J. Membrane Biol. 44, 321–330 (1978)

Metabolic Stimulation of Polymorphonuclear Leucocytes: Effects of Tetravalent and Divalent Concanavalin A

D. Romeo*, G. Zabucchi, G. Berton, and C. Schneider

Departments of Biochemistry and General Pathology, University of Trieste, 34127 Trieste, Italy

Received 15 May 1978; revised 14 August 1978

Summary. Polymorphonuclear leucocytes (PMNL) undergo a marked activation of their oxidative metabolism upon interaction with surface-reactive soluble stimuli as well as with phagocytosable objects. To get some insight into the mechanism of this stimulation, we have compared the stimulatory activity of the tetravalent lectin concanavalin A (Con A) with that of the divalent derivative succinyl-Con A (S-Con A). Both lectins bind to the PMNL surface to the same extent. S-Con A, however, is much less efficient in stimulating the PMNL metabolism. When S-Con A-treated PMNL are further reacted with antiserum to Con A, a potentiation of the metabolic stimulation is observed. Normal serum or addition to PMNL of antiserum to Con A in the absence of lectin has no effect. Furthermore, if S-Con A is displaced from its receptors on the cell membrane with α-methyl mannopyranoside, the addition of antiserum fails to cause a respiratory stimulation. These results suggest that the initial triggering of the metabolic stimulation of PMNL is in part accomplished through cross-linkage of membrane constituents.

A perturbation of the surface of cells, such as that due to the binding of a ligand to specific receptors of the plasma membrane [3, 11, 12, 33], may lead to an alteration of intracellular metabolic pathways. A well-known example of cell response to surface perturbation is the stimulation of the oxidative metabolism of the polymorphonuclear leucocytes (PMNL) provoked by their interaction with a variety of surface-reactive stimuli as well as with phagocytosable objects [1, 8, 17, 19–27, 29, 32, 35]. The mechanism by which the perturbation of the PMNL surface gives rise to the metabolic activation is not clear. After studying the stimulatory activity of multivalent ligands such as antibodies against PMNL surface antigens and either soluble or immobilized concanavalin A (Con A) [19, 22, 27], we suggested that the triggering of the metabolic activation of

* *To whom reprint requests should be addressed:* Istituto di Chimica Biologica, Università di Trieste, 34127 Trieste, Italy.

0022-2631/78/0044-0321 $02.00

PMNL is not merely provided by the ligand-receptor interaction but by a surface redistribution of the ligand-receptor complexes [20, 23]. Grounds for support of this hypothesis are furnished by the observation that receptor crosslinkage may play a role in the effects of some ligands on cells. In fact, fragments of antibodies or lectin derivatives with reduced valency, but with unaltered receptor binding properties, usually induce modified cell responses with respect to the parent molecule [5–7, 11, 18]. For example, divalent succinyl-Con A stimulates lymphocyte growth but, unlike the parent tetravalent lectin, shows no inhibition of mitogenesis [6, 7, 33]. Unlike Con A, succinyl-Con A also fails to inhibit virus release from infected cells [18]. Furthermore, while Con A inhibits growth of chick embryo fibroblasts after release from serum starvation, succinyl-Con A does not exhibit this inhibitory effect [11].

To gain some evidence of a possible role of receptor cross-linking in the activation of the PMNL oxidative metabolism, as suggested by our hypothesis [20, 23], we have now compared the stimulatory efficiency of tetravalent Con A and divalent succinyl-Con A. In addition, since it is known that the surface mobility of bound Con A is greatly affected by anti-Con A antibodies [31], we have studied the effect of an anti-Con A serum on the metabolism of PMNL prelabeled with either succinyl-Con A or Con A.

Materials and Methods

Lectins

Con A was purchased from Sigma Chemical Co. and [^{3}H(G)] Con A (38.8 Ci/mmol) from New England Nuclear. Succinyl-Con A (S-Con A) was prepared from native Con A as described by Gunther *et al.* [6], lyophilized and dissolved before use in 0.154 M NaCl. Labeled S-Con A was obtained by succinylation of a mixture of Con A (20 mg) and [^{3}H] Con A (20 μCi). The protein concentration of the lectin solutions was estimated by the method of Lowry *et al.* [10], with bovine serum albumin as standard.

When subjected to polyacrylamide gel electrophoresis, performed according to the method described by Davis [4], S-Con A showed a major sharp band and a very minor faint one with lower mobility, corresponding to that of the bulk of native Con A. Densitometric scanning revealed that this minor band accounted for less than 2% of the total protein of the samples.

Comparative assays of mitogenic properties of Con A and S-Con A carried out with human blood lymphocytes provided results similar to those reported by Wang *et al.* [33] and by Hadden *et al.* [7].

The anti-Con A goat serum (Miles-Yeda) contained 2.7 mg antibody per ml; before being used, it was extensively dialyzed against isotonic NaCl at 4 °C to get rid of merthiolate, which inhibits PMNL oxidative metabolism. Under all the experimental conditions employed, the complex formed between the lectin and its antibody did not form a visible precipitate.

Cell Metabolism

Guinea pig peritoneal exudate cells were obtained as previously described and suspended in calcium-free Krebs-Ringer phosphate (KRP) [34]. As judged by differential counts of May-Grünwald-Giemsa stained smears, PMNL were more than 90% of the total white cells.

PMNL respiration was monitored at 37 °C with a Clark-type oxygen electrode [21, 22, 25, 27, 34, 35]. The oxygen consumption by untreated PMNL is inhibited by cyanide, whereas the stimulated respiration is insensitive to this inhibitor [20, 23, 29]. To better show the respiratory enhancement, we thus incubated the cells for 5 min with 1 mM KCN before their exposure to the stimulants. These were directly added to the cell suspensions in the electrode vessel [34], and, as reported in previous publications [20, 22], maximal stimulation of the rate of PMNL respiration was observed within about 1 min after their addition to the cells.

The activity of the hexose monophosphate (HMP) pathway was assayed by measuring the production of $^{14}CO_2$ from 1-^{14}C-glucose [22], which was added to PMNL suspended in KRP containing 0.2 mM glucose, in the absence or in the presence of either Con A or S-Con A. The increased yield of $^{14}CO_2$ from 1-^{14}C-glucose was proved to be due to the activation of the HMP pathway and not to increased glucose uptake after establishing that the rate of oxidation of 6-^{14}C-glucose was not modified by the exposure of PMNL to the lectins [22].

Lectin Binding to the Cells

Either [^{3}H] Con A plus 80 μg unlabeled Con A (1850 cpm/μg) or 80 μg [^{3}H] S-Con A (400 cpm/μg) were dissolved in 0.65 ml KRP, containing 0.2 mM glucose and 1 mM KCN, and incubated at 0 °C in plastic vials. 1×10^7 PMNL in 0.35 ml of the same medium were added to the lectin solutions; after 10 min at 0 °C the suspensions were diluted with 9 ml of cold KRP and PMNL sedimented (200 g, 5 min). After two washings with KRP, the pellets were dissolved in 5 ml of Aqualuma (Lumac System AG) and counted in a Beckman LS-230 liquid scintillation counter. The low temperature for the binding assays was chosen to minimize cell aggregation and possible trapping of free lectin molecules within the cell aggregates. Specific binding was determined by the difference between total binding of Con A or S-Con A and binding obtained in the presence of 0.1 M α-methyl mannopyranoside (α-MM, Sigma Chemical Co.).

Cell aggregation was monitored by light microscope observation of PMNL incubated at 37 °C for 4 min with either Con A or S-Con A (100 μg/ml) and then diluted with a solution of trypan blue (0.1% in isotonic NaCl).

Results

Activation of PMNL Oxidative Metabolism

As shown in Fig. 1, at any concentration tested S-Con A appears to be much less efficient in stimulating the oxygen uptake by PMNL than the parent lectin. Similar results are found when comparing the stimulatory activity of Con A and S-Con A on the rate of 1-^{14}C-glucose oxidation through the HMP pathway of PMNL (Fig. 2).

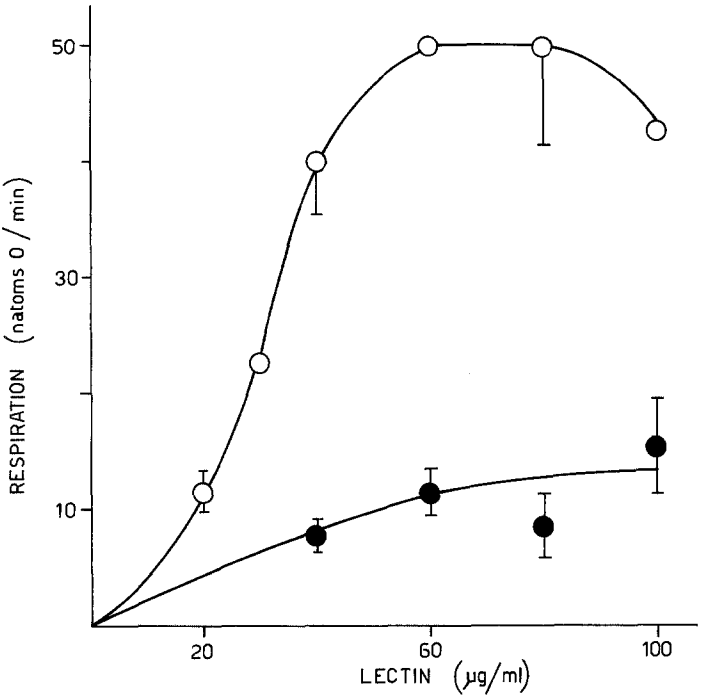

Fig. 1. Dose-response curve of stimulation of PMNL respiration by concanavalin A (—○—) and by succinyl-concanavalin A (—●—). The rate of oxygen consumption by 2×10^7 guinea-pig PMNL, suspended in KRP with 0.2 mM glucose and 1 mM KCN, was monitored at 37 °C with a Clark-type electrode. The rate of respiration of untreated cells, which was less than 15 natoms 0/min, was subtracted from that of stimulated cells. The points are means of at least three experiments ±SEM

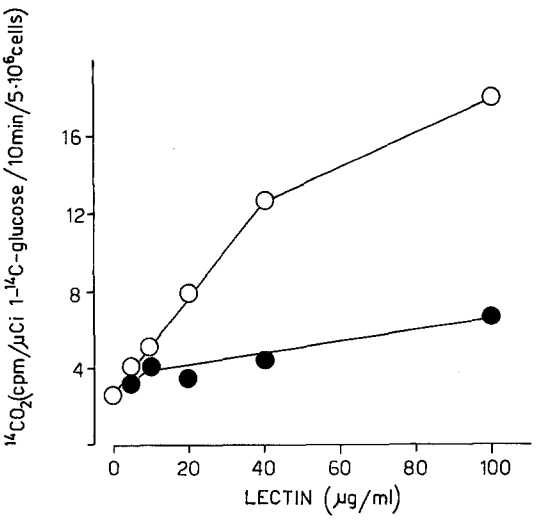

Fig. 2. Dose-response curve of stimulation of 1-^{14}C-glucose oxidation by guinea-pig PMNL treated with concanavalin A (—○—) or succinyl-concanavalin A (—●—). 5×10^6 PMNL in 1 ml of KRP with 0.2 mM glucose were incubated at 37 °C; the lectins and 0.25 μCi of 1-^{14}C-glucose were added, and the incubation was continued for additional 10 min. $^{14}CO_2$ was trapped in KOH and counted by liquid scintillation [22]

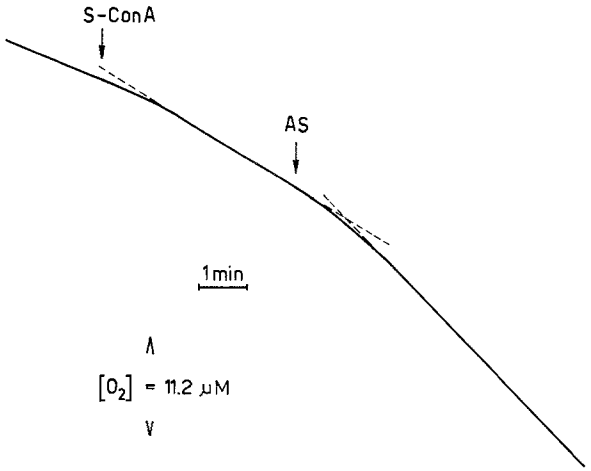

Fig. 3. Polarographic trace of oxygen consumption by PMNL. 2×10^7 guinea-pig PMNL in 2 ml of KRP with 0.2 mM glucose and 1 mM KCN (37 °C) were stimulated with S-Con A (80 µg/ml), followed by anti-Con A serum (AS, 27 µg antibody/ml). Tangential lines (interrupted) were drawn to better show the change in the rate of cell respiration

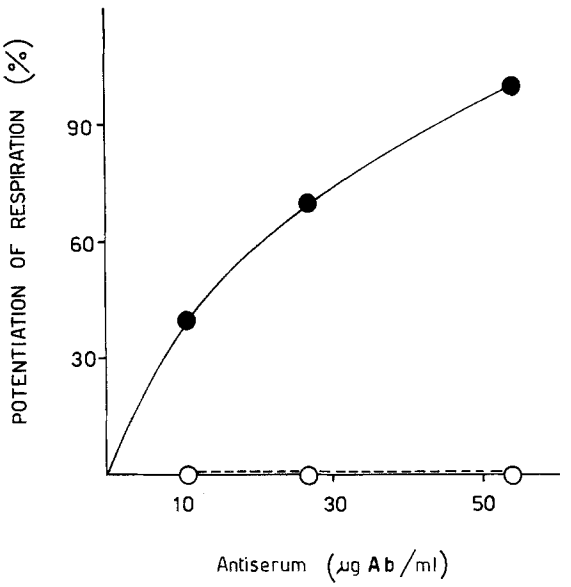

Fig. 4. Potentiation of respiration of PMNL treated with concanavalin A (—○—) or succinyl-concanavalin A (—●—) by anti-Con A serum. 2×10^7 guinea-pig PMNL in 2 ml of KRP with 0.2 mM glucose and 1 mM KCN (37 °C) were stimulated with 80 µg of Con A or S-Con A; when cell respiration reached a steady rate (*see* Fig. 3), the anti-Con A serum was added. The potentiation of PMNL respiration is expressed as % increase with respect to the rate of respiration recorded before the addition of the anti-Con A serum

Table 1. Effect of anti-Con A serum on PMNL respiration stimulated by S-Con A or Con A

Lectin concentration (μg/ml)		Rate of respiration (oxygen natoms/min)	
		Before AS	After AS
S-Con A	40	8.0	15.2
	60	12.0	18.8
	80	14.3	32.2
	120	17.9	32.6
	180	26.0	41.2
Con A	10	3.6	9.8
	20	9.8	17.9
	40	32.2	32.2
	60	45.7	45.7
	80	63.6	63.6

2×10^7 guinea-pig PMNL in 2 ml of KRP with 0.2 mM glucose and 1 mM KCN were stimulated at 37 °C with either S-Con A or Con A. When cell respiration reached a steady rate, anti-Con A serum (AS) was added at a final concentration of 27 μg of antibody/ml. Data are means of two experiments (the rates of respiration of untreated cells were subtracted).

Since it is known that the biological activity of divalent Con A is increased after reaction with anti-Con A antibody [6, 30], PMNL treated with S-Con A were further exposed to anti-Con A serum. By increasing the amount of antibody added to the S-Con A treated cells, a gradual potentiation of the respiratory stimulation is observed (Figs. 3 and 4). When Con A replaces its succinyl derivative (at the same concentration of 40 μg/ml), the anti-Con A serum does not produce any potentiation of the stimulus (Fig. 4). As shown in Table 1, an increase of the stimulatory effect of Con A by the antiserum occurs only at low lectin concentrations, whereas a potentiation of S-Con A stimulatory activity by antibody is obtained in a wide range of concentrations of the divalent ligand in the medium.

That the observed potentiation of the PMNL respiration by anti-Con A serum is due to a specific interaction between antibodies and surface-bound lectins, was confirmed by three types of control experiments. First, the addition of antiserum to cells in the absence of lectins has no effect on the basal respiration of PMNL. Second, comparable amounts of normal goat serum do not increase the respiratory stimulation exerted by S-Con A. Third, when S-Con A or Con A are displaced

from the cell surface by α-MM, a procedure which rapidly abolishes the respiratory stimulation [20, 22], the subsequent addition of antiserum does not modify the cell metabolism.

Lectin Binding

PMNL incubated at 0 °C for 10 min with either the tetravalent or the divalent lectin (80 μg/ml) bind 0.47 ± 0.12 μg Con A/1×10^7 cells and 0.48 ± 0.07 μg S-Con A/1×10^7 cells, respectively (means of three determinations ±SEM; difference of the means not significant by Student's "*t*" test). This result is consistent with the unaltered carbohydrate binding properties of S-Con A reported by others [6, 18, 30].

An evaluation of cell aggregation by lectins offers an indirect indication of their ability to cross-link receptors on the cell surface [6, 30]. At 100 μg/ml and 37 °C both Con A and S-Con A cause aggregation of PMNL, as judged by light microscope observation. In the presence of the divalent lectin, however, the aggregates are smaller and about five times fewer cells are agglutinated as in the presence of Con A.

Discussion

Succinylation of tetravalent Con A produces a dimeric lectin with unmodified carbohydrate binding properties but with altered biological activities [6, 11, 18, 30, 33]. The S-Con A derivative is capable of binding to PMNL membrane and occupies the same number of surface receptor sites as the intact molecule. However, S-Con A has reduced capacity to agglutinate PMNL as compared to intact Con A, and causes a stimulation of the PMNL oxidative metabolism, whose intensity is much lower than that exerted by tetravalent Con A. Addition of anti-Con A serum to PMNL previously reacted with S-Con A, greatly potentiates the respiratory activation produced by the lectin alone. The effect of the serum is due to the presence of specific antibodies against Con A and can be abolished by dislodging the lectin from its surface binding sites by means of α-methyl mannopyranoside. We thus conclude that the metabolic activation of PMNL is triggered by lectin molecules bound at the exterior surface of the cell [20], and that the potentiation of the metabolic activation by anti-Con A serum is caused by the interaction between the antibody and receptor-bound lectins.

Con A-receptor complexes are known to aggregate on the cell surface with a reduction in their lateral mobility [6, 22, 28, 30, 31]. Schlessinger *et al.* [31] have shown that the rate of lateral transport decreases more rapidly the higher the Con A dose and that S-Con A-receptor complexes are very mobile, their mobility being rapidly abolished by anti-Con A antibodies.

These observations might be utilized to postulate that also on the surface of PMNL the S-Con A-receptor complexes incur little aggregation and that the metabolic stimulation of PMNL by ligands such as Con A is proportional to the extent of receptor cross-linkage. The marked potentiation of the metabolic activation of S-Con A-treated PMNL by anti-Con A antibodies is consistent with this assumption.

In this view, the failure of antibodies to modify the respiration of PMNL reacting with tetravalent Con A at concentrations $\geqq 20$ μg lectin/ml might be ascribed to at least two events. First, concentrations of Con A $\geqq 20$ μg/ml might produce a rapid and extensive formation of clusters of Con A-receptor complexes, whose topographical distribution on the PMNL surface would not allow further aggregation by the antibody molecules. Secondly, Con A, which agglutinates PMNL more efficiently than S-Con A, might be particularly concentrated in regions of interactions between cells, thus being not accessible to the antibody. In both cases, further addition of Con A would cross-link still uncomplexed receptors and, in contrast to the antiserum, increase the metabolic activation.

It is possible that surface aggregation of macromolecules might also provide the initial trigger of metabolic activation of PMNL by other stimulants such as phospholipase C [17], La^{3+} [21], Ca^{2+} ionophores [21, 23], amphipathic molecules [8, 35] or phagocytosable objects [1, 23, 24, 26, 29]. In fact, phospholipid splitting and charge neutralization by appropriate counterions are known to cause an aggregation of negatively charged macromolecules in the plane of the membrane [13, 14]. Furthermore, an increase in cytosol concentrations of Ca^{2+} may cause sequestration of proteins into specific membrane domains by promoting clusters of membrane lipids [14, 15]. Finally, phagocytosis alters the surface topography of PMNL as indicated by a rapid decrease in plasma membrane viscosity, probably linked to a lipid phase separation, and by a specific redistribution of lectin receptors [2, 16].

This work was supported by grants from the National Research Council of Italy, CNR (Nos. 76.01498.04 and 76.01317.04).

Note Added in Proof

T. Yasaka and T. Kambara have recently shown that S-Con A is about one half as active as the parent lectin in stimulating the respiration of guinea pig PMNL (Fig. 1, *Biochim. Biophys. Acta* **508**:306, 1978). In their experiment they have used a rather high lectin/cell ratio (100 μg Con A or S-Con A and 4.4×10^6 PMNL/ml), i.e., a concentration of Con A corresponding to the descending limb of the dose-response curve. Their conclusion that the valency of the lectin is not essential for the metabolic activation appears, therefore, to have no grounds.

References

1. Babior, B.M., Kipnes, R.S., Curnutte, J.T. 1973. Biological defense mechanisms: The production by leukocytes of superoxide, a potential bactericidal agent. *J. Clin. Invest.* **52**:741
2. Berlin, R.D., Fera, J.P. 1977. Changes in membrane microviscosity associated with phagocytosis: Effects of colchicine. *Proc. Nat. Acad. Sci. USA* **74**:1072
3. Burger, M.M., Noonan, K.D. 1970. Restoration of normal growth by covering of agglutinin sites on tumour cell surface. *Nature (London)* **228**:512
4. Davis, B.J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**:404
5. Fanger, M.W., Hart, D.A., Wells, J.V., Nisonoff, A. 1970. Requirement for cross-linkage in the stimulation of transformation of rabbit peripheral lymphocytes by antiglobulin reagents. *J. Immunol.* **105**:1484
6. Gunther, G.R., Wang, J.L., Yahara, I., Cunningham, B.A., Edelman, G.M. 1973. Concanavalin A derivatives with altered biological activities. *Proc. Nat. Acad. Sci. USA* **70**:1012
7. Hadden, J.W., Hadden, E.M., Sadlik, J.R., Coffey, R.G. 1976. Effects of concanavalin A and a succinylated derivative on lymphocyte proliferation and cyclic nucleotide levels. *Proc. Nat. Acad. Sci. USA* **73**:1717
8. Kakinuma, K. 1974. Effects of fatty acids on the oxidative metabolism of leukocytes. *Biochim. Biophys. Acta* **348**:76
9. Loor, F., Forni, L., Pernis, B. 1972. The dynamic state of the lymphocyte membrane. Factors affecting the distribution and turnover of surface immunoglobulins. *Eur. J. Immunol.* **2**:203
10. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265
11. McClain, D.A., D'Eustachio, P., Edelman, G.M. 1977. Role of surface modulating assemblies in growth control of normal and transformed fibroblasts. *Proc. Nat. Acad. Sci. USA* **74**:666
12. Mirelman, D., Galun, E., Sharon, N., Lotan, R. 1975. Inhibition of fungal growth by wheat germ agglutinin. *Nature (London)* **256**:414
13. Nicolson, G.L. 1973. Anionic sites of human erythocyte membranes. I. Effects of trypsin, phospholipase C, and pH on the topography of bound positively charged colloidal particles. *J. Cell Biol.* **57**:373
14. Nicolson, G.L. 1976. Transmembrane control of the receptors on normal and tumour cells. I. Cytoplasmic influence over cell surface components. *Biochim. Biophys. Acta* **457**:57
15. Ohnishi, S., Ito, T. 1974. Calcium-induced phase separations in posphatidylserine-phosphatidylcholine membranes. *Biochemistry* **13**:881
16. Oliver, J.M., Ukena, T.E., Berlin, R.D. 1974. Effect of phagocytosis and colchicine on the distribution of lectin-binding sites on cell surfaces. *Proc. Nat. Acad. Sci. USA* **71**:394

17. Patriarca, P., Cramer, R., Marussi, M., Moncalvo, S., Rossi, F. 1971. Phospholipid splitting and metabolic stimulation in polymorphonuclear leukocytes. *J. Reticuloendothel. Soc.* **10**:251
18. Poste, G., Alexander, D.J., Reeve, P., Hewlett, G. 1974. Modification of Newcastle disease virus release and cytopathogenicity in cells treated with plant lectins. *J. Gen. Virol.* **23**:255
19. Romeo, D., Jug, M., Zabucchi, G., Rossi, F. 1974. Perturbation of leukocyte metabolism by nonphagocytosable concanavalin A-coupled beads. *FEBS Lett.* **42**:90
20. Romeo, D., Zabucchi, G., Jug, M., Miani, N., Soranzo, M.R. 1975. Concanavalin A as a probe for the study of the mechanism of metabolic stimulation of leukocytes. *In:* Concanavalin A. T.K. Chowdhury and A.K. Weiss, editors. p.273. Plenum, New York
21. Romeo, D., Zabucchi, G., Miani, N., Rossi, F. 1975. Ion movement across leukocyte plasma membrane and excitation of their metabolism. *Nature (London)* **253**:542
22. Romeo, D., Zabucchi, G., Rossi, F. 1973. Reversible metabolic stimulation of polymorphonuclear leucocytes and macrophages by concanavalin A. *Nature New Biol.* **243**:111
23. Romeo, D., Zabucchi, G., Rossi, F. 1977. Surface modulation of oxidative metabolism of polymorphonuclear leucocytes. *In:* Movement, Metabolism and Bactericidal Mechanisms of Phagocytes. F. Rossi, P. Patriarca and D. Romeo, editors. p. 153. Piccin Medical Books, Padova
24. Root, R.K., Metcalf, J., Oshino, N., Chance, B. 1975. H_2O_2 release from human granulocytes during phagocytosis. I. Documentation, quantitation, and some regulating factors. *J. Clin. Invest.* **55**:945
25. Rossi, F., Romeo, D., Patriarca, P. 1972. Mechanism of phagocytosis-associated oxidative metabolism in polymorphonuclear leucocytes and macrophages. *J. Reticuloendothel. Soc.* **12**:127
26. Rossi, F., Zatti, M. 1966. Effect of phagocytosis on the carbohydrate metabolism of polymorphonuclear leucocytes. *Biochim. Biophys. Acta* **121**:110
27. Rossi, F., Zatti, M., Patriarca, P., Cramer, R. 1971. Effect of specific antibodies on the metabolism of guinea pig polymorphonuclear leucocytes. *J. Reticuloendothel. Soc.* **9**:67
28. Ryan, G.B., Borysenko, J.Z., Karnovsky, M.J. 1974. Factors affecting the redistribution of surface-bound concanavalin A on human polymorphonuclear leukocytes. *J. Cell Biol.* **62**:351
29. Sbarra, A.J., Karnovsky, M.L. 1959. The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. *J. Biol. Chem.* **234**:1355
30. Schekman, R., Singer, S.J. 1976. Clustering and endocytosis of membrane receptors can be induced in mature erythrocytes of neonatal but not adult humans. *Proc. Nat. Acad. Sci. USA* **73**:4075
31. Schlessinger, J., Koppel, D.E., Axelrod, D., Jacobson, K., Webb, W.W., Elson, E.L. 1976. Lateral transport on cell membranes: Mobility of concanavalin A receptors on myoblasts. *Proc. Nat. Acad. Sci. USA* **73**:2409
32. Tedesco, F., Trani, S., Soranzo, M.R., Patriarca, P. 1975. Stimulation of glucose oxidation in human polymorphonuclear leukocytes by C3-Sepharose and soluble C567. *FEBS Lett.* **51**:232
33. Wang, J.L., McClain, D.A., Edelman, G.M. 1975. Modulation of lymphocyte mitogenesis. *Proc. Nat. Acad. Sci. USA* **72**:1917
34. Zabucchi, G., Romeo, D. 1976. The dissociation of exocytosis and respiratory stimulation in leukocytes by ionophores. *Biochem. J.* **156**:209
35. Zatti, M., Rossi, F. 1967. Relationship between glycolysis and respiration in surfactant treated leucocytes. *Biochim. Biophys. Acta* **148**:553