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Activation of human neutrophils by organic polymer surfaces

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By means of lucigenin-dependent chemiluminescence it has been demonstrated that films of organic polymers activate human neutrophils. The strongest response was always induced by polyvinyl chloride-D and the smallest by polyethylene. No significant differences were found between the number of adherent cells on the surfaces of the investigated materials. The stimulatory capacity of the organic polymers were changed after adsorption of some plasma proteins. These effects were not connected with similar alterations of cell attachment. A second stimulation by opsonized zymosan was only possible for neutrophils adherent to polyether urethane and polyvinyl chloride-D.

1. Introduction

Clinical observations and laboratory findings have shown that alterations of humoral factors and blood cells do occur as a consequence of the implantation or temporal contact of the body with artificial materials [1–3]. In particular, it was demonstrated by some investigators that the altered activity of isolated peripheral neutrophils from patients during haemodialysis corresponds to the extent of complement activation induced mainly by the alternative pathway [4]. However, knowledge of the direct interaction of neutrophils as the dominant immune cells in the circulation with organic polymers is limited. No studies exist evaluating the membrane regions engaged in the binding of phagocytic cells on organic polymers.

After stimulation by immunological or non-immunological activators, neutrophilic granulocytes react by an increased production of reactive oxygen species (ROS) and the liberation of enzymes with degradative potential. ROS are formed by the membrane-bound NADPH-oxidase and the arachidonic acid metabolism [5]. Differences in the portions of O_2^- and H_2O_2-production after stimulation by soluble, particulate and solid-phase materials (tissue culture plate) were published by Hoffstein *et al.* [6], who postulated that neutrophils may directly synthesize both H_2O_2 and O_2^- because surface stimuli induce their release in stimulus-specific relations. Neutrophils possess receptors for the Fc-part of IgG and some complement components which are engaged in the elimination of bacteria and other micro-organisms. Furthermore, neutrophilic receptors were characterized which bind distinct plasma proteins and accomplish the attachment of cells to these extracellular matrices and other cells [7, 8]. The capacity of phagocytic cells to recognize and eliminate foreign materials is one fundamental of animal life. This cellular response is involved in defense mechanisms but also in immunopathogenic reactions [9]. This process is not only dependent on the presence of antibodies or other mediators. Materials with a size greater than the cells are not ingestable and lead to a process named frustrated phagocytosis that is connected with the loss of cellular integrity. However, insufficient knowledge exists on the contribution of these cells in the multifactorial processes of acceptance or rejection of biomaterials by the recipients. The increasing demand for substitution of organs with impaired functional activity by prostheses made from organic polymers and other materials requires such studies. Direct evidence for an activation of neutrophils by hollow fibres *in vitro* was published by Nguyen *et al.* [10] and Kuwahara *et al.* [11]. These authors found a different stimulation of ROS-formation after incubation of neutrophils with dialysis membrane materials. However, the information from these studies was rather limited because only the peak chemiluminescence (CL) was measured and the different effective surfaces as well as the number of adherent cells were not considered. The aim of our studies was, therefore, to apply the lucigenin-dependent integral-CL, because in contrast to luminol the reactivity of this compound only with O_2^- is well known. Additionally we intended to determine the relation of adhered cells to the integral CL-response and to investigate the effects of different plasma proteins on neutrophilic binding and activation.

2. Materials and methods

Lucigenin, luminol, human serum albumin, dimethyl sulphoxide, and *p*-nitrophenyl phosphate were purchased from Sigma (USA). Fibrinogen, fibronectin, human IgG and zymosan were purchased from Serva (Germany). MEM (phenolred-free) was obtained from Sifin (Germany). Opsonized zymosan was prepared with pooled serum and stored at $-20\,^{\circ}C$.

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Standard organic polymers in the form of films were kindly provided by Dr Lemm (Klinikum Rudolf Virchow, FU Berlin, Eurobiomat Programe, Berlin): polyethylene (PE), batch number MÖS 2720, thickness 50 μm; polyether urethane (PEL), pellethane 2363-90 AE, medical grade, Dow Chemical, batch number 910428 CENTR, thickness 250 μm; polypropylene (PP), batch number 1350248002, thickness 50 μm; polyvinyl chloride with di-(ethyl-2-hexyl)-phathalate as plasticizer (PVC-D), batch number 298585, thickness 50 μm; polyvinyl chloride with tri-(ethyl-2-hexyl) trimellitate as plasticizer, (PVC-T) batch number 29859, thickness 50 μm. All materials were manufactured by Rehau AG, except the polyether urethane which was obtained from Frontline Filmbläsning.

2.1. Cell preparation and CL measurement

Blood from healthy volunteers (age 20–30 y) was drawn by venipuncture into a syringe containing 20 U heparin/ml. For separation of neutrophils from other blood cells we used the one-step procedure of Ferrante and Thong [12]. After harvesting of the neutrophilic granulocytes, the contaminating erythrocytes were lysed with distilled water. The neutrophils were suspended to 5×10^6 ml^{-1} in MEM and stored at 4 °C for 1–4 h. For measurement of CL response, 0.5×10^6 cells in 0.6 ml MEM and 10 μl lucigenin (5 mg ml^{-1} in dimethyl sulphoxide) were transferred to vials precoated with albumin. A stable basal activity of CL was observed after a preincubation of 20 min at 37 °C. The activation of cells was started by the addition of polymer pieces with an effective surface of 50 mm^2 mounted on steel wires. The distance from the bottom was 2–3 mm. The amplified CL was recorded using a six-channel luminometer LB 9505 (Berthold, Germany). The results are represented in counts min^{-1}. Three parameters were recorded: (1) the integral response for 20 min, (2) the peak response, and (3) the kinetics graphically. All CL measurements were carried out with cells from a minimum of four individuals. All tests were repeated three times with the cells of each donor to collect a sufficient number of data. In a separate set of experiments the discs with the adhering cells were transferred after the first activation to a second vial with 0.6 ml MEM containing 1 mg opsonized zymosan and 10 μl lucigenin.

2.2. Adhesion of neutrophils

After testing the CL response, all discs were washed three times with phosphate-buffered saline (PBS) and stored at 4 °C. The number of adhering cells was determined by the procedure published by Santini *et al.* [13]. In control experiments, the polymer films were fixed exactly on the bottom of Teflon tubes. In this case the effective surface was 100 mm^2 and the ratio of free and bound cells were measured in the same manner after incubation for 20 min at 37 °C.

2.3. Adsorption of proteins

After a preincubation in PBS, the organic polymers were in contact for 1 h at 4 °C with the proteins in the specified concentrations (autologous plasma, undiluted; fibrinogen, 1 mg ml^{-1}; fibronectin, 50 μg ml^{-1}; human serum albumin, 10 mg ml^{-1}; human IgG, 1 mg ml^{-1}). The dilutions were prepared with PBS. The same solution was used for rinsing the discs. The influence of the different treatments of the surfaces on CL response is expressed in relation to CL stimulation by untreated films. The changes are represented in per cent.

3. Results

In the first experiments we compared the luminol- and lucigenin-dependent CL response of neutrophils induced by five different organic polymers. A remarkable CL response for all materials was only induced in the presence of lucigenin. A typical example is presented in Fig. 1. As can be seen from this picture, an increase of the ROS production was induced immediately after addition of the different organic polymers to the cell suspensions. The strongest CL activation was caused always by PVC-D and the smallest by PP and PE. The rank of activation by these polymer surfaces was reproduced with neutrophilic cells of nine individuals. However, a problem was the large differences between strong and weak responders in the

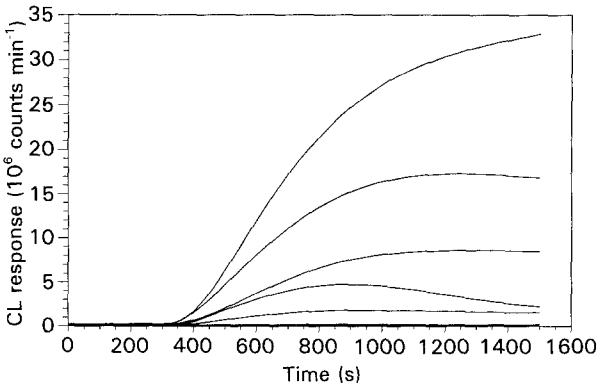

Figure 1 Time course of lucigenin-amplified chemiluminescence of neutrophils activated by surfaces of different organic polymers. After a preincubation of neutrophils for 20 min at 37 °C, the organic polymers were added. Thereafter the integral CL response was measured for a further 20 min. Curves from top to the bottom represent stimulation by PVC-D, PEL, PVC-T, PE, PP and control.

TABLE I Lucigenin-mediated CL response of neutrophils from different individuals

Material	Range (10^7 counts min^{-1})	Mean (10^7 counts min^{-1})	CL_{PVC-D} (%)
PVC-D	20.75–81.39	48.31	100.0
PEL	7.96–30.97	17.85	37.0
PVC-T	4.69–38.03	15.43	31.9
PE	0.93–6.97	2.98	6.2
PP	0.20–8.00	2.34	4.8

Range and mean values are represented ($n = 9$). Normalization was achieved by setting the strongest response (always with PVC-D) at 100%.

range of factor five. Therefore we decided to normalize the results by setting the CL response of PVC-D at 100% (see Table I). A similar pattern of CL activation was found using diluted blood, but also only in the presence of lucigenin (results not shown here).

By determination of alkaline phosphatase activity associated with the polymer pieces after accomplishing the CL measurement, the number of adhering cells was assessed. For an independent confirmation of these results, we repeated the adhesion experiments with an adhesion chamber (see Section 2). Under these conditions, the expected two-fold increase of adhering cells was found (Table II).

Fig. 2 shows that after a preincubation of the organic polymers with autologous plasma, all surfaces acquired a diminished adhesion of neutrophils. PEL, PE and PP express a strong decreased capacity to stimulate a neutrophilic CL response. But PVC-D and PVC-T demonstrated no decrease of their stimulatory capacity. However, no correlations were seen between the changes of cell adhesion and CL response. The preadsorption of fibronectin weakly influenced the adhesion of neutrophils to the different materials (see Fig. 3). The preadsorption of fibrinogen diminished both adhesion and O_2^- formation for all the tested organic polymers except PVC-D, as indicated in Fig. 4. Opposite effects were observed after the incubation of the discs with solutions of human IgG or albumin. Surfaces treated with IgG induced CL responses more than four times higher than in the controls. However, only small changes were observed for cell adhesion as shown in Fig. 5. A strong decrease of the capacity to stimulate a CL response was observed if the polymers were preincubated with serum albumin. A comparable decline of cell adhesion was only observed for PEL and PP (Fig. 6). In an attempt to estimate the cellular reactivity of adhering preactivated cells, we investigated additionally the CL response to a second stimulator. After accomplishment of the first CL activation, the discs were removed from the cell suspension. Thereafter the adhered cells were stimulated by opsonized zymosan. Under these conditions, cells adhering to PVC-D and PEL were again able to respond strongly compared with nuetrophils adhering to discs made from PVC-T, PP and PE, which reacted only moderately (Table III).

TABLE II Dependence of the adherence of neutrophils on surface area

Material	50 mm²	100 mm²
PVC-D	8.8 ± 5.1	24.7 ± 6.4
PEL	10.0 ± 7.2	19.2 ± 8.2
PVC-T	9.5 ± 4.9	25.2 ± 5.5
PE	9.6 ± 5.5	17.1 ± 4.3
PP	11.0 ± 7.0	18.8 ± 5.1

The percentage of attached cells were determined after registration of CL response. Under these conditions the effective surface was 50 mm². For confirmation, the adherence was measured additionally on films in adhesion chambers with an effective surface of 100 mm².

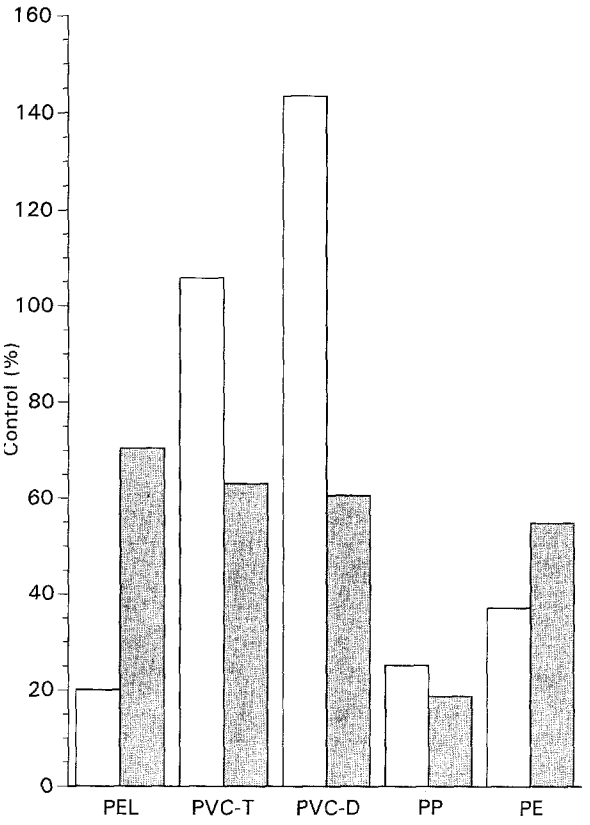

Figure 2 CL response and adherence of neutrophils in contact with plasma-treated organic polymers. White column, CL response; shaded column, cell adherence.

4. Discussion

The measurement of ROS production by activated phagocytes can be done by different physical and chemical procedures. The large number of such methods is an indication of some restrictions [14]. In the presence of lucigenin, the production of O_2^- can be estimated by CL in the integral mode and is therefore preferable against the chemical end-point methods [15, 16]. In comparison with luminol, all five organic polymers always induced a measurable neutrophilic CL response only in the presence of lucigenin (Fig. 1). We found different stimulatory capacities for the studied organic polymers. This pattern of CL response stimulated by these different materials was confirmed with neutrophils from various individuals. It was questionable whether the different CL responses were caused by differences in the number of adherent neutrophils or the cellular O_2^- production. Therefore, we determined the portion of cells fixed to the 50 mm² surfaces after termination of CL measurements. In all cases, only small differences in the number of cells adhering to the organic polymers were seen (Table II). By statistical analysis it was found that the parameters cell adherence and CL response were not correlated (correlation coefficient always < 0.2). Consequently it can be deduced that the considerable differences observed in the CL response cannot be simply explained by different cellular adhesion rates. This conclusion is supported by adhesion experiments with films of double size (100 mm²). Under these conditions we found nearly the expected size-related increase of cell

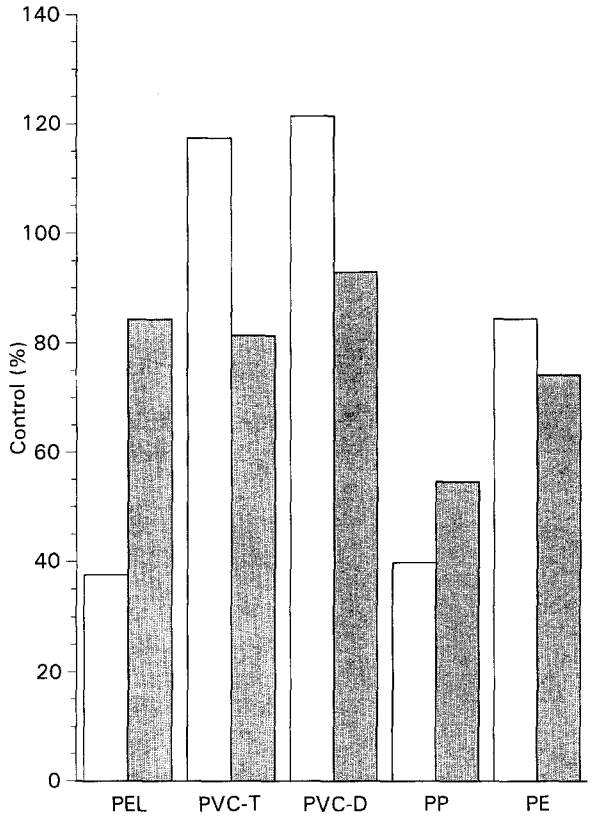

Figure 3 CL response and adherence of neutrophils in contact with fibronectin-treated organic polymers. White column, CL response, shaded column, cell adherence.

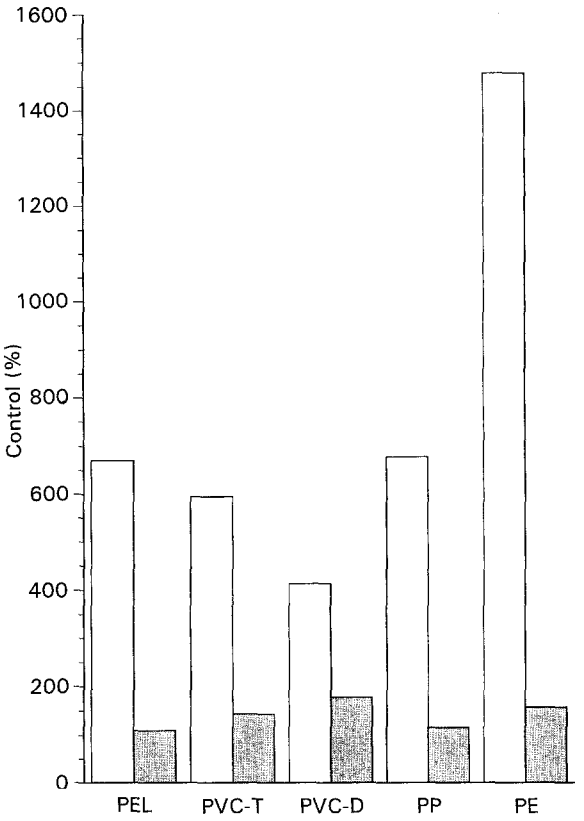

Figure 5 CL response and adherence of neutrophils in contact with IgG-treated organic polymers. White column, CL response; shaded column, cell adherence.

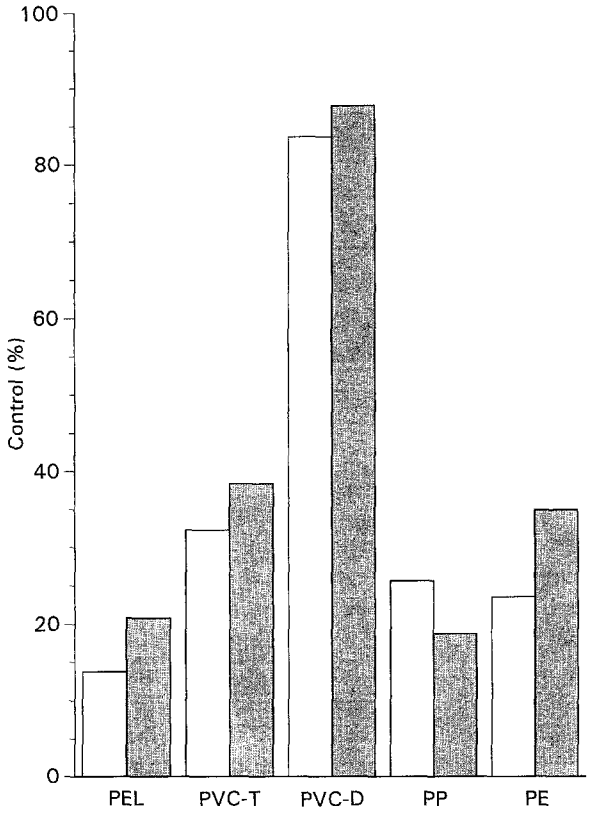

Figure 4 CL response and adherence of neutrophils in contact with fibrinogen-treated organic polymers. White column, CL response; shaded column, cell adherence.

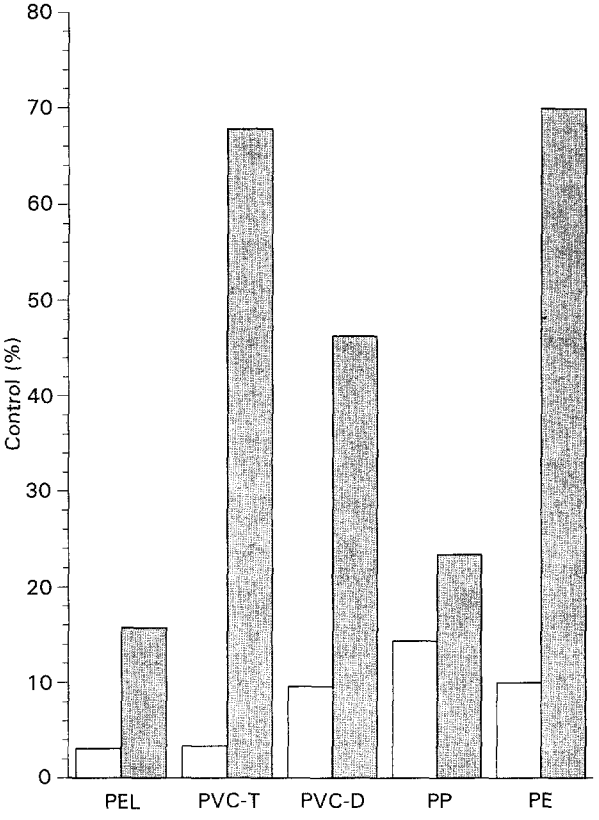

Figure 6 CL response and adherence of neutrophils in contact with serum albumin-treated polymers. White column, CL response; shaded column, cell adherence.

TABLE III Stimulation of adherent neutrophils with opsonized zymosan

Material	First CL response (10^7 counts min^{-1})	Second CL response (10^7 counts min^{-1})
PVC-D	124.0	79.0
PEL	76.2	59.8
PVC-T	30.3	4.2
PE	1.1	0.3
PP	6.2	1.4

0.5×10^6 neutrophils were incubated with the different organic polymers. After registration of the first CL response, the unattached cells were removed. The adhering cells were thereafter stimulated with opsonized zymosan (presented are mean values, $n = 11$).

adhesion, although the arrangement of the adherence substrates was not identical. The origin of the observed differences for the CL stimulation of neutrophils by these organic polymers is unknown. Many groups have reported that the amount of cellular ROS production is dependent on the type of activator. From our results no conclusion can be drawn at present as to the different cellular or biochemical mechanisms. The contribution of electrostatic forces for the activation of neutrophils by polymeric surfaces was suggested [11]. The remarkable CL activation of neutrophils with both PVCs could be conditioned by the high content of plasticizer. However, it was confirmed with different *in vitro* methods that these additives were not leachable and cytotoxic [17]. From our experiments it can be concluded that the adhesion of neutrophils is not strongly related to the chemical composition of these polymers.

In contact with blood, surfaces from organic polymers are quickly covered with a mixture of proteins. The quantity of a single adsorbed protein species is not simply related to their content in the fluid phase. Upon adsorption they can display some conformational changes associated with increased cell stimulation. By elution of used haemodialysis membranes with sodium dodecyl sulphate (SDS), it was found that the protein composition was qualitatively different, depending on the investigated membrane types [18]. Also important is the generation of activated complement products by the alternative pathway, as well as the induction of fibrinolytic and coagulation processes by surfaces. Differences in the capacity of biomedical polymers to activate complement factors are known and accepted as parameters of biocompatibility [4]. In recent years it was stated that some serum proteins, for example, C3b, fibrinogen and fibronectin, are ligands of the integrin family which participate in adhesion of phagocytes to extracellular matrices and cell activation [19]. Neutrophils express the equivalent receptors; however, their importance in relation to adhesion of cells on biomaterials is not completely known.

Preincubation of organic polymers with autologous plasma diminished the capacity of CL stimulation for surfaces made from PEL, PE and PP, but not for the both PVCs (Fig. 2). However, the alterations of cell adhesion, with the exception of PP, were small. It was reported that the superoxide production of neutrophils shows comparable alterations if dialysis membranes made from different organic polymers were preincubated with plasma [11]. This pretreatment increased the stimulation by cellulose acetate but not for polycarbonate. It was discussed that these contrary effects were mediated by differences in complement activation and deposition of C3b.

We have carried out experiments with preadsorbed proteins to test the role of some plasma constituents on neutrophilic adherence and activation. Adsorption of fibronectin and fibrinogen on the different organic polymers caused no unique effects, although these substances possess the tripeptide sequence (arginine-glycine-aspartic acid) sequence and both are ligands for neutrophils. Fibronectin diminished only the CL activation for PEL and PP and affected merely the cell adhesion (Fig. 3). In the case of fibrinogen, cell attachment and CL stimulation were unchanged only for PVC-D (Fig. 4). The other four materials show a clear decrease for both parameters in proportional extent. The preferable adsorption of fibrinogen on PP and PE in comparison with the both types of PVC has been reported [20]. In contrast, we observed in our experiments no equivalent consequences for cell adhesion and cell response (Fig. 4). However, if the organic polymers were precoated with IgG, a huge increase of CL stimulation for all proved materials was found (Fig. 5). This effect was not connected with similar alterations of cell adhesion. The strong CL activation is not surprising, because neutrophilic IgG receptors are participating effectively in the basic defence process of immune phagocytosis, which is coupled with enhanced ROS production. It was shown in many other studies that adsorbed albumin passivates plastic surfaces of artificial organs [21]. This was confirmed also for the organic polymers investigated here. (Fig. 6). Especially important, however, is that the reduction of integral CL response is considerably stronger than the decrease for cell binding.

Recently, Kaplan *et al.* [22] have described that neutrophils adherent to PE exhibit a reduced superoxide production compared with PEL. Furthermore, it was noted that cells in contact with PEL show, after stimulation with FMLP, an increased ROS formation compared with PE and polystyrene. However, the number of attached cells on the different materials was not determined. We observed similar effects for neutrophils adherent to PE and PEL after a second activation with opsonized zymosan and measurement of CL response (Table III). If it is taken into consideration that the number of fixed cells on the different materials is similar, it must be regarded that the expression of neutrophilic receptors for C3b and/or IgG can be altered. This topic deserves special attention in relation to the problematic bacterial infection of medical prostheses. Our findings demonstrate that the number of adherent neutrophils on surfaces is not simply correlated with the extent of the O_2^--dependent CL response. It can be concluded that the adherence process induces secondary changes related to cell activation. From our experiments no conclusions on the nature of cellular binding sites at the surfaces from organic polymers can be drawn. How-

ever, it can be summarized that the primary and secondary activation of attached neutrophils is influenced by the chemical nature of adherence substrates. In future investigations, more information may be gathered by enumeration of neutrophilic receptors of adhered cells.

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