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Wheat germ agglutinin potentiates uptake of bacteria by murine peritoneal macrophages

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Summary. Exposure of thioglycollate-elicited murine peritoneal macrophages to wheat germ agglutinin (WGA) increased markedly the uptake of six different bacteria, which have surface receptors for the lectin. Uptake of *Staphylococcus aureus* H was higher by 3–5-fold, of *S. aureus* 52A2 by 1·8-fold, of *S. aureus* 52A5 by 1·7-fold, of *S. albus* by 2·3-fold, of *Shigella flexneri* by 6-fold and of *Micrococcus luteus* by 6·5-fold. *Klebsiella pneumoniae*, devoid of receptors for WGA, was not phagocytosed following pretreatment of macrophages with the lectin. Pretreatment of the bacteria with the lectin also resulted, in most cases, in an increase in phagocytosis. Interaction of WGA with the macrophages and with the bacteria, as well as the potentiation of phagocytosis, was abolished by tri-*N*-acetylchitotriose, a saccharide that binds specifically to WGA, but not by monosaccharides which do not interact with this lectin. With non-elicited macrophages, enhancement of phagocytosis by WGA was less pronounced, probably because of the higher number of lectin-binding sites (5-fold) on the elicited cells. Peanut agglutinin and soybean agglutinin, that bind to macrophages but not to the bacteria studied, lack the ability to potentiate phagocytosis. Macrophage surface sugars thus appear to play an important role in phagocytosis by serving as receptors for lectins that form bridges between the macrophages and the microorganisms.

Abbreviations: FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PNA, peanut agglutinin; SBA, soybean agglutinin; TG, thioglycollate; WGA, wheat germ agglutinin. All sugars are of the *D* configuration, unless otherwise noted.

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INTRODUCTION

It is well established that the capacity of macrophages to engulf and inactivate the causative microorganisms of infectious diseases is a crucial factor in the ability of the host to overcome such a challenge (Laskin & Lechevalier, 1972; Mackaness, 1970). Thus, potentiation of macrophage activities, including phagocytosis, has broad implications for the resistance capability of animals.

Since phagocytosis requires the formation of close contacts between macrophages and target particles, it might be potentiated by agents that permit the formation of such contacts. Bar-Shavit & Goldman (1976) have indeed demonstrated that concanavalin A (Con A), a lectin specific for mannose and glucose, which binds to sugar residues on both mouse peritoneal macrophages and on yeast cells, enhanced markedly phagocytosis of the latter cells.

We have examined the effect of several other lectins on the *in-vitro* phagocytosis by non-elicited and

thioglycollate (TG)-elicited murine peritoneal macrophage of seven bacterial strains and species that differ in their lectin-binding properties. A remarkable and specific increase in phagocytosis was observed with wheat germ agglutinin (WGA, specific for *N*-acetylglucosamine and its $\beta_{1\to4}$-linked oligomers), when both the bacteria and the macrophages possess receptors for this lectin.

MATERIALS AND METHODS

Mice

C57BL/6 female mice, 10–15 weeks old, were used as donors of macrophages.

Lectins and inhibitors

Wheat germ agglutinin (WGA) (Lotan *et al.*, 1973), peanut agglutinin (PNA) (Lotan *et al.*, 1975) and soybean agglutinin (SBA) (Gordon *et al.*, 1972) were prepared by affinity chromatography, according to published procedures and were dissolved before use in phosphate-buffered saline (0·14 M NaCl, 0·0018 M KH_2PO_4 and 0·0082 M Na_2HPO_4, pH 7·0 (PBS)). Derivatives of the lectins, labelled with fluorescein isothiocyanate (FITC) were prepared according to the method of Clark & Shepard (1963) and repurified by affinity chromatography. For FITC-WGA, the dye: protein ratio was calculated as 1·84 from absorbance measurements at 280 and 493 nm. Tri-*N*-acetylchitotriose ($[GlcNAc]_3$) was isolated from chitin (Rupley, 1964) and further purified by gel filtration on Sephadex G-15; it was dissolved in PBS at a concentration of 3·3 mM. Galactose (Pfanstiehl), glucose (BDH, Analar), mannose (Sigma) and *L*-fucose (Sigma) were used as 0·2 M solutions in PBS. All other materials were commercial products of the highest quality available.

Bacteria

Staphylococcus aureus H, *S. aureus* 52A2 and *S. aureus* 52A5 (Chatterjee *et al.*, 1969; Lotan, Sharon & Mirelman, 1975a) were a gift from Dr David Mirelman (Department of Biophysics, Weizmann Institute of Science). *Staphylococcus albus*, *Shigella flexneri* and *Klebsiella pneumoniae* were clinical isolates from human pathological material used in our laboratory. *Micrococcus luteus* ATCC 2665 was a gift from I. Ginsburg (Hebrew University, Jerusalem, Israel). *S. aureus* and *S. albus* were grown overnight with shaking at 37° in 1% bactopeptone, 0·5% yeast extract and 0·5% NaCl; the other bacteria were grown in brain heart infusion. Labelling of the bacteria with [^{3}H]TdR was as described (Gallily, Douchan & Weiss, 1977) to a level of about 10^3 c.p.m./10^6 bacteria.

Harvesting and culture of macrophages

Isolation of macrophages from the peritoneal exudates of untreated C57BL/6 mice (non-elicited macrophages) or thioglycollate-treated animals (TG macrophages) and removal of non-adherent cells were carried out as described (Gallily *et al.*, 1977). The adherent cells consisted of > 96% macrophages, identified by morphological and phagocytic criteria. The number of adherent cells per plate was determined by counting either the cells in 20 microscopic fields (Geiger & Gallily, 1974), or their nuclei following treatment of the cells with the non-ionic detergent NP40 (Unkeless & Eisen, 1975). The macrophages were cultivated in Hanks' balanced salt solution containing 2% v/v newborn calf serum.

Treatment with lectins

Macrophage monolayers were exposed to the lectins in PBS for various periods of time at 4 or 22° with or without the specific sugar inhibitor. The cells were then thoroughly rinsed to remove the unbound lectin; [^{3}H]TdR-labelled bacteria at a density of 250 Klett units (at the log phase) were suspended in PBS, incubated with the lectin for 30 min at 22° and then centrifuged once to remove unbound WGA.

Adsorption and phagocytic assays

The [^{3}H]-labelled bacteria were added to macrophage monolayers pre-treated with the lectin at a rate of 50 bacteria per macrophage. In the adsorption assay both the treatment of the cells with the lectin and the incubation with the labelled bacteria were at 4°. The exposure to the bacteria was for 75 min. For the phagocytic assays either the macrophages or the bacteria were pretreated with the lectin at 4 or 22° followed by incubation of the macrophages with the bacteria at 37° for 45 min. In some experiments different conditions were used, as specified under results.

At the end of the incubation with the bacteria, the macrophage monolayers were thoroughly rinsed with PBS and lysed with 1% sodium dodecylsulfate. The radioactivity in the trichloroacetic acid-precipitable material was determined as described (Gallily *et al.*, 1977). Radioactivity in the cell lysate was compared with that of the added bacteria and served as a

measure for the number of bacteria attached (at 4°) or phagocytosed (at 37°) per macrophage.

When lysostaphin (Schwarz/Mann) was employed it was added at the end of incubation time of macrophages and the bacteria (3 U/ml for 15 min at 37°). The macrophages were then washed and lysed with 0·1% Triton X-100 and the number of viable bacteria were determined by plating serial dilutions of the lysate (in PBS) on brain heart agar. Bacterial colonies were counted after 24 hr incubation of the plates at 37°.

Lectin binding

Fluorescein isothiocyanate (FITC)-labelled lectins were used to examine the presence of lectin-binding sites on the macrophages and the bacteria. For microscopic observation, the cells were routinely incubated with the FITC-lectin (30–90 μg/ml) at 22° for 30 min. The macrophages were rinsed with PBS and the bacteria were washed by centrifugation also with PBS. The cells were then examined under a fluorescence microscope. In control experiments, specific inhibitory sugars were used routinely: $(GlcNAc)_3$ (3·3 mM) for WGA and galactose (0·2 M) for PNA and SBA.

For quantitative estimation of the binding of WGA to macrophages, the cells (10^6/well) (Linbro) were incubated for 15 min at 4° with increasing amounts of FITC-WGA (ranging from 3 to 120μg FITC-WGA/well). Non-specific binding of FITC-WGA was tested in cells where the inhibitor $(GlcNAc)_3$ was added 15 min prior to the addition of the lectin. Macrophages without any treatment were also prepared as control for light scattering and autofluorescence. Following rinsing with cold PBS, 0·5 ml of 0·2% NP40 was added to the wells, and lysis was carried on for 10 min at 4°. The lysates were transferred to Beckman microfuge tubes, and after centrifugation (10,000 r.p.m., 10 min) 0·4 ml of each supernatant was diluted with 1·6 ml PBS. The fluorescence of the supernatant was determined with spectrofluorimeter (Perkin Elmer MPF 44A). The excitation wave length was 493 nm and scanning was performed from 510 to 530 nm. Slits were 10 nm for excitation and 14 nm for emission.

Experimental design

For each experiment, peritoneal macrophages were pooled from 10 to 12 normal or two to four TG-elicited donor mice and plated in Petri dishes. Data shown in the graphs represent the mean values of two to six experiments, each performed on triplicate macrophage plates. Variation from the triplicate means usually did not exceed 10%. Differences between experimental and control group values in individual experiments were analysed for significance by the one-tail distribution-free Mann–Whitney *U* test. For cumulative analysis of similar experiments, the exact probabilities for the sum of the *U* values were computed.

RESULTS

When TG-elicited murine macrophages were pretreated at 22° with different concentrations of WGA and then incubated with *S. aureus* H at 37°, a 3–5-fold increase in bacteria uptake was observed (Fig. 1), from two to three bacteria per macrophage in the absence of the lectin to eight to ten in its presence. The results were not affected by varying the incubation time from 30 to 100 min. Similarly, treatment of elicited macrophages for 75 min at 22° with varying concentrations

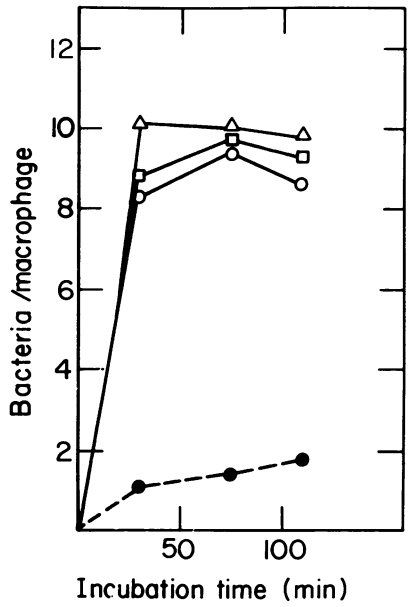

Fig. 1. Effect of pretreatment at 22° of TG-elicited murine macrophages with different concentrations of WGA for various periods of time on the uptake of *S. aureus* H at 37°. Untreated macrophages, ●---●; pretreatment with WGA: ○——○, 30 μg/ml; □——□, 60 μg/ml; △——△, 90 μg/ml.

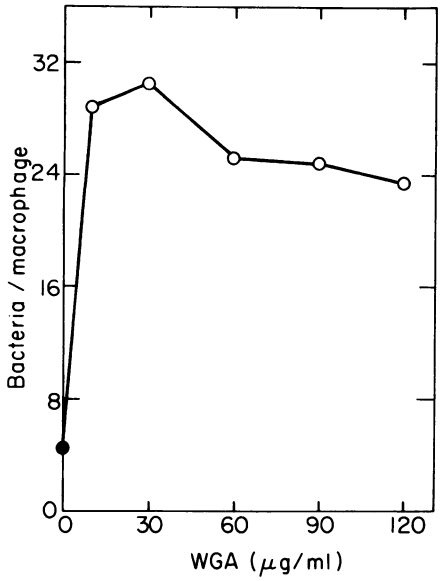

Fig. 2. Effect of pretreatment of TG-elicited macrophages with various concentrations of WGA (for 75 min at 22°) on the uptake of *Micrococcus luteus* at 37°. ●, Untreated macrophages; ○, macrophages pretreated with WGA.

of WGA prior to their interaction with *M. luteus* also demonstrated a marked increase (up to 6·5-fold) in bacterial uptake (Fig. 2).

The effect of WGA on the adsorption to TG-elicited macrophages of the three strains of *S. aureus* at 4°, conditions under which no phagocytosis occurs, is summarized in Fig. 3a. The lectin increased markedly average 3–4-fold (Fig. 3a) and maximally 8-fold (data not shown) the binding to macrophages of *S. aureus* H and 52A5, previously shown to bind WGA (Lotan *et al.*, 1975a), whereas no increase was observed with *S. aureus* 52A2, on which binding of WGA was not detected earlier (Lotan *et al.*, 1975a) under the fluorescence microscope (see however below). The effect of WGA is sugar-specific, as it is completely inhibited by $(GlcNAc)_3$.

Pretreatment of *S. aureus* H and 52A5 with the lectin at 22°, followed by incubation of the bacteria with the macrophages at 37°, conditions that favour phagocytosis, resulted in the uptake of a considerable number of bacteria by the macrophages (eight to nine bacteria per macrophage), which was 1·2–1·8 times higher than in the absence of the lectin, or in the presence of the lectin and its inhibitor (Fig. 3b). Under these conditions, WGA had no effect on the phagocytosis of *S. aureus* 52A2.

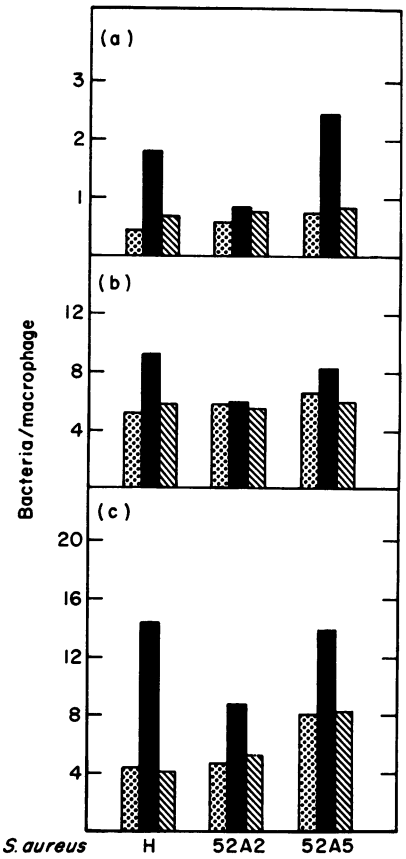

Fig. 3. Effect of WGA (60 μg/ml) on the binding of bacteria to and phagocytosis by TG-elicited murine peritoneal macrophages. (a) Macrophages pretreated with WGA at 4°, followed by incubation with the bacteria at the same temperature. (b) Bacteria pretreated with WGA at 22° and then incubated with the macrophages at 37°. (c) Macrophages pretreated with WGA at 22°, then incubated with the bacteria at 37°. Conditions: ▩, control; ■, +WGA (60 μg/ml); ▧, +WGA (60 μg/ml) and $(GlcNAc)_3$ (3·3 mM). Each experiment was performed 3–6 times in triplicate. Effect of WGA is statistically significant ($P \leqslant 0{\cdot}002$ according to the Mann–Whitney *U* test), except with *S. aureus* 52A2 in experiments a and b where it is not significant ($P > 0{\cdot}05$). For further experimental details, see text.

As can be seen from Fig. 3c, the strongest potentiation of phagocytosis (average 3·5-fold and up to 5–6-fold) was observed with *S. aureus* H, when incubated at 37° with macrophages that had been pretreated with the lectin at 22°. In this series of experiments, more than 14 bacteria per macrophage were taken up on the average (range 10·7–17·6 bacteria per macrophage in six experiments performed). With

S. aureus 52A5, the increase was considerably smaller (only 1·7-fold), presumably because the total number of bacteria internalized in the absence of the lectin was higher than with the other strains. When *S. aureus* 52A2 was incubated with WGA-pretreated macrophages, there was 1·5–2-fold increase in the phagocytosis.

Preliminary experiments, in which the amount of FITC-WGA bound was examined spectrofluorimetrically (with a method similar to that used for lectin binding to macrophages), small but significant binding of the lectin to *S. aureus* 52A2 was observed, which was about one-fifth of that bound to *S. aureus* H, and about one-third of that bound to *S. aureus* 52A5.

Experiments with lysostaphin established that the bacteria were indeed internalized by the WGA-treated macrophages since they were not affected by the enzyme (Table 1).

Potentiation of phagocytosis of *S. aureus* H by WGA was sugar-specific, since it was inhibited only by $(GlcNAc)_3$ (3·3 mM) and not by any other sugar tested even at much higher concentrations (100 mM) (Fig. 4). The same specificity pattern was observed with the other strains of *S. aureus* tested (data not shown).

Pretreatment of normal, non-elicited macrophages with WGA at 4° for 15 min increased binding of *S. aureus* H and 52A5 by 2·3- and 1·4-fold, respectively (Fig. 5a). Treatment of the non-elicited macrophages with WGA at 22°, followed by incubation with the bacteria at 37°, gave only a small increase in phagocytosis (Fig. 5b). No increase in *S. aureus* 52A2 binding or uptake was noted in similar experiments.

Pretreatment of the macrophages with PNA or SBA (30 or 60 μg/ml) for 75 min at 22°, followed by incubation with the three strains of staphylococci did not result in enhancement of uptake (data not shown).

Table 1. Uptake of *S. aureus* H by WGA-treated macrophages following incubation with lysostaphin

Lysostaphin (3 U/ml for 15 min at 37°)	Number of bacteria/macrophage			
	Untreated macrophages		WGA-treated macrophages	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
–	6	3·6	13·1	14·9
+	3·1	2·3	15·5	15·1

* Lysostaphin treatment was carried out as described (Gallily *et al.*, 1977).

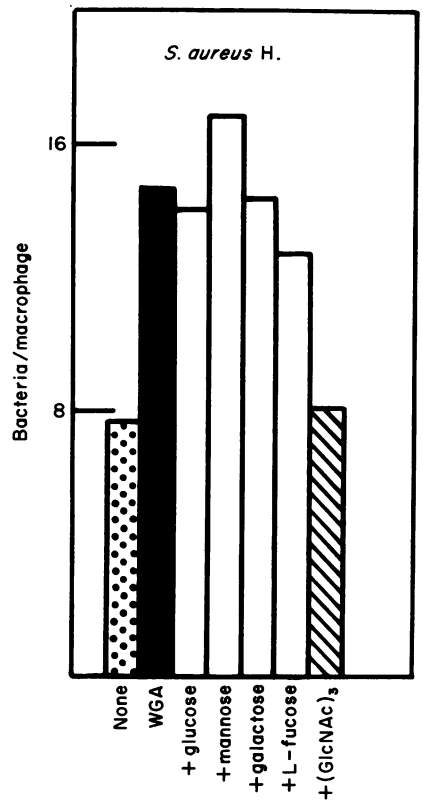

Fig. 4. Effect of different sugars on the uptake of bacteria *S. aureus* H. by TG-elicited macrophages. The macrophages were incubated at 22° for 75 min with WGA alone or together with one of the following sugars: 0·1 M glucose, mannose, galactose, or *L*-fucose, or 3·3 mM $(GlcNAc)_3$. Following rinsing, the bacteria were added and incubation proceeded at 37° for 45 min. The results are the average of two experiments, each run in quadruplicate. Inhibition by $(GlcNAc)_3$ was significant ($P \leqslant 0{\cdot}002$); no inhibition by other sugars was noted ($P > 0{\cdot}05$).

Examination of macrophages stained with FITC-PNA and FITC-SBA, confirmed the earlier finding of the presence of receptors for these lectins on the cells (Goldman, Sharon & Lotan, 1976). However, no binding of these two fluorescent lectins to any of the bacteria studied was observed, which is in accord with the carbohydrate composition of their outer surface (Lotan *et al.*, 1975a).

When macrophages were pretreated with 60μg WGA at 22° and then incubated with four other bacteria, enhancement of phagocytosis of three different bacteria (*S. albus*, *M. luteus* and *Sh. flexneri*) was noted (Fig. 6). *K. pneumoniae* on the other hand was

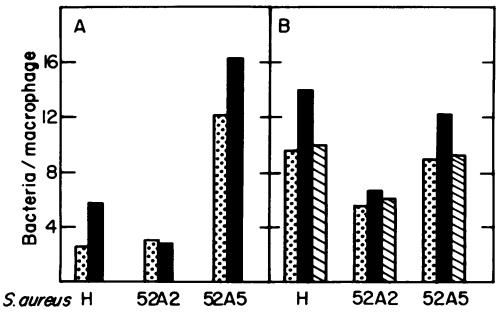

Fig. 5. Effect of WGA (60 μg/ml) on the uptake of bacteria by non-elicited peritoneal macrophages. A Macrophages pretreated with WGA at 4° for 15 min and then incubated with the bacteria at 37° for 45 min. B Macrophages pretreated with WGA at 22° for 75 min, then incubated with the bacteria at 37° for 45 min. Conditions: ▩, control; ■, +WGA (60 μg/ml); ▧, +WGA (60 μg/ml) and $(GlcNAc)_3$ (3·3 mM). Each experiment was performed 3 times. Effect of WGA is statistically significant ($P < 0.05$) except with *S. aureus* 52A2 in experiments A and B, where it is not significant ($P > 0.05$).

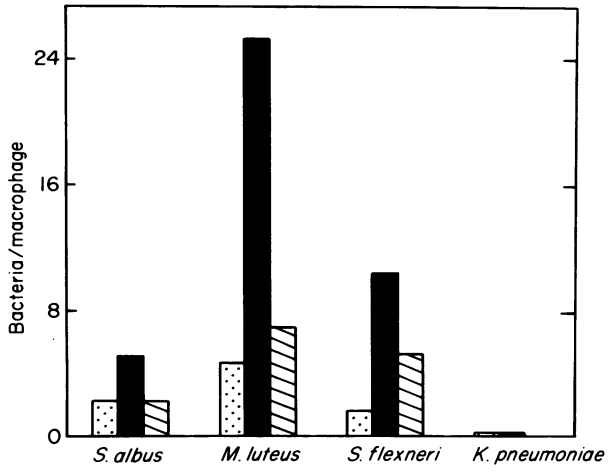

Fig. 6. Effect of WGA (60 μg/ml) on the uptake of four different bacteria by TG-elicited macrophages. Macrophages were pretreated with WGA for 75 min at 22°, washed and incubated with the bacteria at 37° for 45 min (ratio of bacteria:macrophage, 50:1). Conditions: ▩, control; ■, +WGA (60 μg/ml); ▧, +WGA (60 μg/ml) and $(GlcNAc)_3$ (3·3 mM/ml).

not phagocytosed by elicited macrophages even after pretreatment with WGA (60 μg/ml).

Thioglycollate (TG)-elicited macrophages possess 42×10^6 WGA-binding sites/cell (Fig. 7) with association constant $Ka = 0.46 \times 10^6 M^{-1}$ (Fig. 8). The non-elicited macrophages have 9×10^6 binding sites with association constant of $1.36 \times 10^6 M^{-1}$.

DISCUSSION

Lectins bind specifically to saccharides on the surface of cells and cause cell agglutination as well as a variety of other effects (Lis & Sharon, 1977). Treatment with Con A markedly enhanced the attachment and ingestion of yeast cells by murine macrophages (Goldman & Bar-Shavit, 1982). Little is known on the effects of lectins on phagocytosis of bacteria. Recently it was demonstrated that PNA and SBA, as well as *Erythrina cristagalli* agglutinin, enhance phagocytosis of sialidase-treated rat erythrocytes by rat peritoneal macrophages (Müller *et al.*, 1983). We have investigated the effect of WGA, PNA and SBA on the interaction between bacteria and macrophages using bacteria which differ in their lectin-binding properties: all of these bind the former lectin, but not the latter ones. Our findings clearly show that the uptake of the six bacteria by TG-elicited macrophages was increased by the former lectin, but not by the two latter ones. Potentiation of up to 5–7-fold was observed in the uptake of *S. aureus* H, *Sh. flexneri* and *M. luteus*.

Our findings further show that as a result of the binding of WGA to non-elicited or TG-elicited macrophages under conditions where no phagocytosis occurs, there is a significant increase in the attachment of bacteria (*S. aureus* H and 52A5) that bind readily to the lectin. A similar increase in phagocytosis is observed when these bacteria are pretreated with WGA at 22° and incubated with the macrophages at 37°, or when the macrophages are pretreated with the lectin prior to their incubation with the bacteria. Under the latter conditions, however, increase in phagocytosis of *S. aureus* 52A2, a mutant that binds the lectin to a lower degree, by TG-elicited macrophages is also observed. The variation in the extent of enhancement of phagocytosis observed in different experiments, appears to be mainly due to variation in the rate of phagocytosis in the absence of the lectin, which might be caused by the physiological state of animals used.

That the bacteria were indeed localized inside the macrophages rather than attached to their surfaces, was demonstrated by experiments with lysostaphin. Lysostaphin is known to kill staphylococci very rapidly (Weston, Dustin & Hecht, 1975). The number of bacteria/cell (determined by plating dilutions of cell lysates (Gallily *et al.*, 1977)) in WGA-treated macrophages was similar in lysostaphin-treated and untreated cells indicating that the bacteria were indeed phagocytosed by these cells. The effect of WGA on

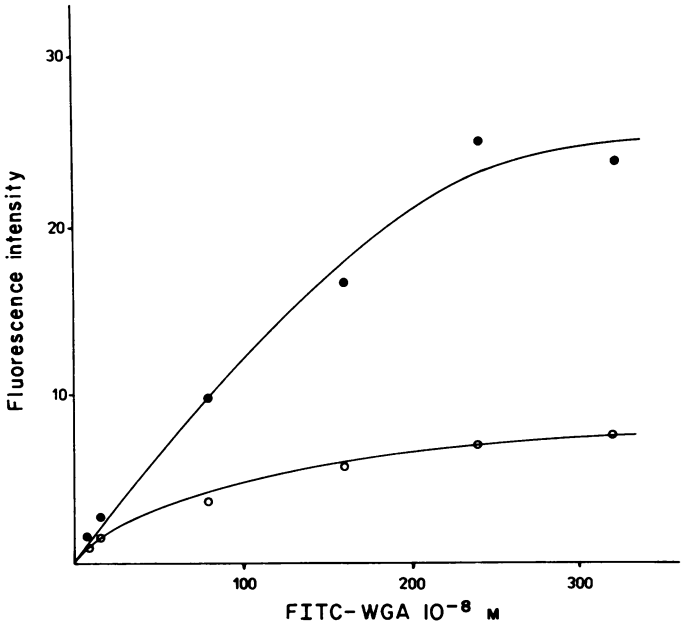

Fig. 7. Binding curves of FITC-WGA to thioglycollate elicited mouse macrophages (●) and to resident macrophages (○). Non-specific binding (< 2%) is subtracted. In ordinate, the amount of bound FITC-WGA has been converted to number of binding sites.

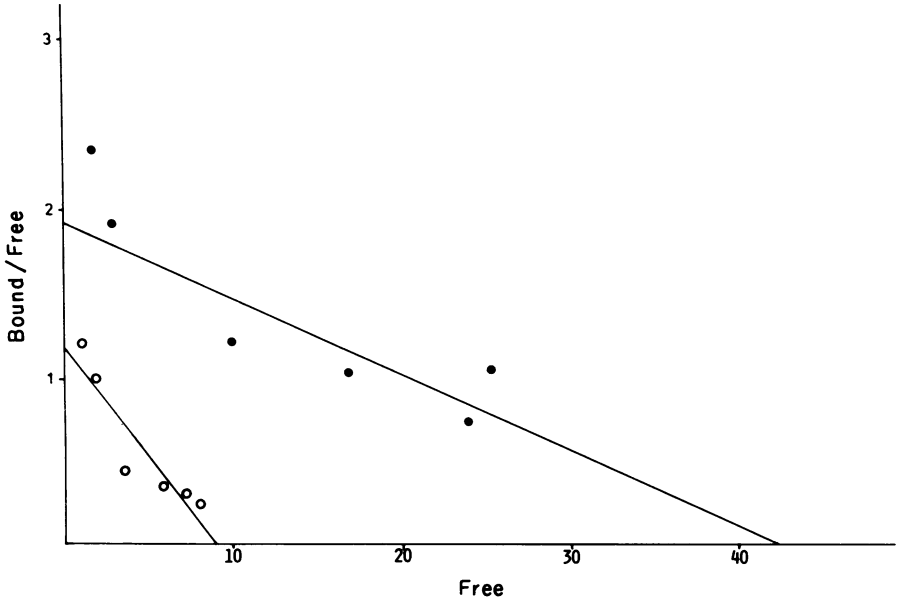

Fig. 8. Scatchard plots calculated from the curves in Fig. 7. Correlation coefficients were 0·895 and 0·937 respectively for elicited (●) and non-elicited (○) mouse macrophages. The straight lines represent linear fits through the data:

$$\frac{B}{F} = \frac{\text{FITC-WGA bound to the macrophages}}{\text{FITC-WGA free}},$$

Where F = increasing amounts of free FITC-WGA ($\times 10^{-8}$M) added to the macrophage.

macrophages was sugar-specific, being inhibited only by $(GlcNAc)_3$ and not by any other sugar tested. The higher phagocytic potentiation values by WGA when TG-elicited macrophages were employed rather than non-elicited ones, might be attributed to the high number of WGA binding sites of the lectin on the former cells compared to the latter ones.

Our study further demonstrates that PNA and SBA lectins that do not bind to the different strains of *S. aureus* or to *S. albus*, do not affect phagocytosis, even though they bind to the surface of macrophages (Goldman *et al.*, 1976). It may thus be concluded that macrophage surface sugars may play an important role in phagocytosis by serving as receptors for lectins that form bridges between the macrophages and microorganisms. Similar enhancement of phagocytosis may also occur in the absence of immune factors *in vivo*, since lectins are present also in animal tissues.

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