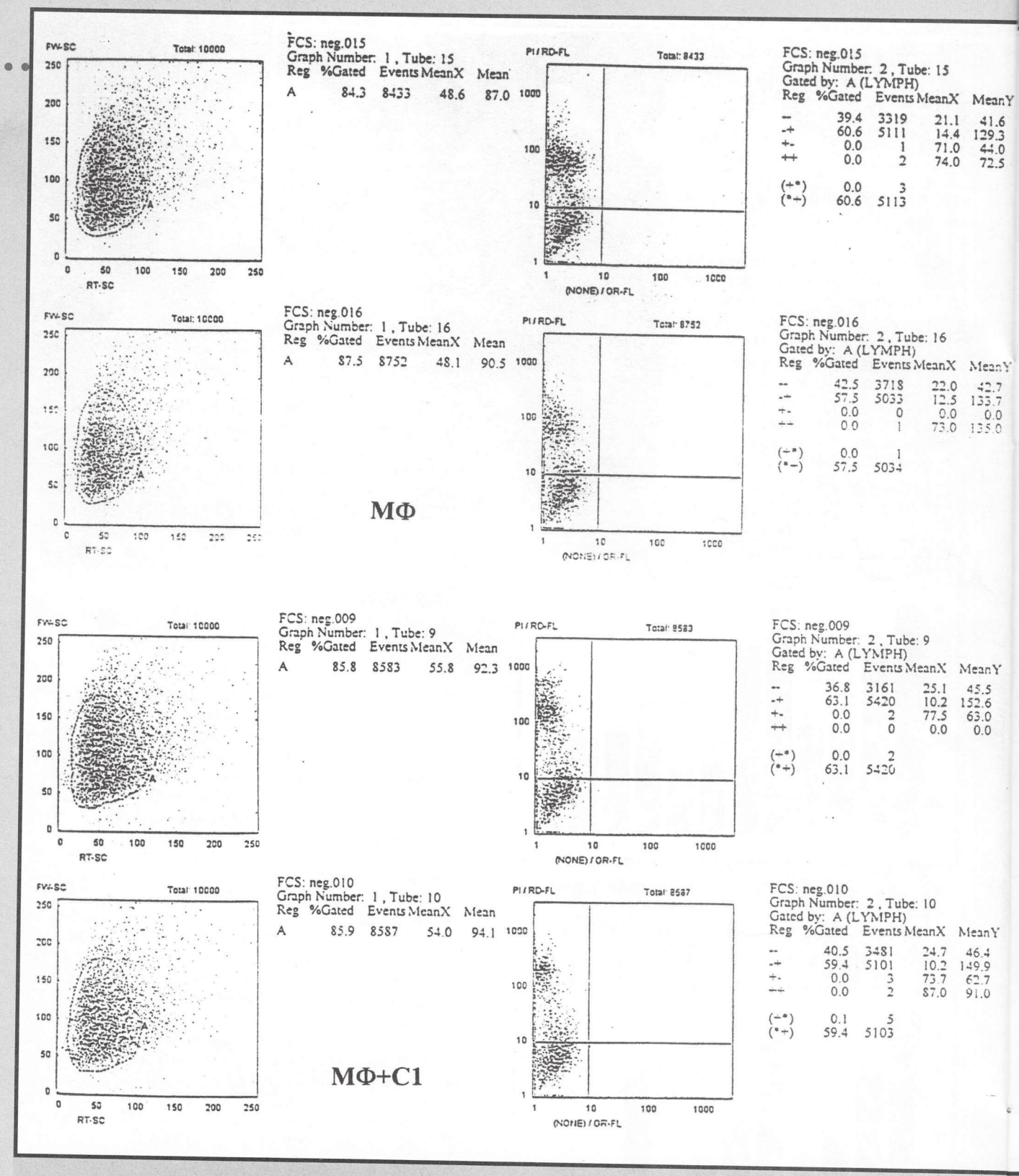


TABLE 1. Flow cytometry result after 3 day culture macrophages with carbon fibres C1, C2 and C3.

these numbers are 40% and 55% respectively. After three days 27%, 25% and 17% of viable cells remain in the presence of C1, C2, and C3 materials respectively.

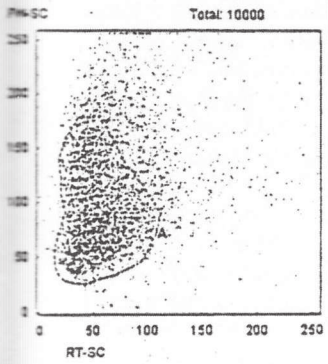
IL-6 measurement in the supernatants after a 24 hour growth of macrophages on the carbon materials (TABLE 2) shows that all the three types of the material studied induce the formation of the high level of IL-6.

Determination of NO

None of the materials studied induced the synthesis of NO in J774 nor in peritoneal macrophages.

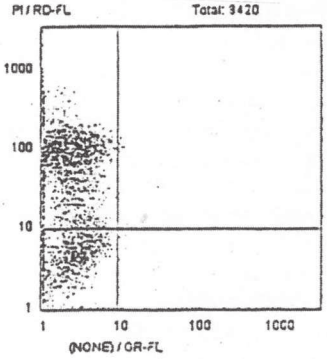
Sample	IL 6 release from [pg/ml]	
	MΦJ 774	pm*MΦ
MΦ	105 ± 20	216 ± 20
MΦ + C1	7940 ± 50	3900 ± 50
MΦ + C2	5410 ± 50	2500 ± 50
MΦ + C3	3500 ± 50	2000 ± 50
* peritoneal mouse macrophag		

TABLE 2. IL-6 release in supernatants after 24 hours cultures by ELISA test.



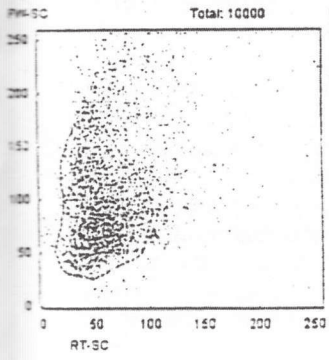
FCS: neg.013
Graph Number: 1 , Tube: 13

Reg	%Gated	Events	MeanX	Mean
A	84.2	8420	52.7	83.6



FCS: neg.013
Graph Number: 2 , Tube: 13
Gated by: A (LYMPH)

Reg	%Gated	Events	MeanX	MeanY
--	27.5	2315	30.3	47.1
-+	72.3	6084	24.3	136.6
+-	0.1	5	77.6	62.6
++	0.2	16	78.0	115.1
(+*)	0.2	21		
(*+)	72.4	6100		



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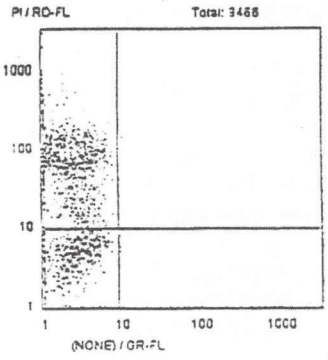
Graph Number: 1 , Tube: 14

Reg	%Gated	Events	MeanX	Mean
A	84.7	8466	52.2	82.5

MΦ+C2



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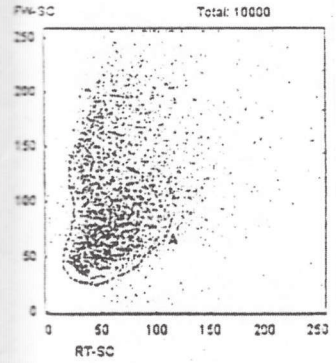
Graph Number: 2 , Tube: 14
Gated by: A (LYMPH)

Reg	%Gated	Events	MeanX	MeanY
--	25.5	2158	31.3	47.2
-+	74.3	6292	24.8	136.1
+-	0.1	7	74.1	56.3
++	0.1	9	76.8	127.9
(+*)	0.2	16		
(*+)	74.4	6301		



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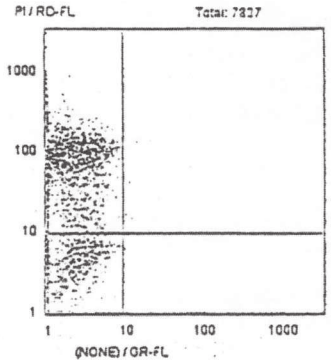
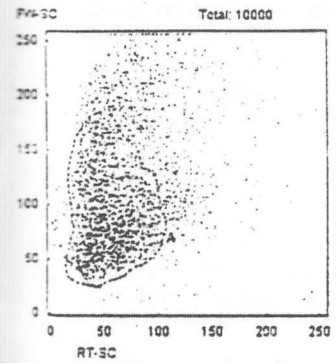
Reg	%Gated	Events	MeanX	Mean
A	78.4	7837	53.9	75.9



FCS: neg.011
Graph Number: 2 , Tube: 11
Gated by: A (LYMPH)

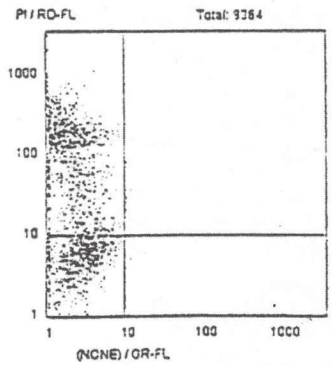
Reg	%Gated	Events	MeanX	MeanY
--	19.7	1543	28.4	46.4
-+	80.2	6282	23.7	137.5
+-	0.0	2	73.5	63.5
++	0.1	10	77.4	127.3
(+*)	0.2	12		
(*+)	80.3	6292		



FCS: neg.012
Graph Number: 1 , Tube: 12

Reg	%Gated	Events	MeanX	Mean
A	83.6	8364	53.2	87.4

MΦ+C3



FCS: neg.012
Graph Number: 2 , Tube: 12
Gated by: A (LYMPH)

Reg	%Gated	Events	MeanX	MeanY
--	26.8	2240	31.2	50.0
-+	73.0	6105	16.3	151.1
+-	0.1	5	74.4	59.6
++	0.2	14	78.5	114.2
(+*)	0.2	19		
(*+)	73.2	6119		

Discussion

Carbon materials studied differed in the amount of oxygen connected with carbon structure. Oxygen forms with carbon different types of bonds: C=O, C::O, C-O. It is frequently assumed that these bonds are a part of lactone, carboxyl or phenol groups whose properties are similar to the ones known in organic chemistry. The three samples studied differ in the chemical composition of the surface. C1 contains a small amount of oxygen with respect to the other two types of materials and it forms mainly single bonds and radical ions. The C2 sample contains oxygen in double bonds, radical ions and single bonds. The absence of the band due to the OH groups in the IR spectrum of this sample indicates that the carbon-oxygen bonds are a part of lactone groups. The C3 sample - apart from the the groups present in the C2 sample - contains also phenol and carboxyl groups. From the results presented it can be concluded that the carboxyl and phenol groups present in the highest amount on the surface of the C3 sample cause the inhibition of the cells divisions and their death in the case of the J774 macrophages and the faster death in the case

of the peritoneal macrophages with respect to the blank culture . All the materials studied induce evolution of a high level of IL-6 which shows that the materials studied cause the inflammation state. The lowest level of IL-6 observed in the supernatants from the culture grown in the presence of the C3 material can be explained either by the lower number of cells (because of the inhibition of their proliferation) or by the different type of interactions of this material with surface structures of macrophages. On the other hand, the absence of NO induction indicates that the cells do not use their strongest defence mechanisms in the reaction with the materials studied since the synthesis of this toxic radical is under strict control and it is applied in the cases when other defence mechanisms fail [16,17].

The results presented do not allow to draw conclusions concerning the applicability of the materials studied as biomaterials. It is generally assumed that a given material is biocompatible when it stimulates the cells to play their role in the system while acting as a catalyst which activates the cells [18-20]. Thus in our case a strong activation of the macrophages to release proinflammatory IL-6 as well as the inhibition of the divisions of the J774 cells, death of the mature cells previously activated by treatment with thioglycolate by the C3 material makes it interesting for further studies. It has been shown before [4] that the material (in the form of the powder) of similar surface composition is intensively phagocytize by macrophages. Hence, taking into account also the present results, it can be concluded that the cells react with different surface groups of carbon material and are activated at different ways (phagocytosis, death, evolution of IL-6). Increased phagocytosis, inhibition of proliferation and finally the death of some cells in the presence of the C3 material indicate a strong reaction of these cells with its surface. However, the possible applications of these materials require further studies. It is known that in the chronic inflammatory states the MGC multinucleated giant cells) are formed. They originate from macrophages and they are responsible for granulomatous decease. There are reports from studies in vitro that the macrophages fusion is preceded by their proliferation [21]. Our studies have shown that the carbon materials, especially the ones containing on their surface carbon-oxygen or carbon-oxygen-hydroxyl bonds inhibit proliferation while simultaneously activating the IL6 evolution. As it is known IL-6 induces also the synthesis of acute phase proteins whose aim is to limit the inflammation state. Thus its high level in the macrophages cultures seems to be advantageous. Studies in vivo (unpublished data) showed the induction of the strong inflammation state in rats which stopped after two weeks. The C1 material caused the chronic inflammatory state which lasted for six weeks. From our work it can be concluded that the number and type of hydroxyl-oxygen-carbon groups on the carbon surface influences the cellular response. Hence, it seems possible that the preparation of a material of a surface modified in an appropriate way will make it possible to control the induction of the local inflammation state as well as the inhibition of the development of chronic inflammation.

Acknowledgements

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