Phagocytosis by Human Blood Leukocytes Measured by the Uptake of \(^{131}\)I-Labeled Human Serum Albumin: Inhibitory and Stimulatory Effects of Cytochalasin B

NICHOLAS A. CANNAROZZI AND STEPHEN E. MALAWISTA

Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

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INTRODUCTION

In the study of phagocytosis, the experimental systems used must be able to distinguish among various aspects of the phagocytic process, such as chemotaxis, adherence of the ingestate, engulfment, fusion of phagosome and lysosome, metabolic perturbations, and extracellular release of various substances. Chang introduced a method for measuring the degree of ingestion of starch particles by rabbit exudate granulocytes in vitro, by the concomitant uptake of radioiodinated \(^{131}\)I human serum albumin (1). This method was sensitive and quantitative, and was particularly useful for distinguishing adherent from ingested particles, since, even if surface particles could not be dislodged, radioactive label in the media could be washed away.

We have modified this method in a study of phagocytosis by human blood leukocytes in vitro, and employed it to emphasize the importance of the particle ingested, in the results obtained. In using the mold metabolite, cytochalasin B, to modify ingestion, we have found particle-dependent stimulatory as well as inhibitory effects of the drug, which have similarities to effects of cytochalasin B in other systems, but were not detected in previous studies of the effects of that drug on phagocytosis (2–7).

MATERIALS AND METHODS

Human blood leukocytes. As described previously (8), heparinized venous blood was sedimented in 2 volumes of 3% dextran, and the leukocyte-rich supernatant

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2 Associate Professor of Medicine, Yale University; recipient of NIH Research Career Development Award AM-19864.

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was sedimented and washed in modified Krebs-Ringer phosphate buffer, pH 7.4. The cells were osmotically shocked (to lyse red cells), restored to isotonicity, washed once more in buffer, counted, and adjusted to about $3 \times 10^7$ white blood cells (WBC) per ml. Differential counts were performed.

Radio-iodinated human serum albumin ($^{131}I$-HSA). $^{131}I$-HSA contained HSA, 10 mg per ml, and (initially) either 100 (Abbott Laboratories, Chicago, Ill.) or 500 (Squibb, New Brunswick, N.J.) μCi per ml.

Particles. *Staphylococcus aureus* strain 502A (9) was shaken overnight in beef heart infusion broth at 37°C, centrifuged, and washed once in normal saline, and the bacteria were suspended in buffer. The concentration of staphylococci was estimated from a constructed curve of transmittance at 600 nm and confirmed by quantitative plating in agar. Bacteria were heat-killed (8) and frozen in aliquots containing $1.8 \times 10^9$ or $3.5 \times 10^9$ bacteria per ml saline. "Staphylococci" refers to heat-killed bacteria, except as noted.

Starch particles, generously provided by Dr. K. J. Goering of Montana State University, were isolated from the seed of *Saponaria vaccaria* or Montana "cow soapwort" (10). They were chosen for their small size and relative uniformity (0.5-1.6 μm in diameter). For each experiment 5 mgm of starch (6.7X$10^8$ granules per mg [1]) were suspended in 1 ml containing equal volumes of fresh human serum and heparinized buffer, and left at room temperature for at least an hour before use. The starch, unlike the bacteria, was thus pre-opsonized.

Drugs. Cytochalasin B (Imperial Chemical Industries, Ltd., Macclesfield, Cheshire, England; MW 479) was dissolved in dimethylsulfoxide (DMSO spectralized, Fisher Scientific Co., Pittsburgh, Pa.) at a concentration of 3 mg per ml. Aliquots were stored at minus 70°C until use, at which time they were diluted in aqueous media. In experiments with cytochalasin B, controls received corresponding dilutions of DMSO (cytochalasin B, 5 μg per ml, the highest concentration used, contained 0.17% DMSO).

Phagocytosis. Details of the phagocytic system evolved as noted in the Results, but except for alterations attendant upon the addition of $^{131}I$-HSA, the conditions of incubation were similar to those used previously in studies of ingestion that dealt with the recovery of live bacteria from supernatants and cells (8, 11, 4). In general, leukocytes in at least 12% autologous serum-heparinized buffer, in 25-ml Erlenmeyer flasks, were pre-incubated in an Eberbach Incubator Model 6250 (Eberbach Corp., Ann Arbor, Mich.), 100 reciprocations per min, at 37°C for 15 min. Then 6 μCi of $^{131}I$-HSA were added, followed immediately by particles (zero time) at the ratio required (particle:PMN). The total incubation volume was about 2.8 ml. When cytochalasin B was used, it was added after the $^{131}I$-HSA, and pre-incubated for 10 min before the addition of particles.

After a given period of incubation, phagocytosis was stopped by placing the flasks in ice. The contents of the flasks were transferred to 10-ml screw-cap tubes, the flasks rinsed with 5 ml of buffer to which EDTA had been added (0.5%), and the rinses added to the tubes. The cells were sedimented for 10 min at 1,500 rpm in an International Centrifuge Model PR-2 (International Equipment Co., Needham Hts., Mass.). The supernatants were discarded, the cells resuspended in 1 ml EDTA-buffer, and the contents transferred with Pasteur pipettes to another sets of tubes that had been rinsed with 50% serum-buffer (to minimize adsorption of $^{131}I$-HSA). Seven ml of EDTA-buffer were added, and the cells were resuspended and sedimented as before; this procedure constituted the first wash. Subse-
sequent washes, usually to a total of four, were done in the same set of tubes, by
discarding the supernatants, adding 8 ml EDTA-buffer, and resuspending and re-
sedimenting the cells. Cell-associated radioactivity of pellets was determined after a
given wash by placing the tubes in a gamma well counter (Nuclear Chicago,
Waltham, Mass.), counting the pellets, and subtracting background counts from
the empty well (usually in the range of 150–300 cpm).

All glassware was siliconized, sterile, and pyrogen-free. All flasks receiving par-
ticles were run at least in quadruplicate; those containing resting cells, at least in
triplicate.

Statistics. The t test for paired variates was used throughout.

RESULTS

The Effects of Washing and of Serum Concentration on Cell- and Particle-
Associated Radioactivity

Resting cells (Fig. 1). With washing, cell-associated radioactivity generally de-
creased until the third or fourth wash. To be on the safe side, four washes were
chosen for subsequent experiments, except as noted.

For a given wash, the addition of serum 60 min before the addition of $^{131}$I-HSA,
resulted in considerably diminished cell-associated radioactivity. Since there was
no significant transport of radioactivity into the cell in this system (see below, and
Fig. 2A), serum presumably blocked the binding of HSA to the cell surface. Al-
though the serum effect was dosage-dependent, decrements in cell-associated radio-
activity were small beyond 12% serum, and 12–17% serum was used in subsequent
experiments. We also determined that a 60-min preincubation with serum was un-
necessarily long (not shown in Fig. 1), and a 15-min period became the standard.

Particles. To distinguish between particles that are adherent to cells and those
that are intracellular, one must be able to wash the non-ingested particles relatively
free of radioactivity. To see if particles could be cleansed of radioactivity by wash-
ing, we incubated serum-buffer containing $^{131}$I-HSA, with either live staphylococci,

![Graph](image-url)

Fig. 1. The effects of washing and of serum concentration on cell-associated radioactivity.
Four sets of quadruplicate flasks each containing $4.0 \times 10^7$ human leukocytes, PMN 73%,
were pre-incubated at 37°C for 60 min in buffer and autologous serum, 0, 12, 22 or 46%
total volume 2.6 ml). $^{131}$I-HSA was added, the flasks were incubated for 60 min, and
cell-associated radioactivity was determined after successive washes (see Methods).
heat-killed staphylococci, or starch (five flasks of each), or with no particles (four flasks). After 60 min, the flasks were placed in ice and their contents washed as if they contained cells (see Methods), except that the sedimentations were carried out at 2,000 rpm for 20 min (to bring down most of the particles). Enough live staphylococci were added initially \((1.7 \times 10^9)\) so that, after the incubation, transfers, and four washes, the five tubes still contained a mean of \(5.9 \times 10^8 (\pm 0.2 \times 10^8)\) live bacteria per tube (determined by quantitative plating in agar). However, in terms of radioactivity, those same tubes after four washes contained only \(30 \pm 12\) cpm more than controls given no particles. Since in subsequent studies of phagocytosis the entire inoculum is usually only about \(5 \times 10^8\) particles (at a ratio of \(20:1\), particle:PMN), adherent live staphylococci should not contribute significantly to cell-associated radioactivity. Radioactivity associated with heat-killed staphylococci and with starch after four washes was similarly insignificant (less than \(50\) cpm per estimated \(5 \times 10^8\) particles).

In summary, under appropriate conditions of washing and of preincubation with serum, cell-associated radioactivity was low, and particle-associated radioactivity was trivial.

**The Effect of Time on the Uptake of Radioactivity by Resting and by Phagocytizing Human Leukocytes** (Fig. 2)

**Resting leukocytes.** Cell-associated radioactivity did not increase with time between 10 and 60 min (Fig. 2A). Thus there is no evidence either for progressive

![Fig. 2. The effect of time on the uptake of radioactivity by resting and by phagocytizing human leukocytes.](image-url)
binding of $^{131}$I-HSA to the cell surface, or for pinocytosis or other means of transport of radioactivity into resting leukocytes.

**Phagocytizing leukocytes.** When leukocytes were given heat-killed staphylococci (Fig. 2A) or starch particles (Fig. 2B), cell-associated radioactivity increased with time over 60 min. The largest increases occurred early in the hour, when we know from other studies (11) that most of the ingestion is also taking place. When live staphylococci were used (not shown in Fig. 2), the results were similar to those with heat-killed staphylococci.

When three washes were used instead of the usual four (Fig. 2A), there was more cell-associated radioactivity at each interval, but the relative rates of uptake were similar.

**The Effect of Ratio (Particle:PMN) on the Uptake of Radioactivity by Phagocytizing Human Leukocytes (Fig. 3)**

Because the uptake of cell-associated radioactivity varies among leukocytes from different donors under similar conditions, the effects were studied of variable ratios of both staphylococci and starch to PMN, using leukocytes from a single donor, incubated concurrently for 40 min. For each particle, cell-associated radioactivity increased with the dose of particles, but at a given ratio there were progressively more cell-associated counts with staphylococci than with starch. This effect could not be attributed to a differential survival of cells: in 11 experiments the number of leukocytes counted after four washes was $67 \pm 1\%$ of those at the start; recovery was not significantly different for resting cells compared to cells given staphylococci or starch. In addition, wet preparations examined after four washes, revealed only minimal clumping of cells with either particle at the various ratios.

When three washes were used instead of the usual four, there was more cell-
associated radioactivity at each ratio, but the relationships between ratios were similar.

**The Effect of Temperature on the Uptake of Radioactivity by Human Leukocytes Shaken with Particles (Fig. 4)**

When leukocytes were incubated at different temperatures for 40 min, with and without staphylococci or starch, there were temperature-dependent differences in cell-associated radioactivity between resting cells and those given particles.

Like the studies shown in Fig. 3, those in Fig. 4 were done concurrently, with cells from a single donor. Again the importance is emphasized of the type of particle used.

**Studies with Cytochalasin B**

The results so far suggest that this system can distinguish ingested particles from those on the cell surface: the former have associated radioactivity that is dosage-dependent and cannot be washed away; the latter, if not washed off the surface themselves, can be stripped of significant radioactivity by washing. We decided to test cytochalasin B in this system, a drug that (at 5 μg per ml) makes human leukocytes unable to ingest significant numbers of staphylococci which are nevertheless adherent to the cell surface (4).

After both 20 and 40 min of incubation, the uptake of radioactivity during phagocytosis of live staphylococci was diminished by 5 μg per ml of cytochalasin B (Fig. 5A). Cytochalasin B was more inhibitory, at a given concentration, when the particles for ingestion were starch rather than staphylococci. In two experiments, one of which is shown in Fig. 5B, the uptake of radioactivity during phagocytosis of starch was suppressed almost completely by 5 μg per ml of cytochalasin B.

Cytochalasin B, or the corresponding concentration of its initial solvent, DMSO,
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Fig. 5. The effect of cytochalasin B, 5 μg/ml, on the uptake of radioactivity by phagocytizing human leukocytes. The mean differences are shown between cells with and without particles after 20 and 40 min of incubation, for both live staphylococci (A) and starch (B). (A): 3.0 × 10⁷ WBC per flask, PMN 63%, ratio 17:1 (staph:PMN). Standard errors of the means are indicated (n = 4, except for the drug-treated flasks at 40 min, where n = 3). At 20 min, p < .01. (B): 3.9 × 10⁷ WBC per flask, PMN 82%, ratio 16:1 (starch:PMN). Standard errors of the means are indicated (n = 4). At 20 min, p < .01; at 40 min, p < .001.

had no effect on the cell-associated radioactivity of resting cells, or on the numbers of leukocytes recovered after the four washes.

Paradoxical effect: When the concentration of cytochalasin B was decreased to 2 μg per ml, the uptake of radioactivity during phagocytosis of live staphylococci was increased rather than decreased (Fig. 6A). This finding was confirmed at different ratios (Fig. 6A; Table 1), and after both 20 and 40 min of incubation (Table 1).

However, when starch was used, 2 μg per ml of cytochalasin B inhibited the uptake of radioactivity during phagocytosis (Fig. 6B). Thus, that concentration of cytochalasin B was either stimulatory or inhibitory, depending upon the particle employed. By decreasing the concentration of cytochalasin B further, one could also demonstrate stimulation of uptake during phagocytosis of starch (Fig. 6B).

TABLE 1

THE STIMULATORY EFFECT OF CYTOCHALASIN B, 2 μg/ml, ON THE UPTAKE OF RADIOACTIVITY BY HUMAN LEUKOCYTES GIVEN LIVE STAPHYLOCOCCI

| Ratio | Staph:PMN | WBC per flask | PMN per cent | Minutes of incubation | Cyt B, 2 μg/ml | Control | n | p |
|-------|------------|---------------|--------------|----------------------|---------------|---------|---|---|
| 10:1  | 3.9 × 10⁷  | 72%           | 40           | 4,561 ± 170          | 3,582 ± 151   | 4       | < .05 |
| 18:1  | 3.6 × 10⁷  | 70%           | 20           | 3,286 ± 162          | 2,683 ± 141   | 4       | < .02 |
| 18:1  | 3.6 × 10⁷  | 70%           | 40           | 5,052 ± 818          | 3,560 ± 146   | 4       | < .02 |

* Mean differences between cells with and without particles.
**Fig. 6.** Inhibitory and stimulatory effects of cytochalasin B, with live staphylococci and starch. The mean differences are shown between cells with and without particles after 40 minutes of incubation. Standard errors of the means are indicated. (A): $3.0 \times 10^7$ WBC per flask, PMN 75%, ratio 39:1 (staph:PMN). (B): $3.8 \times 10^7$ WBC per flask, PMN 77%, ratio 20:1 (starch:PMN).

**Fig. 7.** Inhibitory and stimulatory effects of cytochalasin B, with heat-killed staphylococci. The mean differences are shown between cells with and without particles after 40 min of incubation. Standard errors of the means are indicated. (A): $3.4 \times 10^7$ WBC per flask, PMN 70%, ratio 22:1 (staph:PMN). (B): $3.6 \times 10^7$ WBC per flask, PMN 69%, ratio 21:1 (staph:PMN).
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TABLE 2
THE INHIBITORY AND STIMULATORY EFFECTS OF CYTOCHALASIN B, MEASURED BY THE ENUMERATION OF CELL-ASSOCIATED, HEAT-KILLED STAPHYLOCOCCI

|               | Number of bacteria per cell ± SEM (n = 20) |
|---------------|-------------------------------------------|
|               | Before washing                             | After four washes                        |
| Cyt B, 5 µg/ml| 14.3 ± 1.5                                 | 13.8 ± 1.4                               |
| Control       | 22.0 ± 1.3                                 | 22.3 ± 1.3                               |
| Cyt B, 0.5 µg/ml| 28.6 ± 1.7                     | 29.6 ± 2.1                               |
| Control       | 21.8 ± 1.8                                 | 21.0 ± 1.5                               |

*p < .01
**p < .001

*3.6 × 10⁷ WBC per flask, PMN 76%, ratio 26:1 (staph:PMN), time of incubation 40 min. Samples were centrifuged onto glass slides and stained, and the bacteria per cell counted for 20 consecutive cells, without knowledge of which flask they were from.

When live staphylococci were used, the increased concentrations of drug required for either inhibition or stimulation (compared to effects with starch) could not be attributed to direct inactivation of cytochalasin B by the live bacteria. Pre-incubation of cytochalasin B, 2 µg per ml, with live staphylococci for 1 hr at 37°C, with or without serum, did not affect the ability of the supernatant to inhibit the uptake of radioactivity by leukocytes given a fresh inoculum of heat-killed staphylococci.

Whether the staphylococci were live or heat-killed had not been found to affect subsequent cell-associated radioactivity. Nevertheless, we wondered if some heat-sensitive product of live staphylococci might have been responsible for the particle-dependent differences seen in the effects of cytochalasin B (Fig. 6). Therefore, we did similar experiments using heat-killed bacteria (Fig. 7). With heat-killed staphylococci, the range of inhibition and stimulation by cytochalasin B was more like that with starch (Fig. 6B) than with live staphylococci (Fig. 6A).

We confirmed both the inhibitory and stimulatory effects of cytochalasin B microscopically, by counting cell-associated bacteria both directly after incubation and after four washes (Table 2).

DISCUSSION

The Measurement of Phagocytosis

This system appears to provide a sensitive measure of the extent of phagocytosis by human blood leukocytes, within the limits studied. At a ratio of 20:1, particle:PMN, cell-associated radioactivity increased with time over 60 min (Fig. 2). The largest increases occurred early in the hour, when most of the ingestion of staphylococci takes place in this system (11). Cell-associated radioactivity was temperature-dependent (Fig. 4), and varied with the ratio of particles to PMN (Fig. 3). In resting leukocytes, cell-associated radioactivity did not increase with time between 10 and 60 min (Fig. 2); thus there is no evidence either for progressive binding of ¹³¹I-HSA to the cell surface, or for pinocytosis or other means of transport of radioactivity into resting leukocytes. These results confirm and extend the findings of Chang (1), who used ¹³¹I-HSA under similar conditions to study phagocytosis of starch by rabbit exudate granulocytes.

The ¹³¹I-HSA most likely gains access to the cell with the particle, and some of it may be bound to the particle itself or to the cell membrane that becomes inter-
iorized to form the phagocytic vacuole, or both. In addition, we cannot rule out the possibility that some of the increase in cell-associated radioactivity during phagocytosis may be due to some form of transport remote from the particle, e.g., pinocytosis stimulated by the process of phagocytosis.

The Uses of This System

This system has some advantages over others in studying the effect of drugs on phagocytosis, and especially for distinguishing particles adherent to cells, but not ingested, from those that are intracellular. Some known effects of cytochalasin B are illustrative. In human PMN treated with cytochalasin B and given staphylococci, the microorganism may adhere to the cell, which may then flow around it but stop short of the membrane fusion that would produce the intracellular (endo
cytic) phagosome (see Fig. 8 of Ref. 4). The particle lies then in a crypt, surrounded by the cell but not intracellular. In the light microscope it would seem intracellular. Even in a thin section cut across the bottom of the crypt, one would see what appeared to be a staphylococcus within a phagosome. If the particle itself were labelled, and remained in the crypt, it would be counted as intracellular. However, if the label were in the medium, as in the present system, the radioactivity could presumably be washed out.

Another advantage of this system is that any particle can be used, so long as it can be washed relatively free of radioactivity. This advantage has permitted recognition of the critical importance of the particle used in studies of phagocytosis, in the results obtained. Some studies of colchicine effects on phagocytosis are illustrative. For example, in the system on which the current method is modelled, colchicine was shown to suppress phagocytosis of starch granules or of Pseudomonas aeruginosa by rabbit exudate granulocytes at a concentration of $1 \times 10^{-4}$ M, but not to affect phagocytosis of Staphylococcus aureus at concentrations as high as $2.5 \times 10^{-3}$ M (12). This work was subsequently confirmed by other methods: in a study with human leukocytes, colchicine, $6 \times 10^{-3}$ M, had little effect on the uptake of heat-killed Staphylococcus aureus or heat-killed Candida albicans, but blocked that of plastic microspheres almost completely (13). Such studies might explain previous conflicting reports of systems in which colchicine did inhibit phagocytosis (urate crystals [14], paraffin oil emulsified with bovine albumin [15]), or did not (staphylococci [11, 16], E. Coli [16], Serratia marcescens [16]).

Studies with cytochalasin B. The importance of the particle is emphasized further by the present studies with cytochalasin B. At $5 \mu g$ per ml, the uptake of radioactivity during phagocytosis of live staphylococci was diminished somewhat, but with starch was suppressed almost completely (Fig. 5). At $2 \mu g$ per ml, there was still inhibition of uptake when starch was used (Fig. 6B), but stimulation when live staphylococci were used (Fig. 6A; Table 1). Thus the same concentration of cytochalasin B was either inhibitory or stimulatory, depending upon the particle employed. By decreasing the concentration of cytochalasin B further (to $0.5 \mu g$ per ml), one could also demonstrate stimulation of uptake during phagocytosis of starch (Fig. 6B).

These particle-dependent differences in the effects of cytochalasin B can be at least partially attributed to a heat-sensitive product of the live staphylococcus, which has raised the threshold of cellular response both for inhibition and for stim-
ulation. With heat-killed staphylococci, the range of inhibition and stimulation by cytochalasin B was more like that with starch than that with live staphylococci (Fig. 7). The inhibitory and stimulatory effects on uptake of radioactivity were confirmed microscopically, by actual counts of cell-associated bacteria (Table 2).

The biphasic effect of cytochalasin B on phagocytosis has not been reported previously (2–7), probably because sufficient distinction was not made between adherent and intracellular particles, as discussed earlier, or because the concentrations of cytochalasin B used were too high. However, biphasic effects of the drug have been seen on other cellular functions. For example, cytochalasin B, 2–4 μg per ml, reversibly inhibited the chemotactic response of human and rabbit PMN to a bacterial chemotactic factor or to the complement-derived factor, C5a; at lower concentrations (0.1–1.0 μg per ml), the drug stimulated chemotaxis (17). Although our shaking phagocytic system is designed to provide easy contact between particle and cell, a similar mechanism to the one operant in the chemotactic system might obtain, with the range of response dependent upon the amount of bacterial product available.

In another study, cytochalasin B, 5 μg per ml, appeared to inhibit the synthesis of DNA by normal rat lymph-node cells following stimulation by phytohemagglutinin or by suboptimal doses of concanavalin A; in the range 0.2–1.0 μg per ml, the drug stimulated DNA synthesis under the same conditions (18). However, in our system the PMN are post-mitotic cells, and, unless other (dividing) leukocytes in the media can rapidly modify the function of PMN, DNA synthesis is not likely to be involved.

The reasons for these biphasic effects are as yet unknown. They could result from (a) facilitation (stimulation) and, at higher doses, disorganization (inhibition) of a single function that is initially somewhat suppressed, (b) different effects predominating on the same cell at different concentrations (e.g., on glucose transport [19–21], functions of microfilaments [22], membrane fusion [23]), or (c) effects on different cell types or sub-types present in the media.

**SUMMARY**

The concomitant uptake of radioiodinated (\(^{131}\)I) human serum albumin (\(^{131}\)I-HSA) appears to provide a sensitive measure of the extent of phagocytosis by human blood leukocytes. Cell-associated radioactivity varied with the ratio of particles to polymorphonuclear leukocytes (PMN), and with the time and temperature of incubation. In resting leukocytes, cell-associated radioactivity did not increase with time, thus providing no evidence for pinocytosis or other means of transport into resting cells.

The particle employed in studies of phagocytosis was of critical importance in the results obtained. Cytochalasin B, μg/ml, diminished somewhat the uptake of radioactivity during phagocytosis when live staphylococci were used, but inhibited uptake almost completely when starch particles were used. Two micrograms per milliliter still suppressed uptake with starch, but stimulated uptake with live staphylococci. Thus, the same concentration of drug was either inhibitory or stimulatory, depending upon the particle employed. A still lower concentration of cytochalasin B, 0.5 μg/ml, stimulated uptake when starch was used. With staphylococci that were heat-killed, the range of inhibition and stimulation by cytochalasin B was more like that with starch than that with live staphylococci. The increased threshold of cellular
response seen with live staphylococci both for inhibition and for stimulation, can be at least partially attributed to a heat-sensitive product of the live bacteria. Both the inhibitory and the simulatory effects of cytochalasin B were confirmed microscopically, by the actual counts of cell-associated bacteria.

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