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Superoxide production from human polymorphonuclear leucocytes stimulated with immunoglobulins of different classes and fragments of IgG bound to polystyrene dishes

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Summary. Polystyrene surfaces coated with proteins at alkaline pH were found to be useful to investigate the stimulation of neutrophils independently of phagocytosis. Human neutrophils, when exposed to appropriate stimuli, release superoxide anion (O_2^-). We have measured superoxide dismutase-inhibitable cytochrome c reduction by cells stimulated with various kinds of protein coated on polystyrene dishes.

Native immunoglobulins in solution did not stimulate O_2^- generation in neutrophils. IgG and IgA adherent to polystyrene dishes stimulated O_2^- generation in neutrophils, but IgM, IgD, IgE, albumin, Fab, and Fc of IgG on polystyrene dishes did not.

INTRODUCTION

Polymorphonuclear leucocytes (PMNs) have receptors for immunoglobulins and complement (Lay & Nussenzweig, 1968; Messner & Jelinex, 1970). Through these receptors PMNs are stimulated to exhibit biological activities: phagocytosis (Ehlenberger & Nussenzweig, 1977; Mantovani, 1975), release of granular enzyme (Henson, Johnson & Spiegelberg, 1972; Henson & Oades, 1975), and generation of superoxide anion (O_2^-) (Babior, Kipnes & Curnutte, 1973; Curnutte & Babior, 1974a), hydrogen peroxide (H_2O_2) (Klebanoff, 1975) and chemiluminescence (Johnston, Lehmeyer & Guthrie, 1976a). These activities have important roles in bacterial killing (Babior *et al.*, 1973; Curnutte & Babior, 1974a), and immune complex diseases (Johnston & Lehmeyer, 1976b). In certain conditions, release of granular enzyme or activation of the hexose monophosphate pathway (HMP) of PMNs occurs independently of phagocytosis. (Henson & Oades, 1975; Goldstein, Roos, Kaplan & Weissmann, 1975; Goldstein, Kaplan, Radin & Frosch, 1976). PMNs from patients with chronic granulomatous disease (CGD) can phagocytose bacteria, but can not kill certain strains of bacteria because of their inability to generate O_2^- (Curnutte, Whitten & Babior, 1974b; Johnston, Keele, Misra, Lehmeyer, Webb, Baehner & Rajagopalan, 1975).

The present study was undertaken to determine the ability of immunoglobulins of different classes to stimulate O_2^- release by PMNs, and to determine the effector site of IgG to stimulate PMNs. To eliminate the effect of phagocytosis on the O_2^- release, we developed a new method to stimulate PMNs without phagocytosis. Stimulation of PMNs

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with aggregated IgG on millipore filters has been already reported by others (Henson *et al.*, 1972; Henson & Oades, 1975; Goldstein *et al.*, 1975, 1976). We used unaggregated immunoglobulins for the stimulation of PMNs. Polystyrene dishes coated with proteins without aggregation have been found useful to stimulate PMNs.

MATERIALS AND METHODS

Isolation of human peripheral polymorphonuclear leucocytes (PMNs)

Human PMNs were isolated aseptically according to the method of Boyum (1968). After hypotonic lysis of contaminating erythrocytes, PMNs were suspended in Hanks' balanced salt solution (HBSS) without phenol red, with heparin (10 iu/ml). The mean cell viability counted by trypan blue dye-exclusion test was 96%. Of viable cells, 97% were PMNs and 3% were lymphocytes, monocytes and erythrocytes.

Myeloma proteins and fragments of IgG

The myeloma proteins of different classes were isolated from serum and plasma of patients with multiple myeloma according to published procedures (Spiegelberg, Prahl & Grey, 1970; Shimizu, Watanabe, Yamamura & Putnum, 1974; Ishizaka, Ishizaka & Lee, 1970). IgG was digested with papain (Porter, 1959), or with pepsin (Turner, Bennich & Natvig, 1970), and Fab, Fc, and $F(ab')_2$ were isolated by diethylaminoethyl (DEAE) cellulose ion-exchange chromatography and gel-filtration on Sephadex G-200. IgA dimer and $F(ab')_2$ were further purified by passing through a protein A-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) column to remove contaminating IgG.

The purity of all proteins and fragments was checked either by immunoelectrophoresis or by Ouchterlony's double diffusion technique. The following antisera were used; rabbit anti-IgG kappa prepared in our laboratory, goat anti-IgD and anti-IgE obtained from Roswell Park Memorial Institute (Buffalo, New York), and commercially available anti human whole serum, anti-IgA and anti-IgM (Behringwerke, Marburg-Lahn, West Germany). All myeloma proteins and fragments gave a single precipitin band except IgA Hi dimer which contained a very small amount of albumin.

Preparation of nonphagocytosable surfaces

Generally proteins have the property to adhere to polystyrene surfaces at alkaline pH. This property has been applied to the method of solid-phase radio-immunoassay (Catt & Tregear, 1967). We attached proteins to polystyrene surfaces and these surfaces were used for the stimulation of PMNs. Polystyrene dishes (Falcon, Oxnard, California, No. 3001, 35 mm in diameter) were treated with a saturated solution of potassium dichromate in sulphuric acid overnight and thoroughly washed with deionized water. After adding 2 ml of a protein solution (500 μg/ml in 0·05 M sodium carbonate bicarbonate buffer pH 9·8), these dishes were placed in a incubator at 37° for 2 h. After three washes with 0·9% NaCl, they were used as non-phagocytosable surfaces. The pH of HBSS did not change in these dishes. To evaluate the amount of proteins adherent to the dishes, ^{131}I labelled (New England Nuclear, Boston, Massachusetts) proteins were used. Proteins were labelled with ^{131}I by the Chloramine T method (Hunter & Greenwood, 1962). A constant amount of the radioactive ^{131}I labelled protein was supplied to the dishes, containing 2 ml of the protein solution (10, 50, 100, 500 and 1000 μg/ml in 0·05 M sodium carbonate bicarbonate buffer pH 9·8). These dishes were placed in a incubator at 37° for 2 h. After three washes with saline, these dishes were assayed for radioactivity. Since only about 4% of the radioactivity was washed out after seven additional washes, protein firmly attached to the dishes.

Stimulation of PMNs

PMNs were stimulated either with soluble proteins, or with nonphagocytosable surfaces. According to Babior *et al.* (1973), the amount of O_2^- released from PMNs during incubation was calculated from the amount of superoxide dismutase (SOD)- inhibitable cytochrome c reduction. ΔE mM (ferrocytochrome c minus ferricytochrome c) at 550 nm was taken as 18·5. Released O_2^- was expressed as n moles cytochrome c reduced per 5×10^5 PMNs per 30 min or 2 h.

(a) *Stimulation with soluble proteins.* Protein solutions (500 μg/ml in HBSS) were centrifuged at 175,000 *g* for 2·5 h to remove aggregated proteins, and the upper half of each solution was used as the soluble protein fraction. Ultracentrifugation showed that about 10% of each protein fraction aggregated.

PMNs (5×10^5) were incubated in 1 ml of each soluble protein solution with 80 μM cytochrome c, with or without 30 μg/ml SOD at 37° for 30 min. After centrifugation of PMNs, the absorbance of each solution was scanned from 560 nm to 540 nm with a spectrophotometer (Cary 17 or Hitachi 124).

(b) *Stimulation with nonphagocytosable surfaces.* PMNs ($2{\cdot}5 \times 10^5$) in 1·5 ml of HBSS with 80 μM cyt. c, with or without 30 μg/ml SOD were put into a polystyrene dish coated with each of the myeloma proteins and fragments. These dishes were placed in a 5% CO_2-atmosphere at 37° for 2 h to settle PMNs onto protein-coated surfaces. After incubation, the amount of cytochrome c reduced was measured as above. Throughout the experiments the reduction of cytochome c at zero time was negligible, and the reduction which could not be inhibited by SOD was less than 10% of the total reduction. After removal of the medium, the viability of the cells attached to each surface was measured by the trypan blue dye-exclusion test.

RESULTS

Amount of proteins adherent to dishes

Figure 1a and b show the relationship between the amount of proteins adherent to dishes, and the concentration of proteins used for the treatment of dishes. The amount of adherent protein was proportional to the total concentration added. The average amount of each protein adherent per dish at the starting concentration of 500 μg/ml was 195 μg for IgG Yo, 115 μg for IgA Hi, 80 μg for IgD Ko, 157 μg for IgE Ps and 109 μg for IgM Ga.

Stimulation of PMNs

(a) *Stimulation with soluble proteins.* As shown in Table 1, soluble IgG, fragments of IgG, and HSA did not induce O_2^- release. Cell viability slightly decreased during incubation with IgG Yo and Fab Yo.

(b) *Stimulation with nonphagocytosable surfaces.* The ability of immunoglobulin classes on polystyrene surfaces to induce O_2^- release is shown in Table 2.

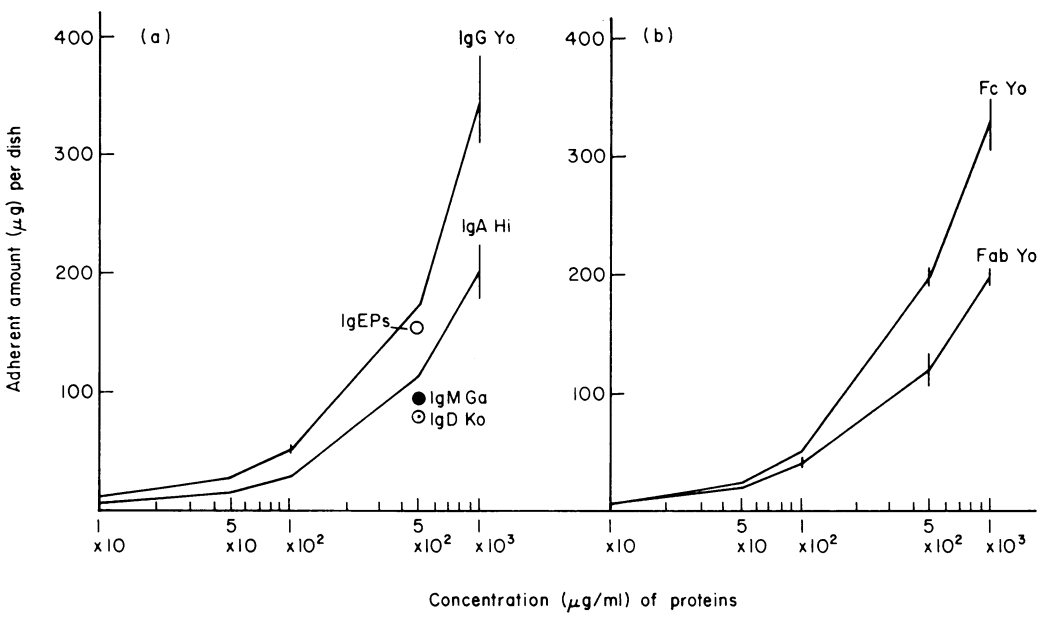

Figure 1. Amounts of immunoglobulins adherent to polystyrene dishes vs concentration of protein solutions used for the treatment. A constant amount of the ^{131}I labelled protein and 2 ml of various concentrations of the protein solution in 0·05 M sodium carbonate bicarbonate buffer pH 9·8 was supplied to dishes. The dishes were placed in a incubator at 37° for 2 h. After three washes with 0·9% NaCl, radioactivity remained on each dish was counted. Vertical bars show ± standard deviation. (n = 2).

Table 1. Stimulation of PMNs with soluble protein solutions

Soluble protein	PMN	O_2^- released: n moles cyt c reduced /5 × 10⁵ PMNs/30 min	Viable cells before, and after incubation (%)
		mean ±SD	95·3
—	—	0·36 ±0·50	
—	+	1·40 ±0·05	98·3
IgG Yo	+	0·00 ±0·00	89·3
Fab Yo	+	0·00 ±0·00	86·4
Fab Ky	+	0·47 ±0·05	98·4
Fc Yo	+	0·18 ±0·25	96·4
HSA	+	0·00 ±0·00	95·3

PMNs (5 × 10⁶) in 1 ml of soluble protein solution (500 μg/ml in HBSS with 80 μM cyt.c and 10 iu/ml heparin, with or without 30 μg/ml SOD) were incubated at 37° for 30 min. Soluble protein solutions were obtained after ultracentrifugation at 175,000 *g* for 2·5 h. Four duplicate experiments were done.

The stimulations with IgG1 Yo, IgG2 Ky, IgA Hi nomomer, and IgA Hi dimer on surfaces were much higher than those with IgD Ko, IgE Ps, IgM Ga, and HSA. Cytochrome c was not reduced in the buffer control. Polystyrene surfaces not coated with proteins stimulate PMNs slightly in many cases, but sometimes stimulate to a rather high level. The stimulation of PMNs with IgG and IgA on surfaces was significantly higher than those with non-coated dishes. In all experiments IgD, IgE, IgM, and HSA on surfaces did not stimulate PMNs. These proteins seemed to inhibit O_2^- release from PMNs. Since cell viability did not change much during incubation with proteins on surfaces, the inhibitory effect of these proteins may be due to prevention of PMNs making contact with polystyrene surfaces.

Table 2. Stimulation of PMNs with non-phagocytosable surfaces coated with immunoglobulins

Protein on surfaces	PMN	O_2^- released: n moles cyt. c reduced /5 × 10⁵ PMNs/2 h	Viable cells before, and after incubation (%)
		mean ±SD	93·7
—	—	0·87 ± 0·25	
—	+	31·27 ±10·01	97·2
IgG1 Yo	+	53·39 ± 5·38	93·5
IgG2 Ky	+	67·21 ±12·22	96·9
$(IgA\ Hi)_1$	+	68·25 ± 9·52	98·9
$(IgA\ Hi)_2$	+	63·15 ± 3·05	98·9
IgD Ko	+	0·78 ± 0·61	99·1
IgE Ps	+	2·42 ± 0·74	98·2
IgM Ga	+	1·13 ± 0·12	99·2
HSA	+	12·78 ± 8·31	99·0

PMNs (2·5 × 10⁶) in 1·5 ml of HBSS (with 80 μM cyt.c and 10 iu/ml heparin, with or without 30 μg/ml SOD) were settled onto surfaces of polystyrene dishes coated with a protein at 37° for 2 h in a 5% CO_2-atmosphere. Six duplicate experiments were done.

Table 3. Stimulation of PMNs with non-phagocytosable surfaces coated with IgG and fragments of IgG

Protein on surfaces	PMN	O_2^- released: n moles cyt. c reduced /5 × 10^5 PMNs/2 h	Viable cells before, and after incubation (%)
		mean ± SD	93·1
—	—	0·31 ± 0·43	
—	+	50·11 ± 1·73	92·6
IgG1 Yo	+	97·56 ± 16·42	95·3
IgG2 Ky	+	99·91 ± 6·19	88·3
IgG*	+	100·32 ± 1·29	93·1
Fab Yo	+	28·82 ± 0·14	93·6
Fab Ky	+	9·58 ± 0·29	—
Fc Yo	+	53·88 ± 0·71	73·9
(Fab + Fc) Yo	+	26·99 ± 1·87	86·7

PMNs ($2·5 \times 10^5$) in 1·5 ml of HBSS (with 80 μM cyt.c and 10 iu/ml heparin, with or without 30 μg/ml SOD) were settled onto surfaces of polystyrene dishes coated with a protein at 37° for 2 h in a 5% CO_2-atmosphere. Six duplicate experiments were done. * Normal pooled human IgG.

Table 3 shows the stimulation of PMNs with non-phagocytosable surfaces of IgG and fragments of IgG. IgG Yo, IgG Ky, and pooled human IgG induced O_2^- release, but Fab Yo, Fab Ky, Fc Yo, and mixtures of Fab Yo and Fc Yo did not. In other experiments $F(ab')_2$ and Fc Ky did not stimulate PMNs. Because pooled human IgG stimulated PMNs, stimulation of PMNs is not limited to IgG myeloma proteins. Since Fc, Fab, and mixtures of Fc and Fab on surfaces did not stimulate PMNs, the intact whole molecule of IgG is required for the stimulation.

DISCUSSION

The results reported here indicate that soluble monomeric IgG and IgA, when incubated with PMNs in suspension, do not induce O_2^- release from PMNs, but IgG and IgA without aggregation, when bound to surfaces, stimulate O_2^- released independently of phagocytosis. Immunoglobulin-class-specific stimulation of granular enzyme release from PMNs was reported by Henson *et al.* (1972) who showed that aggregated IgG and IgA, in suspension or on surfaces, induced release of lysosomal enzyme from PMNs. Stimulation of PMNs with aggregated IgG was also reported by Goldstein *et al.* (1975) who found that insoluble aggregated IgG, in suspension or on surfaces, induced both lysosomal enzyme release and HMP activation. They also found that soluble aggregated IgG, when incubated with PMNs in suspension, induced only HMP activation, but, when bound to surfaces, induced both lysosomal enzyme release and HMP activation. Johnston *et al.* (1976a and, b) reported that aggregated IgG on millipore filters stimulates PMNs to generate O_2^-, H_2O_2 and chemiluminescence. As nonphagocytosable surfaces, the above reporters used aggregated immunoglobulins on millipore filters. We prepared nonphagocytosable surfaces by coating the surfaces of polystyrene dishes with non-aggregated immunoglobulins. Since no aggregation was required to induce O_2^- release, similar comformational changes as seen when immunoglobulins form immune complexes or are aggregated, may occur in the molecules on polystyrene surfaces. Since immunoglobulin is stable between pH 4 and 10 (Gould, Gill & Doty, 1964; Takahashi, Hirai, Azuma, Hamaguchi & Migita, 1970), it is unlikely that immunoglobulins undergo denaturation or aggregation in the carbonate bicarbonate buffer pH 9·8 used for the treatment of dishes. Because IgG or IgA on surfaces induced the activation, PMNs may be stimulated through the receptors for IgG and IgA. It was not clear whether PMNs have two different receptors or one common receptor for IgG and IgA.

The present study has also shown that whole molecules of IgG were necessary to induce O_2^- release from PMNs, because fragments of IgG: Fc, Fab, $F(ab')_2$, and mixtures of Fc and Fab on surfaces did not stimulate. It is known that PMNs have an IgG Fc receptor, and that the receptor participates in phagocytosis (Ehlenberger & Nussenzweig, 1977; Mantovani, 1975), but the participation of the Fc receptor in causing granular enzyme release or O_2^- generation without phagocytosis is not clear. Henson *et al.* (1972) also reported that aggregated Fc or $F(ab')_2$ of IgG induced less granular enzyme release than did the whole molecules, and reduction and alkylation of IgG before aggregation reduced its ability to stimulate enzyme release from PMNs. So, it is probable that not only Fc is required for the binding site, but also a certain conformation for the stimulator is required to induce granular enzyme release or O_2^- release. This conformation may be retained in the intact whole molecule of IgG on surfaces, and may disappear after reduction and alkylation, or digestion of IgG.

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