INDUCTION OF VACUOLATION IN THE MOUSE PERITONEAL MACROPHAGE BY CONCANAVALIN A

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1. Introduction

Surface macromolecules appear to be randomly distributed in the membranes of lymphocytes, and other cell types grown in tissue culture. Upon binding of specific ligands such as antigens or of non-specific ligands such as multivalent antibodies and plant lectins, they undergo a striking redistribution, the sequence of which involves passive clustering into patches and sometimes active coalescence to form a 'cap' at one pole of the cell. Following this redistribution the networks of interacting surface immunoglobulin molecules and antibodies and those of lectin binding sites and lectins are pinocytosed [1–4]. Huet and Bernhard [4] infer differences in the surface movements of normal and virus transformed cells in culture from the rate of disappearance of Con A/ peroxidase label from the cells. Measurements of radioactive Concanavalin A (Con A) showed that most of the lectin penetrated into the cell and that the shedding of labelled cell coat material into the medium was minimal [4]. Karsenti and Avrameas [5] report a rapid disappearance of Con A molecules from the surface membranes of lymphocytes, possibly as a result of induced pinocytosis.

Con A was shown [6] to bind to mouse peritoneal macrophages in a specific and dose dependent way. Romeo et al. [7] have shown that upon interaction with Con A rabbit alveolar macrophages undergo metabolic stimulation parallel to that invoked during phagocytosis. The authors speculate that formation of patches of crosslinked mobile glycoprotein receptors may trigger the onset of the metabolic stimulation.

The above observations, and the notion that cell surface phenomena induced in different cell types upon interaction with antigens, antibodies and lectins exhibit possibly one related general principle prompted the present investigation into the nature of the interaction of Con A with macrophages.

The following sequence of events is suggested from morphological as well as binding studies with tritium labelled Con A: a) surface binding of Con A; b) an energy dependent formation of numerous small Con A containing pinocytotic vesicles; c) intracellular fusion of vesicles to form vacuoles of a remarkable size occupying most of macrophage cytoplasm, and d) vacuole disappearance in a 48 hr period and regeneration of normal morphology.

2. Methods

2.1. *Collection and cultivation of macrophages*

Unstimulated peritoneal macrophages were aseptically collected from BALB/C strain mice, weighing 20–25 g, following essentially the method of Cohn and Benson [8]. Peritoneal exudate cells suspended in Dulbecco's modified Eagle's medium were allowed to attach (1 hr, 37°C) to 25 mm diameter cover glasses (0.5×10^6 mononuclear cells, 0.1 ml) placed in 35 × 10 mm Falcon plastic tissue culture dishes (Falcon Plastics, Div. of Bioquest, Oxnard, Calif.) or directly to the plastic dish at a concentration of 2×10^6 mononuclear cells per dish. The dish was washed in phosphate buffered saline (PBS) to remove non-adhering cells and 2 ml of tissue culture medium consisting of Dulbecco's modified Eagle's medium, 200 units/l of penicillin, 200 μg/l of streptomycin (to be called 'medium') and 20% of heat inactivated new born calf serum (NBCS) (obtained from Grand Island Biological Co., New York) were added. Adhering cells were then incubated for 24–48

hr in a Co_2-incubator (5% CO_2 –air mixture). Lymphocytes were detached from the culture during the incubation. Differential cell counts revealed that only about 40–50% of the peritoneal exudate cells were macrophages.

2.2. *Con A*

Con A, twice crystallized was obtained from Miles-Yeda (Rehovot, Israel). Acetylation of the lectin with ^{3}H-acetic anhydride [9] yielded ^{3}H-Con A with a specific activity of 6.5×10^6 cpm/mg.

2.3. *^{3}H-Con A binding assay*

Cultivated macrophages ($\sim 1 \times 10^6$ cells) were washed 3 times with PBS and then interacted with ^{3}H-Con A–PBS solutions for 30 min at 24°C. Four washes with PBS followed and then the cells were either incubated (30 min, 24°C) in PBS containing 0.1 M of α-methyl-D-mannoside (αMM-PBS), a specific Con A low molecular weight inhibitor, to remove surface bound ^{3}H-Con A, or incubated for 90 min with medium at 37°C preceding the incubation in αMM-PBS. Both the medium and the αMM-solutions were collected after the respective incubations and counted. Radioactivity measures the amount of Con A bound to the surface membrane. ^{3}H-Con A associated with cells and not removed by incubation with αMM-PBS (internalized Con A) was dissolved with the cells from the plastic dish by use of 0.2% sodium dodecyl sulfate (SDS). Blank experiments were carried out in parallel to the various treatments with culture dishes incubated with 20% serum but devoid of cells. Con A binds to plastic dishes in both specific (αMM replaceable) and non-specific (SDS solubilized) way, a binding that is dose dependent and saturable. The corresponding blanks are corrected for.

2.4. *Morphological observations*

Macrophages cultivated on glass treated as specified in the text, were washed in PBS, fixed (1.5% glutaraldehyde, 0.1 M K-phosphate, pH 7.2, 15 min, 4°C), and stained (Giemsa). Photographs were taken with the Zeiss Ultraphot apparatus.

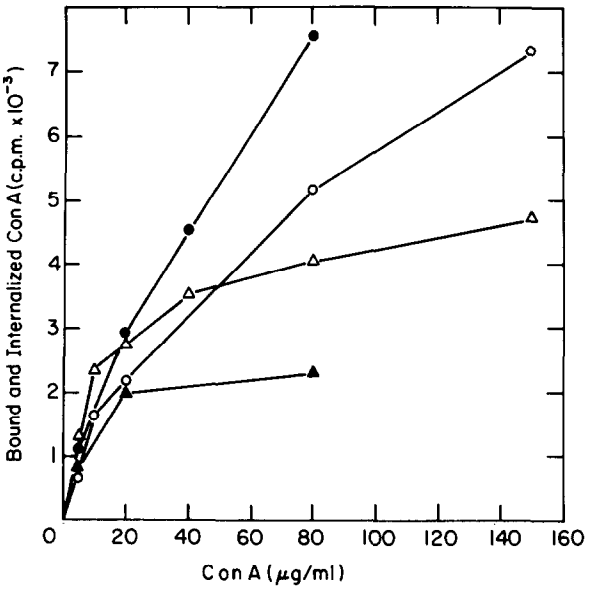


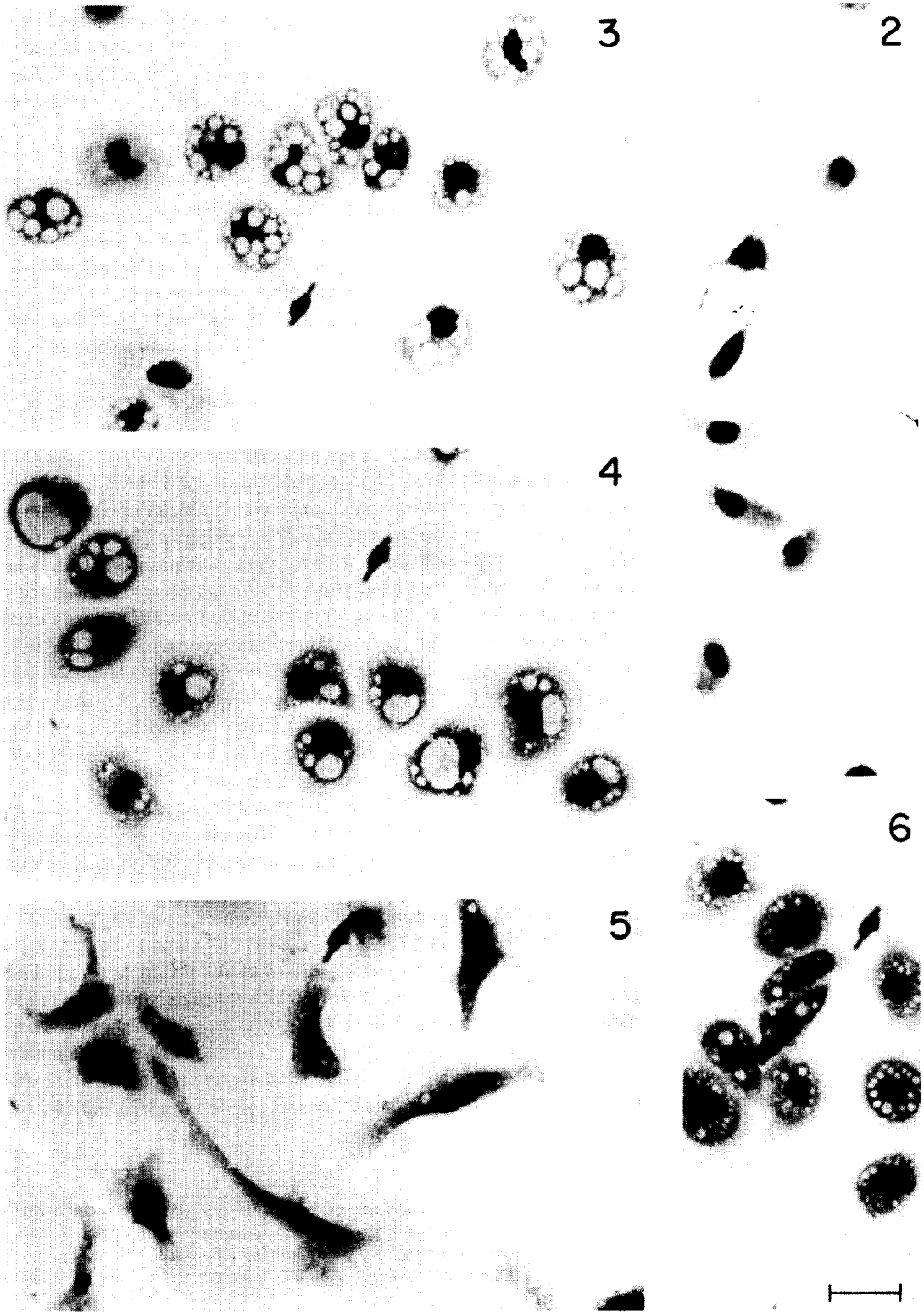
Fig. 1. Dose and time dependence of binding and internalization of Con A. (●——●) and (▲——▲) denote surface-membrane bound and internalized Con A after a 30 min exposure to the lectin. (○——○) and (△——△) denote surface membrane bound and internalized Con A in cultures that were incubated for 90 min, 37°C, in medium prior to αMM-PBS treatment. For experimental details see Methods.

3. Results and discussion

3.1. *Quantitative aspects of ^{3}H-Con A distribution in macrophages*

Several millions of specific binding sites for Con A can be titrated per macrophage. When binding studies are carried out at 24°C for 30 min a fraction of the lectin that becomes associated with the cells cannot be displaced by αMM (see fig. 1). This fraction of ^{3}H-Con A, to be referred to as internalized Con A, undergoes intracellular digestion and all the internalized label appears in the medium after an additional incubation period (checked at 24 hr). The fraction of associated ^{3}H-Con A that can be displaced by αMM, referred to as surface bound Con A, increases in a dose dependent way with the concentration of Con A in the incubation medium (fig. 1). The fraction of bound Con A can be internalized. Fig. 1 shows that an incubation of 90 min at 37°C in medium subsequent to the first binding step and PBS wash, results in a further internalization of ^{3}H-Con A.

The internalization during the two incubation steps described depends at least to a certain extent on energy derived from the respiratory chain. Sodium azide (3×10^{-3} and 10^{-2}M) added to the Con A incubation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mixture (after a 5 min pretreatment with NaN_3-PBS) results in a 45% inhibition in the Con A internalization. Addition of azide during the second incubation, results in a 20% inhibition in the overall internalization. Possible a longer azide pretreatment would have reduced pinocytosis even further.

3.2. *Morphological observations*

Cells incubated with Con A (24°C, 30 min) step one, when fixed and stained, do not exhibit unusual morphology (viewed under the light microscope) except for being slightly more spread. Some aggregation of cells is often observed apparently because cells move on the substratum and crosslinking via Con A might occur. Upon removal of Con A by PBS wash and incubation in medium (60 min, 37°C), step two, remarkable changes in the morphology of the cells take place (fig. 3). In a time dependent process, small pinosome like vesicles appear, and while moving centripetally they fuse and progressively become large vacuoles. At about 45–60 min the big vacuoles are already very pronounced (fig. 3). The periphery is abundant still with very small pinosomes. At a later stage (5 hr, fig. 4) the vacuoles occupy most of the cell volume and the nucleus is frequently displaced to the periphery. After 24 hr, vacuoles can still be detected, and after 48 hr of incubation in 20% serum, the macrophages recover from vacuoles, they do however increase in size as compared to the control (figs. 5 and 2 respectively). The process of vacuole enlargement is temperature dependent; incubation in medium or in 20% serum (serum has some positive effect on vacuole formation) at 24°C for 60 min results in the appearance of few vacuoles of average size. Incubation (60 min, 37°C) in PBS (no energy source supplied) does not enable a full exhibition of vacuole formation (fig. 6). The presence of 10^{-2} M of sodium azide in the Con A binding step does not inhibit Con A association with the cells to a significant extent (^{3}H-Con A labelling experiment). Thus, wash out of NaN_3 and a second step of incubation enables bound Con A to be internalized and vacuole formation is not inhibited. If cells are treated however with NaN_3 during the first binding step, incubated with αMM, and then incubated in medium, almost no vacuole formation can be observed, indicating that internalization during the first step was effectively inhibited by NaN_3 and suggesting that it is an energy requiring step. Control experiments show that the amount internalized during the first step suffices for extensive vacuole formation. Azide presence in the second incubation step has an additional effect the result of which is a generation of vacuoles of significantly smaller size. The results are in accord with the notion that pinocytosis depends on energy from the respiratory chain [10] and with observations that cap formation by lectins [4,11] and antibodies [1,3] is inhibited by azide.

Vacuole formation and size distribution are both time and dose dependent. Cells exposed to 40 μg/ml of Con A for 20 sec and then to a wash period (30 min, 24°C) with 0.1 M of αMM, developed already numerous small pinocytotic vesicles upon further incubation (90 min, 37°C medium). The vacuoles grow progressively with the time of exposure to Con A in the first incubation, other conditions being kept constant. At 20 and 30 min vacuole formation is very extensive and more so when αMM removable Con A is not washed out between the two incubation steps. Incubation for 20 min with 1 μg/ml of Con A and then a washout period in αMM, and step two incubation, is almost without effect on the cells. With 5 μg/ml the effect resembles that of 20 sec with 40 μg/ml.

Cells were 100% viable under conditions of extensive vacuolization. This conclusion was reached on the basis of several observations: a) Trypan Blue was excluded at any stage of the treatments; b) leucine incorporation into trichloroacetic acid insoluble material increased by 17% and by 23% as compared to control values when cells treated with 40 μg/ml of Con A were pulsed with ^{3}H-leucine for 20 hr immediately after the binding of Con A or 24 hr later, and c) glucoseamine incorporation as well as acid phosphatase levels were markedly higher in cultures recovering from Con A effects (to be published). The increase in metabolic activity of re-

Figs. 2–6. The effect of Con A on the morphology of macrophages. Macrophages on coverslips were washed 3 times in PBS and incubated (30 min, 24°C) either in PBS (fig. 2) or in 20 μg/ml of Con A in PBS (figs. 3–6). Cells were washed again 3 times in PBS, and incubated in a CO_2-incubator at 37°C as follows: fig. 2 - 60 min in PBS; fig. 3 - 60 min in Dulbecco's medium; fig. 4 - 5 hr in Dulbecco's medium; fig. 5 - 48 hr in 20% serum and fig. 6 - 60 min in PBS. The bar in fig. 6 represents 20 μ length. All figures are enlarged to the same extent.

covering macrophages, as compared to that of control cultures, might stem from the need for extensive membrane and lysosome regeneration.

4. Concluding remarks

Con A has been shown to interact with cell surface receptors on a variety of cell types, both normal and virus transformed malignant cells. The topographic distribution of Con A binding sites differs markedly in the different cell types studied, this notion being based on fluorescence [12] and electron microscopic observations [2,13] of either random distribution or the induction of clusters and caps of Con A binding sites by suitably labelled Con A. Differences in agglutinability of cells by Con A were also interpreted as due to topographic variations and hindrances to lateral movement of the binding sites [13].

Pinocytosis of clusters of Con A binding sites crosslinked by the lectin has been rather frequently observed [4,5]. No extensive vacuolation beyond the formation of pinosomes has to my knowledge been reported as yet. It is suggested that the induced vacuolation described in the present communication stems from pinosome formation as a first step in Con A internalization. Normally pinocytotic vesicles fuse with primary or secondary lysosomes upon movement towards the cell interior, their size distribution being rather constant [14]. The small pinosome like vesicles induced in macrophages by Con A do reach, however, remarkable dimensions, thus it seems that there is some interference with the dynamics of handling of incoming pinocytotic vesicles, and their normal turnover.

Several hypotheses can be put forward in an attempt to explain this phenomenon: a) The membranes of the pinocytotic vesicles induced by Con A may differ in composition from normal pinocytotic vesicles since clustering of Con A binding sites preceded the internalization. b) The fact that Con A is being internalized implies crosslinking of membrane components, such a perturbation could lead to increased susceptibility for fusion. c) Con A induced pinosome internalization could be at such a rate that the lysosomes of normal macrophages cannot cope with it. It was noticed that during 24–48 hr the vacuoles totally disappear. d) Lysosomal enzymes have been shown to be glycoproteins [15]; at least 5 lysosomal enzymes could be precipitated with Con A from supernatants of frozen–thawed lysosomal preparations [16], without loss of catalytic activity. There exists therefore the possibility that upon fusion of Con A bearing pinocytotic vesicles primary lysosomes, lysosomal enzymes interact with the lectin. This could result in an interference with rates of digestion of the internalized components. e) Membrane components crosslinked via Con A might exhibit a higher resistance to lysosomal enzymes thus leading to reduced rates of its breakdown. Moreover, the lectin molecule could be relatively resistant to enzymic breakdown. f) The unstimulated macrophage has a very active undulating membrane a significant fraction of which can be internalized in a short time (approx. 30 min) during phagocytosis. In this respect the macrophage differs apparently from other cell types that have been studied. The ability to internalize at any time a relatively large fraction of cell membrane could be an additional reason for the formation of big vacuoles. It seems worth mentioning that preliminary experiments with thioglycolate stimulated macrophages indicate that while ^{3}H-Con A was internalized, formation of large vacuoles was not observed.

Several of the hypotheses put forward above as well as Con A distribution as visualized in the electron microscope with ferritin labelled Con A are currently under investigation.

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