An Fc-receptor activity of plasma membranes from guinea-pig peritoneal polymorphonuclear leucocytes

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Plasma membranes prepared from guinea-pig peritoneal polymorphonuclear leucocytes showed an immune-complex-binding activity that corresponded well with the activity in intact cells. The characteristics of this activity were reversible binding, dependence on the Fc portion of antigen-complexed IgG (immunoglobulin G), competition with aggregated IgG and independence from energy metabolism. These results support the conclusion that the binding activity found in the isolated plasma membranes is an Fc-receptor activity of guinea-pig peritoneal polymorphonuclear leucocytes. The activity showed $K_d = 6.7 \times 10^{-8}$ M-IgG and maximum binding of 17 pmol of IgG/mg of membrane protein when measured with an immune complex of α -amylase and homologous guinea-pig anti-(α -amylase) IgG. Inhibition of the Fc-receptor activity by a series of various salts indicated the contribution of the hydrophobic interaction to the binding. Inhibitory effects of salts or metal-chelating reagents on the Fc-receptor activity were also observed on superoxide generation by these cells induced by the immune complex, suggesting a role of the Fc receptor as the immune-complex-binding site responsible for the initiation of superoxide generation.

Polymorphonuclear leucocytes are actively involved in the cellular defence mechanism. The cells are especially active in endocytosis as well as in release of oxygen metabolites (superoxide, hydrogen peroxide etc.), of lysosomal enzymes and of prostaglandins (Theofilopoulos & Dixon, 1979; Badwey & Karnovsky, 1980). Some of the most effective inducers of these responses are IgG-complexed foreign substances, which are recognized by polymorphonuclear leucocytes through IgG receptors on the plasma membranes. The IgG receptor selectively accepts the Fc portion of antigen-complexed IgG in these and other types of cells (Theofilopoulos & Dixon, 1979; Paraskevas *et al.*, 1972; Dickler, 1976). After this binding, the information is transmitted through the plasma membranes of these cells and results in a series of complex events that leads to endocytosis and the accompanying defence mechanism. Thus this cellular defence mechanism induced by immune complex

is a typical example of transmembrane control and provides a good experimental system to use in studies on mechanisms of interaction of cells with their environment, since the whole process shows the existence of specific receptors and the clear responses of endocytosis and the accompanying defence mechanism.

Analytical studies on the cellular defence mechanism are, however, limited by the fact that the overall process is complex and is only observed in intact cells. One approach that has proved most fruitful in many studies on complex metabolic reactions is to investigate partial reactions separately and then to reorganize the whole process as a sum of the elementary processes (Racker, 1976). Our preparation of plasma membranes from guinea-pig polymorphonuclear leucocytes (S. Tsunawaki, D. Mizuno & M. Kasahara, unpublished work) had made it possible to use this approach in studies on the immune-complex binding, the first step in the complex cellular defence mechanism. It was demonstrated for the first time that an Fc-receptor activity was measurable in a plasma-membrane fraction.

Abbreviations used: IgG, immunoglobulin G; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

The present paper describes detailed studies on the immune-complex binding in plasma membranes, where many properties of the binding activity were found to be in common with Fc-receptor activities of these and other cells. These results support the conclusion that the immune-complex-binding activity in plasma membranes is an Fc-receptor activity of guinea-pig peritoneal polymorphonuclear leucocytes. The use of plasma membranes was found to be essential in several experiments on the Fc receptor in which the experimental conditions were inappropriate for use of intact cells.

Superoxide-generating activity was chosen as a representative response to immune-complex binding, since this activity is an important defence activity of polymorphonuclear leucocytes and it can be measured quantitatively. Many properties of the Fc-receptor activity found in the present study were reflected in the superoxide generation induced by immune-complex binding.

Materials and methods

Materials

Guinea pigs (Hartley, male, 250–500 g) and rabbits (Japan White-Sat, 3–4 kg) were purchased from Nippon Animal Supply Center, Tokyo, Japan. Sodium caseinate was from Tokyo Chemicals, Tokyo, Japan, bovine serum albumin (fraction V) from Armour, and α -amylase (crystalline, from *Bacillus subtilis*) from Katayama Chemicals, Osaka, Japan. Complete Freund's adjuvant and ovalbumin were from Difco, DEAE-cellulose (DE-52) and glass-fibre filters (GF/C) from Whatman, and Sephadex G-25 and G-150 from Pharmacia. Pepsin A was from Worthington, human γ -globulin (Cohn fraction II) from Miles, cytochrome *c* from Boehringer, cytochalasin B from Aldrich and ^{125}I from Amersham International. Other reagents used were analytical grade.

Methods

Preparation of polymorphonuclear leucocytes and plasma membranes. Polymorphonuclear leucocytes were collected from guinea-pig peritoneal cavity 14–16 h after injection of sodium caseinate (Sbarra & Karnovsky, 1959). After red blood cells were removed by hypo-osmotic shock, leucocytes were washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate buffered saline (137 mM-NaCl / 2.6 mM-KCl / 8.1 mM- Na_2HPO_4 / 1.5 mM- KH_2PO_4 , pH 7.4) and suspended in a buffer (130 mM-NaCl / 0.5 mM- MgCl_2 / 20 mM-Hepes, pH 7.4). A plasma-membrane fraction was prepared by a nitrogen-cavitation method (S. Tsunawaki, D. Mizuno & M. Kasahara, unpublished work). In brief, after addition of an equal amount of 0.5 M-sucrose, the cells were subjected to 505 kPa (50 atm) for 20 min in a cavitation 'bomb' (Parr,

Moline, IL, U.S.A.). The mixture was recovered dropwise from the bomb and centrifuged at 450 g for 15 min. The supernatant was further centrifuged at 150 000 g for 60 min. The pellet was washed with 10 mM-Hepes buffer, pH 7.4, and was used as the plasma-membrane fraction. Two marker activities for the plasma membranes, the Fc-receptor activity and 5'-nucleotidase activity, and several markers for other organelles showed 20–25% recovery of plasma membranes in this fraction, a 5-fold increase in specific activities of plasma-membrane markers as compared with those of homogenate, and less than 10% of contaminating cellular constituents (9% of 'microsomes', 3–7% of the lysosomes and a negligible amount of mitochondria or cytoplasm).

Preparations of anti-(bovine serum albumin) IgG and anti-(α -amylase) IgG. Rabbits were immunized with bovine serum albumin (2 mg) or α -amylase (2 mg) in complete Freund's adjuvant. The same antigen/adjuvant mixture was injected again 4 weeks later. A week later blood was obtained from an ear vein. IgG was prepared by precipitation with $(\text{NH}_4)_2\text{SO}_4$ and DEAE-cellulose chromatography (McLaury & Racker, 1973). Guinea-pig anti-(α -amylase) IgG was prepared in the same way as rabbit IgG, except that half as much antigen/adjuvant mixture was used for immunization and blood was obtained by cardiac puncture.

Preparation of F(ab')_2 . Guinea-pig anti-(α -amylase) IgG was dialysed against 0.1 M-sodium acetate buffer, pH 4.5, for 12 h at 4°C. Pepsin A (0.45 mg/ml) was added to the anti-(α -amylase) IgG solution (30 mg/ml) and the mixture was incubated for 12 h at 37°C. The mixture was then adjusted to pH 7.0 with 1 M-NaOH and applied to a column of Sephadex G-150 equilibrated with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate buffered saline. The F(ab')_2 fraction recovered showed the same antigen-binding activity as untreated IgG in Ouchterlony double-diffusion tests.

Iodination of IgG and F(ab')_2 . The IgG or F(ab')_2 fragment was radioiodinated with ^{125}I by the chloramine-T method of McConahey & Dixon (1966).

Preparation of human aggregated IgG. Human IgG was aggregated at 63°C for 20 min in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline by the method of Dickler (1974). Before binding assays the aggregated IgG was centrifuged at 600 g for 15 min to remove large aggregates.

Preparation of immune complex. Antigen (bovine serum albumin or α -amylase) and iodinated IgG or F(ab')_2 were mixed at a fixed ratio in buffer A ($\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline containing 16 mg of ovalbumin/ml) and incubated at 4°C overnight. The mixture was centrifuged at 10 000 g for 30 min and the supernatant was used for binding assays.

Binding assay. The assay mixture (500 μ l) contained polymorphonuclear leucocytes [(1–2) $\times 10^7$ cells] or plasma membranes (50–200 μ g of protein) prepared from these cells, 125 I-labelled immune complex and buffer A). Binding assays were always performed in duplicate. Assay mixtures containing formed in duplicate. Assay mixtures containing intact cells were incubated for 3 h at 4°C and those containing plasma membranes for 3 h at 20°C or 5 h at 4°C. Immune complex bound to intact cells was separated from unbound complex by centrifugation. The assay mixture was centrifuged at 450 g for 3 min and the cell pellet was washed three times with buffer A (5 ml). In the case of plasma membranes the separation was performed by filtration. The assay mixture was applied to a glass-fibre filter (GF/C) and washed three times with buffer A. Radioactivity associated with intact cells or plasma membranes was counted in a well-type gamma counter (JDC 751, Aloka, Tokyo, Japan).

Assay of superoxide generation. Superoxide generation was assayed by measuring reduction of cytochrome *c* or 70%-acetylated cytochrome *c*. Acetylated cytochrome *c* is specifically reduced by superoxide anions but not by other intracellular enzymic processes (Kakinuma & Minakami, 1978). Intact cells were suspended in assay medium containing 5 mM-glucose and 10 μ M-cytochrome *c* or 15 μ M-acetylated cytochrome *c* in Ca^{2+} / Mg^{2+} -free phosphate buffered saline at 37°C in a cuvette with an attached cell mixer (Kakinuma *et al.*, 1979). Reduction of either form of cytochrome *c* was recorded in a double-beam spectrophotometer (356, Hitachi) ($A_{350-540}^{\text{PM}} = 19.1$). The initial rate of activity was measured after addition of the immune complex.

Results

Binding of immune complex to guinea-pig peritoneal polymorphonuclear leucocytes

Fig. 1 shows the immune-complex-binding activity of polymorphonuclear leucocytes measured at 4°C with an immune complex of α -amylase and guinea-pig anti-(α -amylase) IgG. Maximum binding was observed when the immune complex was added at a molar ratio of antigen to antibody of about 0.1 (the maximum-binding region). At molar ratios of over 1, the second phase of binding occurred (the antigen-excess region). Since immune-complex binding in these two regions showed different extents of enhancement of superoxide generation (see below) and of other functions in these and other types of cells (Theofilopoulos & Dixon, 1979), the properties of the binding were studied in both the maximum-binding region and the antigen-excess region. The binding of the antigen-complexed F(ab')_2 fragment showed negligible activity, like that of

Fig. 1. *Effect of antigen-to-antibody ratio on immune-complex binding to intact cells*

A 2 μ g portion of 125 I-labelled guinea-pig anti-(α -amylase) IgG or an equimolar amount of F(ab')_2 was mixed with 0.01–5.1 μ g of α -amylase. The antigen/antibody solution was incubated as described in the Materials and methods section. The binding reaction was performed with immune complex containing 1.7 pmol of IgG or an equimolar amount of F(ab')_2 and 2×10^7 intact cells in buffer A (Ca^{2+} / Mg^{2+} -free phosphate-buffered saline containing 16 mg of ovalbumin/ml) at 4°C. The assay mixture was applied to a filter which was then washed three times with 2 ml of buffer A. Radioactivity associated with the filters was counted in a gamma counter. O, Antigen-complexed IgG; ●, antigen-complexed F(ab')_2 .

antigen-free IgG (uncomplexed IgG). These results indicated that the binding activity depends on the Fc portion of antigen-complexed IgG. In following experiments antigen-free IgG or the antigen-complexed F(ab')_2 fragment was used as a control. The binding reaction at 4°C was slow but was almost saturated after 6 h (Fig. 2a). The binding of the antigen-complexed F(ab')_2 fragment was low at all times. The binding activity was inhibited by the immune complex formed with heterologous rabbit IgG (Table 1) and the inhibition was of the same order of magnitude as that observed when the same amount of non-radioactive homologous immune complex was added to the assay mixture. This suggests that at least some of the heterologous immune complex competes for the homologous immune-complex-binding site.

Immune-complex binding to plasma membranes of polymorphonuclear leucocytes

Plasma membranes were prepared from polymorphonuclear leucocytes by a nitrogen-cavitation

Fig. 2. Time course of immune-complex binding to intact cells (a) or plasma membranes (b)

Immune complex was made at a molar ratio of antigen to antibody of 0.12 with α -amylase and guinea-pig anti-(α -amylase) IgG or F(ab')₂. The immune complex of 1.7 pmol of IgG or an equimolar amount of F(ab')₂ was incubated with 2×10^7 intact cells (a) or 50 μ g of plasma membranes (b) at 4°C. Other procedures are described in the Materials and methods section. O, Antigen-complexed IgG; ●, antigen-complexed F(ab')₂.

method. The fraction was reasonably pure and was obtained in relatively large quantity (see the Materials and methods section). The binding reaction on the plasma membranes was slow and showed a similar time course to that in intact cells (Fig. 2b). The binding reaction proceeded faster at 20°C than at 4°C and nearly reached equilibrium after 3 h (results not shown). Since some Fc receptors are known to bind aggregated IgG (Dickler, 1976), the effects of heat-aggregated human IgG on plasma membranes and intact cells were studied. Aggregated IgG cause complete inhibition of both preparations (Fig. 3).

The results described in this and the above subsection indicate that the immune-complex-bind-

Table 1. Competition with immune-complex binding of intact polymorphonuclear leucocytes by non-radioactive homologous or heterologous immune complex

Radioactive immune complex was made with α -amylase and iodinated guinea-pig anti-(α -amylase) IgG at a molar ratio of antigen to antibody of 3. Non-radioactive homologous or heterologous immune complex was made with α -amylase and guinea-pig or rabbit anti-(α -amylase) IgG at the same molar ratio. These two immunoglobulins exhibited the same antigen-binding activity, as determined by Ouchterlony double-diffusion test. The assay mixture contained polymorphonuclear leucocytes (2×10^7 cells), immune complex (1.7 pmol of IgG) and the indicated amount of non-radioactive homologous or heterologous immune complex in buffer A (Ca²⁺/Mg²⁺-free phosphate-buffered saline containing 16 mg of ovalbumin/ml). Other procedures are described in the Materials and methods section. Data are expressed as percentages of the control value (4.9 pmol of IgG/10⁹ cells).

Immune complex (μ mol of IgG/ml)	Binding (% of control)	
	+Guinea-pig IgG	+Rabbit IgG
66	71	71
132	40	40
264	26	31

ing activity is the Fc-receptor activity of polymorphonuclear leucocyte plasma membranes. The properties of the receptors described in the following sub-sections were consistent with this notion. In the present paper we refer to the binding activity observed in intact cells and plasma membranes as 'Fc-receptor activity'.

Affinity and number of Fc receptors

It is known that low concentrations of immune complex are enough to activate cellular defence mechanism of polymorphonuclear leucocytes (Theofilopoulos & Dixon, 1979). A single class of saturable binding site with a K_d of 6.7×10^{-8} M-IgG and a maximum binding of 17 pmol of IgG/mg of protein was observed in plasma membranes (Fig. 4). Intact cells showed a K_d of 6.7×10^{-8} M-IgG and a maximum binding of 0.20 nmol of IgG/10⁹ cells. Since the recoveries of protein and of Fc-receptor activity of the plasma-membrane fraction were 4 and 20–25% respectively, and the protein content of intact cells was about 100 mg/10⁹ cells (S. Tsunawaki, D. Mizuno & M. Kasahara, unpublished work), the maximum binding of 0.20 nmol of IgG/10⁹ cells should correspond to 10–13 pmol of IgG/mg of membrane protein. Thus the observed maximum binding of 17 pmol of IgG/mg of protein to plasma membranes corresponds well with this estimated value. A reasonable estimate of 1.2×10^5 receptor sites/cell is obtained if we assume that the immune complex at antigen-excess region is com-

Fig. 3. Inhibition of immune-complex binding by heat-aggregated IgG

Immune complex was prepared from bovine serum albumin and rabbit anti-(bovine serum albumin) IgG at a molar ratio of antigen to antibody of 0.09. Binding assay was performed as described for Fig. 2, except that incubation was carried out at 4°C for 3 h with intact cells and at 20°C for 3 h with plasma membranes. The binding of immune complex without heat-aggregated IgG was 17 pmol of IgG/10⁹ cells or 1.6 pmol of IgG/mg of membrane protein. O, Intact cells; Δ, plasma membranes.

Fig. 4. Immune-complex binding of intact cells or plasma membranes with various amounts of immune complex

The immune complex was made with α -amylase and guinea-pig anti-(α -amylase) IgG at a molar ratio of antigen to antibody of 3. The assay mixture was incubated for 3 h at 4°C with intact cells (2×10^7 cells) or for 3 h at 20°C with plasma membranes (250 μ g of protein). Data are shown as double-reciprocal plot. O, Intact cells; Δ, plasma membranes.

posed of two antigens and one antibody (Theofilopoulos & Dixon, 1979).

When the immune complex was formed with bovine serum albumin and heterologous rabbit anti-(bovine serum albumin) IgG, intact cells or plasma membranes showed a 10-times-higher K_d than that for the homologous immune complex. But the maximum binding was very similar to that with the homologous immune complex (S. Tsunawaki, D. Mizuno & M. Kasahara, unpublished work). These results are consistent with the above suggestion that the homologous and the heterologous immune complexes both bind to the same Fc receptor. Binding assays with various amounts of cells indicated anti-(bovine serum albumin) IgG contained 20–28% of specific IgG for bovine serum albumin (S. Tsunawaki, D. Mizuno & M. Kasahara, unpublished work). In the present study, however, no correction of the molar ratio of immune complex was made.

Effects of various reagents on the Fc-receptor activity

Among the inhibitors of energy metabolism tested, only moniodoacetate inhibited the binding by intact cells (Table 2), but in plasma membranes no energy inhibitors tested were inhibitory. It is conceivable that the difference in the effect of moniodoacetate is due to the fact that intact cells have residual endocytotic activity during the binding measurement at 4°C. In the category of mobility inhibitors, inhibitors acting on microtubules and microfilaments did not show marked effects on either intact cells or in plasma membranes. The metal-ion chelators EDTA and EGTA inhibited the binding, but addition of Ca²⁺ or Mg²⁺ was not required for the binding. Thus the small amount of metal ion present in the assay mixture may be enough for the binding. Consistent with our results there are reports that addition of Ca²⁺ ions is not required for immune-complex binding by human polymorphonuclear leucocytes (Korchak & Weismann, 1978) or lymphocytes (Dickler, 1976). Monosaccharides (mannose, glucose, galactose, mannose 6-phosphate, *N*-acetylglucosamine) or disaccharides (sucrose, lactose) at 50 mM had no effect. Mild reduction of the immune complex with dithiothreitol is apparently inhibitory for the binding. Rabbit IgG raised against plasma membranes of these cells inhibited the Fc-receptor activity of both intact cells and plasma membranes (results not shown), suggesting that anti-(plasma membrane) IgG contains antibody for Fc receptor.

Effects of various anions on the Fc receptor activity

During studies on the effects of various substances, a prominent effect of inorganic salts was observed. In intact cells the binding was inhibited by

Table 2. *Effect of various reagents on immune-complex binding*

Immune complex was made with bovine serum albumin and rabbit anti-(bovine serum albumin) IgG at a molar ratio of 0.17. Immune-complex binding was measured by incubation with intact cells (1.7×10^7 cells) or plasma membranes (60 μ g of protein) for 3 h at 4°C with the additions indicated. Other conditions are described in the Materials and methods section. Net binding is expressed as a percentage of the control value (7.1 pmol of IgG/ 10^9 cells with intact cells or 0.7 pmol of IgG/mg with plasma membranes).

Reagent	Concn.	Net binding (% of control)	
		Intact cells	Plasma membranes
Iodoacetate	10 mM	27	97
NaN ₃	10 mM	74	116
Dinitrophenol	1 mM	97	123
2-Deoxyglucose	50 mM	87	104
Cytochalasin B	50 μ M	84	80
(Dimethyl sulphoxide)*	1%	85	88)
Colchicine	50 μ M	96	104
EDTA	50 mM	36	26
EGTA	50 mM	58	51
N-Ethylmaleimide	10 mM	105	110
HgCl ₂	200 μ M	99	90
p-Chloromercuribenzoate	200 μ M	68	77
Dithiothreitol	10 mM	32	24
2-Mercaptoethanol	10 mM	67	49

* Dimethyl sulphoxide was used as a solvent for cytochalasin B.

high concentrations of Ca²⁺/Mg²⁺-free phosphate-buffered saline (Fig. 5a). To avoid the complication of an osmotic effect on cell integrity, further experiments were performed mainly with plasma membranes. NaCl strongly inhibited the Fc-receptor activity and the binding of antigen-free IgG (Fig. 5b). As shown in Fig. 5(b), the Fc-receptor activity was most discriminately measurable under the iso-osmotic conditions used in our routine assay. Non-specific binding of IgG to the glass-fibre filter used in the assay was negligible at all salt concentrations. Several anions were used to determine whether the inhibition was anion-specific or was simply due to ionic strength. The anions tested showed the following clear series of inhibitory effects on intact cells and plasma membranes (Table 3):

This series is known as the ‘series of chaotropic ions’ (Hatefi & Hanstein, 1969) or the ‘Hofmeister series’

Fig. 5. *Effects of salt concentration on immune-complex binding to intact cells (a) and plasma membranes (b)* The immune complex was prepared from bovine serum albumin and rabbit anti-(bovine serum albumin) IgG at a molar ratio of antigen to antibody of 0.09. In (a), intact cells (2×10^7 cells) and immune complex (1.7 pmol of IgG) were incubated at 4°C for 30 min in the indicated concentration of Ca²⁺/Mg²⁺-free phosphate-buffered saline. The abscissa shows multiples of the iso-osmotic concentration. O, Antigen-complexed IgG; ●, IgG only. In (b), immune complex (1.7 pmol of IgG) and plasma membranes (50 μ g) were incubated for 3 h at 4°C with the indicated amount of NaCl. Δ, Antigen-complexed IgG; ▲, IgG only; □, antigen-complexed IgG without addition of plasma membranes; ■, antigen-free IgG without addition of plasma membranes.

Table 3. *Effects of various anions on immune-complex binding*

Immune complex was made with α -amylase and guinea-pig anti-(α -amylase) IgG at a molar ratio of antigen to antibody of 3. The incubation mixture contained intact cells (2×10^7 cells) or 100 μ g of plasma membranes, immune complex (1.7 pmol of IgG), 3-fold-diluted Ca²⁺/Mg²⁺-free phosphate-buffered saline, 16 mg of ovalbumin/ml and the indicated sodium salts (100 mM each). Incubation at 4°C was continued for 3.5 h with intact cells and for 7 h with plasma membranes. Other procedures are described in the Materials and methods section.

Anion	Binding	
	Intact cells (pmol of IgG/ 10^9 cells)	Plasma membranes (pmol of IgG/mg of protein)
SCN ⁻	1.0	0.14
I ⁻	1.6	0.19
NO ₃ ⁻	2.1	0.26
Cl ⁻	4.4	0.60
CH ₃ CO ₂ ⁻	5.4	0.69

Fig. 6. Reversal of immune-complex binding by high salt (a) or competition (b)

Immune complex was made with α -amylase and guinea-pig anti-(α -amylase) IgG at a molar ratio of antigen to antibody of 3 (O, antigen-excess region) or 0.12 (Δ , maximum-binding region). Plasma membranes (100 μ g) and immune complex (1.7 pmol of IgG) were preincubated for 5 h at 4°C in buffer A. In (a), after addition of the indicated amount of NaCl, incubation was continued for 7 h at 4°C. Glass-fibre filters were washed with buffer A supplemented with the same concentration of NaCl as in the incubation medium. Data are expressed as percentages of the control, which was incubated without addition of NaCl (0.5 pmol of IgG/mg of protein with immune complex made in the antigen-excess region or 1.6 pmol of IgG/mg of protein with that made in the maximum-binding region). In (b) the immune complex made in the antigen-excess

(von Hippel & Schleich, 1969). This finding suggests that hydrophobic interaction plays some role in the binding reaction.

Inhibitory effects of salts were found reversible. After plasma membranes were preincubated overnight with a 100 mM concentration of each anion, washing of plasma membranes with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline reversed the inhibitory effects of the above salts. Thus the salt-treated membranes showed the same binding activities as the control, which was either not preincubated or preincubated with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline.

The same magnitudes and sequence of anion inhibition were observed when assay mixtures with each anion were preincubated overnight (results not shown). Thus the concentration of anions, not their concentration gradient across plasma membranes, is important for the anion inhibition.

Reversibility of the binding reaction

Fig. 6(a) shows that addition of NaCl reversed the binding of the immune complex both in the maximum-binding region and in the antigen-excess region. In competition experiments, excess non-radioactive immune complex or heat-aggregated human IgG reversed the binding of the radioactive immune complex to plasma membranes which had been preincubated for 5 h (Fig. 6b). Addition of 300 mM-NaCl enhanced the inhibitory effects. At low salt concentration, reversal was rather incomplete (results not shown). The fact that the binding is reversible suggests that endocytotic activity or uptake of immune complex into membrane vesicles is negligible in the present preparation of plasma membranes.

Superoxide-generating activity of polymorphonuclear leucocytes associated with immune-complex binding

The superoxide-anion-generating activities of intact cells on addition of the two types of immune complex (one formed at the maximum-binding

region (1.7 pmol of IgG) was preincubated with plasma membranes as described in (a). After addition of 300 mM-NaCl (final concn.) and the indicated amount of non-radioactive immune complex made in the same way (O) or non-radioactive heat-aggregated human IgG (Δ), incubation was continued for 4 h at 4°C. The ordinate shows values as a percentage of the control, which was incubated with 300 mM-NaCl only (0.4 pmol of IgG/mg of protein). Incubation with buffer A only is also indicated (●).

Fig. 7. Superoxide generation by intact cells with immune complex made in the antigen-excess region (O) and maximum-binding region (Δ)

Immune complex was made with bovine serum albumin and rabbit anti-(bovine serum albumin) IgG at a molar ratio of antigen to antibody of 3 (antigen-excess region) or 0.17 pmol (maximum-binding region). Superoxide generation was monitored at 37°C by measuring the absorbance change in cytochrome *c*. The assay mixture (1.7 ml) contained 5 mM-glucose, 10 μM-cytochrome *c*, 1.6×10^7 intact cells and immune complex in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline. The abscissa shows the concentration of immune complex expressed as the IgG concentration. As controls, antigen-free IgG (●) or bovine serum albumin (▲) was used. In the case of bovine serum albumin, the abscissa indicates the equivalent amount of bovine serum albumin used for immune-complex formation. O, Immune complex made in the antigen-excess region; Δ, immune complex made in the maximum-binding region; ●, IgG only; ▲, antigen (bovine serum albumin) only.

region and the other at the antigen-excess region) were studied. The former complex greatly increased superoxide generation at 0.7 nmol of IgG/ml, but its effect decreased when it was added at high concentration (Fig. 7). Neither antigen-free IgG nor antigen only (bovine serum albumin) induced superoxide generation. The latter complex induced much lower activity than the former, and its effect was observed only over a small concentration range (Fig. 7). Similarly high activity of superoxide generation was found with α-amylase-complexed IgG, but the α-amylase-complexed F(ab')₂ fragment had no effect on this activity (results not shown). These results indicate that binding of the immune complex to the Fc receptor leads to induction of superoxide-generating activity.

The relation of superoxide generation to the immune-complex binding was studied. A marked inhibition of superoxide generation was observed when the salt concentration of the incubation medium was increased by addition of NaCl (Fig. 8). Similar results were obtained by adding $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline instead of NaCl. The data shown in Figs. 8 and 6(a) indicate that superoxide generation was inhibited by salt at the initial immune-complex-binding site. They also indicate involvement of another salt-sensitive site, since superoxide generation was inhibited more than immune-complex binding, and basal superoxide generation without addition of immune complex was also inhibited by higher concentrations of salts. As shown in Table 4, the inhibitory series of anions on superoxide generation were identical with their effects on immune-complex binding (Table 3). The effects of chelating agents such as EDTA and EGTA on superoxide generation were also similar to their effects on immune-complex binding.

Fig. 8. Effect of salt concentration on superoxide generation

Immune complex was made with bovine serum albumin and rabbit anti-(bovine serum albumin) IgG at a molar ratio of antigen to antibody of 0.17. Superoxide generation was measured with 15 μM-acetylated cytochrome *c* and immune complex (1.1 nmol of IgG/ml). The $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline concentration was decreased to one-third and the indicated amount of NaCl was added to the assay mixture. Other conditions were as described for Fig. 7. Superoxide generation with (O) and without (●) immune complex is shown; Δ, net activity due to immune complex.

Table 4. *Effect of various anions and chelating agents on superoxide generation*

Immune complex was made with bovine serum albumin and rabbit anti-(bovine serum albumin) IgG at a molar ratio of 0.17. Superoxide generation was measured with 15 μ M-70%-acetylated cytochrome *c*, intact cells (0.9×10^7 cells/ml) and immune complex (1.1 nmol of IgG/ml). In (a) the indicated sodium salt was added to the assay mixture containing one-quarter the standard concentration of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline. In (b), EDTA or EGTA (sodium salt) was added to the assay mixture. Other conditions for superoxide generation are described in the Materials and methods section.

Addition	Concn. (mM)	Acetyl-cytochrome <i>c</i> reduced	
		(nmol/min per 10^9 cells)	(% of control)
(a) Buffer	(120)	8.3	100
SCN ⁻	120	2.3	28
I ⁻	120	1.9	23
NO ₃ ⁻	120	2.1	25
Cl ⁻	120	8.4	101
CH ₃ CO ₂ ⁻	120	17.7	213
(b) EDTA	12.5	9.8	118
	25.0	3.4	41
	50.0	1.4	17
EGTA	12.5	9.5	114
	25.0	5.7	69
	50.0	5.0	60

Discussion

Guinea-pig peritoneal polymorphonuclear leucocytes exhibited immune-complex-binding activity. Previous reports on polymorphonuclear leucocytes from humans (Theofilopoulos & Dixon, 1979) and animals (Lay & Nussenzweig, 1969; Phillips-Quagliata *et al.*, 1969; Henson, 1969) indicated immune-complex-binding activity that was dependent on the Fc portion of IgG. In previous studies, assays of antibody-sensitized erythrocyte adherence to these cells (rosette formation) was used to detect binding activity. This method is, however, inadequate in analytical studies on binding activity. In the present work we found that the immune-complex binding of guinea-pig polymorphonuclear leucocytes is reversible, dependent on the Fc portion of antigen-complexed IgG, inhibited competitively by aggregated IgG and independent of energy metabolism. These properties are the same as those of Fc-receptor binding in other types of cells (Theofilopoulos & Dixon, 1979; Dickler, 1976). Thus it is reasonable to conclude that the binding activity is due to the Fc-receptor activity of guinea-pig peritoneal polymorphonuclear leucocytes.

We developed a method for preparation of fairly pure plasma membranes from guinea-pig polymorphonuclear leucocytes in relatively large quantity by a nitrogen-cavitation method. Systematic studies indicated that the preparation was suitable for studies on plasma-membrane functions of these cells (S. Tsunawaki, D. Mizuno & M. Kasahara, unpublished work). Use of these membranes enables us to study the properties of the binding activity of the immune complex without interference from the biological activities induced in intact cells after immune-complex binding. Plasma membranes prepared from guinea-pig polymorphonuclear leucocytes exhibited Fc-receptor activity and showed almost the same properties as those of intact cells. The plasma membranes used in the present study showed no appreciable endocytotic activity or phenomena dependent on an immune-complex binding such as the superoxide-generating activity. Therefore the preparation is suitable for use in studies on the Fc receptors.

Several factors prevent the exact measurements of Fc-receptor activity in intact cells. At a physiological temperature, endocytotic activity interferes with the binding activity, and so binding assays on intact cells have to be performed at a low temperature. Furthermore, assays must be performed under iso-osmotic conditions (or very nearly) to prevent shrinkage or swelling of cells. The effects of various salts on the binding activity with plasma membranes suggest that the binding reaction involves hydrophobic interactions. Thus they may provide useful information about the exact binding site on the Fc portion of IgG, which has been suggested to be in the C₁3 or C₂-3 region in other cells (MacLennan *et al.*, 1974; Spiegelberg, 1975). It is very noteworthy that hydrophobic interactions are also implicated at another specific binding site in immunoglobulins, namely the binding sites for haptens (Kabat, 1972; Halsey & Biltonen, 1975).

Our finding of a single class of Fc-receptor activity in intact cells or plasma membranes does not exclude the possibility that several classes of IgG receptors, each corresponding to an individual subclass of IgG, are present on the plasma membranes. Indeed, this possibility has been indicated in the case of macrophages (Shinoyama & Koyama, 1976; Heusser *et al.*, 1977), although conflicting results have also been reported (Leslie & Cohen, 1976). Further experiments using different subclasses of IgG will be necessary to clarify this matter.

The affinity and number of binding sites of the Fc receptor observed are similar to those of other types of cells (Leslie & Cohen, 1976; Knutson *et al.*, 1977; Yagawa *et al.*, 1979). In those studies the affinities are reported to be 3.7×10^{-9} – 2.1×10^{-10} M-IgG and the numbers of sites per cell are

0.9×10^5 – 5.1×10^6 , values that are in good agreement with the affinity and number of Fc-receptor sites observed in the present work, namely 6.7×10^{-8} M-IgG and 1.2×10^5 sites/cell.

Superoxide generation is enhanced by the addition of immune complex, but not of antigen-free IgG or antigen-complexed $F(ab')_2$ fragment. The activity is inhibited by EDTA, EGTA, or anions in the series of chaotropic ions. These properties are essentially the same as those of the Fc receptor of plasma membranes. It is now clear that the initial event in immune-complex binding to the Fc receptor delimits many important properties of superoxide generation, which is the result of complex biological reactions in polymorphonuclear leucocytes.

Finally, it is appropriate to discuss the results of Korchak *et al.* (1980) on the inhibitory effect of hyperosmolarity. They studied the effects of permeant anions on degranulation and superoxide generation of human neutrophils, with special reference to the 'chemiosmotic-swelling' hypothesis for degranulation proposed by Pollard and his colleagues (Pollard *et al.*, 1977; Pazoles & Pollard, 1978). The finding of Korchak *et al.*, (1980) that increase of salt concentration outside the cells inhibits superoxide generation is apparently consistent with our results. However, their basic idea that flux of anions plays a crucial role in degranulation and other functions of neutrophils is not consistent with our finding that the concentration of salts (not the gradient of salts) is important for immune-complex binding and subsequent biological activities. This was unequivocally shown in assays with plasma membranes. Thus the use of plasma membranes may be essential in further studies on the mechanisms of the effects of anions on the cellular defence mechanism.

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