EFFECT OF CONCANAVALIN A ON PHAGOCYTOSIS BY MACROPHAGES

Rachel GOLDMAN
Laboratory of Membranes and Bioregulation, The Weizmann Institute of Science, Rehovot, Israel

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1. Introduction

Macrophage surface receptors for Concanavalin A (Con A) were shown to be internalized during extensive phagocytosis carried out in the absence of Con A. About 32% of the receptors were internalized in a 1 hr period [1]. Berlin [2] looked at the possibility that free Con A receptors on the surface membrane are essential for the phagocytic process. He has shown that binding of Con A to rabbit polymorphonuclear leucocytes brought about a 50% inhibition in the association of latex beads with cell monolayers while it did not affect membrane transport of nonelectrolytes and adenine. It has been suggested [2] that the Con A effect can stem from either a prevention of adsorption of particles to specifie phagocytic sites or from prevention of internalization of membrane subsequent to particle-site interaction. The results favoured the second hypothesis though not unequivocally. In the preceding communication [3] it was shown that upon interaction of macrophages with Con A, the lectin is bound and internalized in minute pinocytic-like vesicles. The vesicles fuse and grow in size until they occupy (within 1-5 hr) most of the cell cytoplasm and the macrophages swell and round up. Washing off surface bound Con A by α methyl-D-mannoside (αMM), a low molecular weight competitor for the binding site [4], a few seconds after exposure of cell monolayers to Con A, does not reverse the effect. The experimental evidence suggests that Con A induces extensive membrane internalization. This internalization may deplete membrane reserves of the macrophage needed for the phagocytic process. To test this notion, a study of the effect of Con A on the two steps involved in phagocytosis; attachment and ingestion was undertaken. The association of latex beads with macrophages was markedly affected by Con A. Using turkey red blood cells (TE) it was possible to show that attachment of TE to macrophages treated with the lectin was somewhat impaired, while ingestion was significantly inhibited.

2. Materials and methods

2.1 *Macrophages*

Collected and cultivated as described in the preceding paper [3].

2.2 *Erythrocytes*

Freshly drawn turkey red blood cells (TE) were washed four times in PBS and resuspended in PBS containing 2.5% of heat inactivated new born calf serum (NBCS) at a concentration of $1-3 \times 10^8$ cells/ml. TE suspensions were aged by incubation at 37°C for 2 hr. ^{51}Cr labelled erythrocytes (TE-^{51}Cr) were obtained by adding 30–100 μCi/ml of $Na_2\ ^{51}CrO_4$ to suspensions of TE simultaneously with the ageing procedure.

2.3 *Procedure for the determination of attached and ingested TE^{51}-Cr*

Macrophages (2.5×10^5 per cover glass) were overlaid with 1 ml of 20% heat inactivated NBCS in Dulbecco's modified Eagle's medium (Grand Island Biological Co., New York) containing 5×10^6 TE-^{51}Cr. Macrophages and red blood cells were incubated for 90 min either 37°C in a CO_2-incubator (5% CO_2-air) or at 24°C in room atmosphere. Attachment and ingestion were separately measured by the following procedure. Macrophage monolayers were thoroughly washed in PBS and incubated for 45 sec in 1 ml of PBS

diluted 1/5 with water to effect lysis of bound but uningested TE. A second wash in 1 ml PBS was also collected. Radioactivity in the hypotonic lysate and the wash was a measure of TE bound to the surface of macrophages. Radioactivity that remained associated with the coverslips, taken as a measure of TE ingestion by the phagocytes, was released into the medium by 2 treatments with 1 ml of 0.2% SDS each. Radioactivity was measured in a gamma counter. The exact number of macrophages per cover glass, the labelling of TE and the extent of the ageing were not kept constant. The radioactivity determination provides therefore relative values of the attachment and ingestion of TE and permits comparison only within a particular experiment on the same day. Moreover, the results cannot reflect rates of adsorption or phagocytosis but only a final value, for the whole time interval. All determinations were made in triplicates. The whole experiment with slight variations was carried out 5 times.

2.4 *Morphological observations*

Coverslips of macrophages incubated with TE were rinsed thoroughly and then fixed in glutaraldehyde (1.5% in 0.1 M K-phosphate buffer, pH 7.2 at 4°C for 15 min) and stained (Giemsa). Surface bound TE kept their characteristic elongated morphology while ingested TE became rounded, often a few appeared together in one very big phagocytic vacuole. Their cytoplasm became yellow-greenish. Ingested and attached TE could be readily differentiated in the light microscope under oil immersion (X 100). 400 cells were counted in each slide. Photographs were taken with the Zeiss Ultraphot apparatus.

2.5 *Con A*

Con A, twice crystallized was obtained from Miles-Yeda (Israel). Experimental details of Con A binding and internalization are given in the legends to the table and figures.

3. Results and Discussion

Latex beads were most often used to assay phagocytosis [5]. Macrophages cultured on glass for 48 hr avidly phagocytosed latex beads, on the average 60 beads were associated per cell in the culture. The preceding communication suggests that one can manipulate the amount of surface membrane available for normal macrophage functions, such as phagocytosis, and possibly also change the distribution of membrane glycoproteins by exposing cell monolayers to Con A.

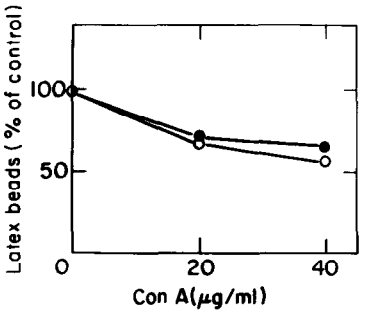


Fig. 1. Latex beads associated with cell monolayers. Expressed in % of control monolayers. Cell monolayers were incubated for 30 min at 24°C in PBS (control) or in Con A-PBS as specified, washed thoroughly and then (●—●) incubated for 90 min at 37°C with a latex suspension (10^8 latex beads/ml of 20% serum-medium). A second series (○—○) was incubated with 20% serum-medium for 60 min at 37°C prior to incubation with the latex suspension. Monolayers on cover glass were agitated in a series of three beakers of PBS and then the cells dissolved in 1 ml of 1% SDS. The number of latex beads (Bactolatex 0.81 μ diameter, Difco) was assessed by counting in a Hemacytometer. Blanks carried out with cover glasses undergoing the same treatment were corrected for. All determinations were carried out in triplicate, and are within a ±10% of the mean. Controls for the first and second series gave 12.7×10^6 and 15×10^6 latex particles per monolayer respectively.

The effect of Con A binding on the uptake of latex beads is shown in fig. 1. Incubation of macrophages with 40 μg/ml of Con A resulted in about 40% inhibition of latex association with cells in the culture. The effect was dose dependent and did not depend on the initial state of the vacuoles in the cells, i.e. when tested immediately after Con A treatment Con A was apparently internalized in minute pinocytotic vesicles, while when latex was applied after a one-hour incubation period at 37°C cells were already filled with large vacuoles.

Using latex particles it is difficult to quantitate and distinguish attached particles from those that have been ingested. Furthermore, the beads that were available were of a small diameter (0.81 μ) and thus needed relatively less membrane for engulfment than

particles of a large diameter would need. A particle with larger dimensions seemed favourable for establishing the details of the effect of Con A on phagocytosis by macrophages.

Macrophages were shown to phagocytose aged and glutaraldehyde treated red blood cells [5–7], and techniques for differentiating attachment from ingestion have been developed [8]. It seemed suitable therefore to use red blood cells as the challenge for phagocytosis of macrophages. The use of turkey red blood cells affords an additional advantage namely the cells are nucleated and nuclei are readily visualized by both light and electron microscopy.

Fresh turkey red blood cells, by all criteria foreign to the mouse macrophage, do not attach to the cell monolayers. After an aging period of about 1–2 hr in PBS or PBS + 2.5% NBCS at 37°C, macrophages are able to attach and ingest TE. The extent of either of these processes varied from experiment to experiment. Counting TE on cells, as described in methods, shows that about 70–90% of the mononuclear cells cultivated on glass have at least one TE attached, while only 40–60% of the cells show ingestion of at least one TE. Practically all the cells in the monolayer are phagocytic towards latex beads. The ability of macrophages under 7 different experimental conditions to attach and ingest TE is summarized in table 1.

Triplicate experiments under exact conditions exhibit

Table 1
Effect of Con A on attachment and ingestion of TE-^{51}Cr

Series	Treatment[a]			Attachment	Ingestion
	PBS±Con A 30 min, 24°C (μg Con A/ml)	20% serum 60 min, 37°C	TE-^{51}Cr 90 min, (°C)	cpm SD	cpm SD
1	–	–	24	27000 ± 6000	16352 ± 2831
	20	–	24	17929 ± 5400	6984 ± 3502
	40	–	24	16374 ± 2735	2469 ± 752
2	–	+	24	30375 ± 830	11689 ± 2533
	20	+	24	29009 ± 5200	8068 ± 721
	40	+	24	20147 ± 1759	3631 ± 639
3	αMM[b]	–	24	31689 ± 1048	9656 ± 1283
	20+αMM	–	24	36611 ± 1059	12321 ± 2548
4	–	αMM[c] 30 min, 24	24	29718 ± 6906	9630 ± 1416
	20		24	32334 ± 6803	7126 ± 1559
5	–	–	37	28386 ± 4450	19226 ± 2614
	20	–	37	29781 ± 3349	8426 ± 592
	40	–	37	24262 ± 2707	6563 ± 2189
6	–	+	37	25278 ± 2473	12460 ± 2337
	20	+	37	25226 ± 2203	8232 ± 856
	40	+	37	25223 ± 1577	5972 ± 731
7	–	48hr[d] 20%– serum	37	16474 ± 5015	9703 ± 924
	20		37	16629 ± 8137	8041 ± 787
	40		37	12758 ± 1846	9270 ± 683

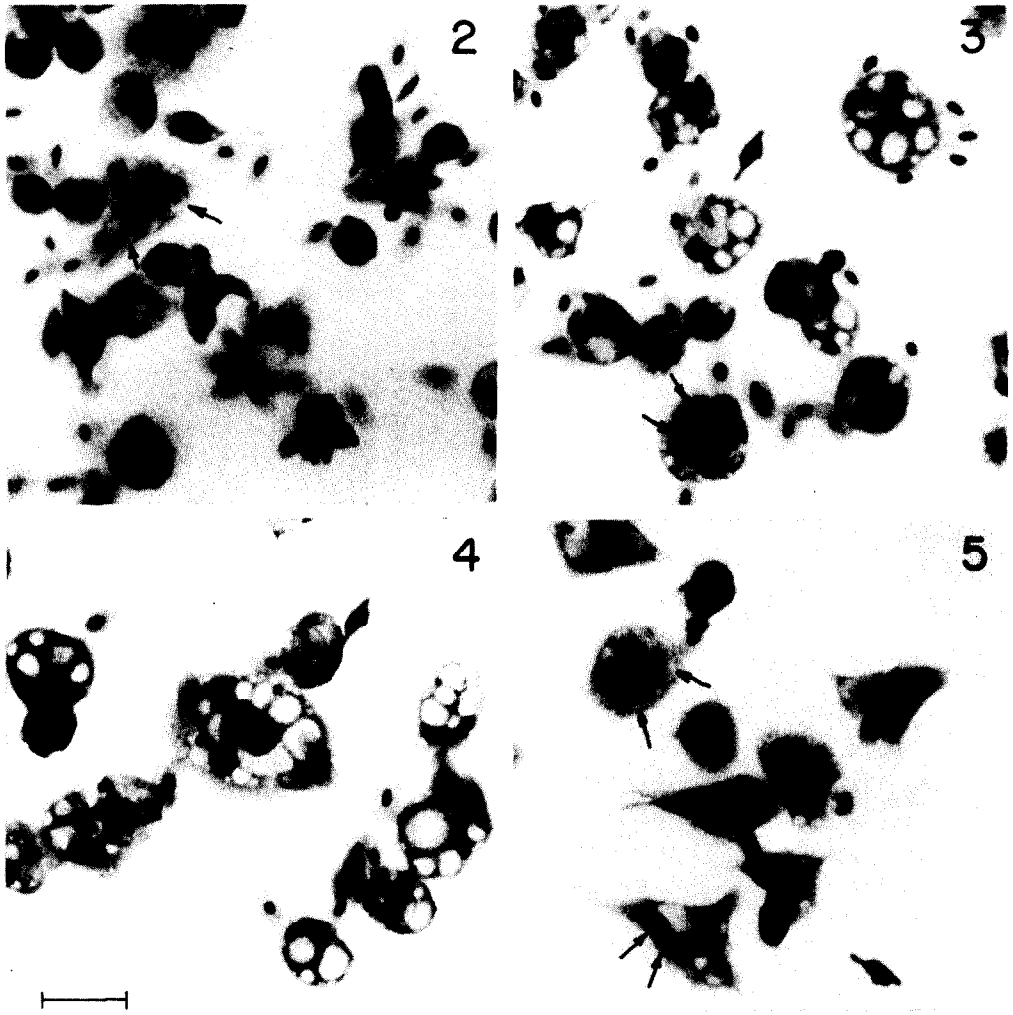
[a] The treatment consisted of 2 to 3 steps in the given order. For details see Experimental Section.

[b] 0.1 M of αMM was included.

[c] Monolayers were incubated in 0.1 M αMM in PBS after washing off the Con A.

[d] Cells were allowed to recover for 48 hr and then challenged with TE. Absolute values of counts cannot be compared with those of former series.

a relatively high standard deviation. Analysis of the effect of Con A on attachment of TE shows that there is a trend of decrease in attachment. There was quite a high ingestion in series 1 and 2 even though the temperature (24°C) was relatively low. This stems most probably from the fact that the assay was done over a rather long time interval (90 min). When incubation was limited to 30 min almost no ingestion could be traced. When added together with Con A to the cells, α-methyl-D-mannoside (αMM) abolishes the effect of Con A on both attachment and ingestion (series 3). When cells were treated with αMM after 30 min incubation with Con A, attachment of TE to the cells was back at its control value but the inhibition of ingestion could not be reversed (series 4). Exposure of Con A treated macrophages to TE suspensions for 90 min at 37°C resulted in an almost unaffected attachment as compared to control cultures while ingestion was inhibited 50–66% (series 5 and 6). Macrophages that have recovered morphologically



Figs. 2–5. Effect of Con A on attachment and ingestion of TE. Figs. 2 and 3 represent conditions given in table 1 series 5. Fig. 2 is that of the control and Fig. 3 is that of a monolayer treated with 20 μg/ml of Con A. Figs. 4 and 5 represent monolayers treated with 20 μg Con A conditions are those of series 6 and 7 respectively. The bar in fig. 4 represents 20 μ. All figures are enlarged to the same extent. The arrows point at examples of ingested TE.

from the effect of Con A (48 hr incubation) have an unimpaired attachment as well as ingestion (series 7). The experiments indicate a dose dependent inhibition by Con A of ingestion and possibly also of attachment. All the experiments indicated in the table (1–6) were carried out on the same day except for series 7 which was carried out 48 hr later.

Different experiments gave slightly higher values for inhibition of attachment especially in cases where ingestion was low. Similar results were obtained when 400 cells in each culture were counted for attached and ingested TE. Figs. 2–5 show the interaction of TE with macrophages. Cells not exposed to Con A (fig. 2) are small and exhibit both attachment and ingestion of TE. Macrophages that have bound Con A for 30 min at 24°C, have not yet developed big vacuoles. When overlaid immediately after Con A treatment with a suspension of TE the big vacuoles induced by Con A were formed preferentially to TE uptake, attachment was not impaired severely (fig. 3). A similar situation arose when the vacuoles were allowed to grow in size (60 min incubation at 37°C) prior to interaction with TE (fig. 4). The recovery of the phagocytic capacity after 48 hr in culture in 20% serum is shown in fig. 5. Several experiments indicate that the ingestion capacity might have even increased in cultures that have recovered from Con A treatment.

It is noteworthy that while treatment in hypotonic PBS lysed the TE and released ^{51}Cr in the solution, the nuclei of most of the attachment TE were apparantly stuck on the macrophage and not released by the procedure. This might indicate that the attachment of the plasma membrane of the TE involves invagination of the surface membrane to an extent that prevents nuclei from diffusing out from the site of attachment upon lysis of the TE. Validation of such a hypothesis must await electron microscope studies.

Previously [3] it was shown that only 25–35% of the bound Con A is being internalized during the half hour of exposure of cells to Con A. An additional amount of Con A can be internalized during a subsequent incubation in the absence of Con A. The effect that Con A has on attachment could possibly reflect a complex interplay of positive and negative parameters. Negative parameters such as inhibition of attachment due to site occupancy by the lectin and steric hindrance at the site, and positive parameters such as attachment via the lectin binding sites on both macrophages and TE. There is no doubt however that ingestion of TE is markedly impaired under all experimental conditions that allow a prior formation of the primary small pinocytotic vesicles.

Results are best explained by the following hypothesis: a certain fraction of the surface membrane of macrophages is available for phagocytosis and pinocytosis. Circumstances leading to utilization of these reserves bring about an apparent inhibition of any further membrane internalization. The inhibition is relieved after regeneration of the depleted membrane. Con A induces extensive vacuole formation with a concomitant rapid depletion of membrane reserves, phagocytosis is thus bound to be arrested. Such a situation should not affect so strongly surface attachment, in accord with experimental results.

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