Variants Deficient in Phagocytosis of Latex Beads Isolated from the Murine Macrophagelike Cell Line J774

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ABSTRACT   Variants that lack the ability to ingest latex beads were isolated from the mouse macrophagelike cell line J774. Carboxylated latex beads were derivatized with polylysine and then daunomycin by a carbodiimide method. Cells that ingested such beads were killed; variants that survived were isolated. Variants were detected at very low frequency and only after nitrosoguanidine mutagenesis. Of 11 independent isolates, 10 showed a lowered rate of uptake of polystyrene beads (without daunomycin). All of these proved normal in rate and extent of Fc-mediated phagocytosis. There was essentially no change in sensitivity to free daunomycin in the variants compared to the parent. These results support the previous hypothesis that there are differences in the metabolic routes of receptor-mediated and nonspecific phagocytosis.

Phagocytosis, one of the important differentiated functions in macrophages, has been grouped into receptor-mediated and receptor-independent or general phagocytosis (1). Among the receptors that mediate the former process are the Fc receptors which recognize the Fc portion of antibody (2, 3), the C3b receptor that recognizes the C3b fragment of the complement protein C3 (4, 5), and a receptor for fibronectin (6); some evidence suggests that the receptor for pinocytosis of mannose, fucose, and N-acetyl-glucosamine-terminated glycoconjugates (7, 8) can also function in phagocytosis (9). Several previous studies suggest that there are some different essential metabolic steps for receptor-mediated and general phagocytosis, in addition to the obvious lack of receptors for the latter. Thus, Michl et al. (10) showed that under certain conditions 2-deoxyglucose inhibited receptor-mediated phagocytosis, in a mannose-reversible process, but did not inhibit either receptor binding or general phagocytosis. In other experiments, Muschel et al. (11) isolated variants of a macrophagelike cell line that were defective in Fc-mediated phagocytosis although they possessed Fc receptors; such variants were able to phagocytose via general phagocytosis. These findings suggest that some of the processes subsequent to receptor recognition differ for each type of phagocytosis. To study this phenomenon it would be helpful to have variants that are the reverse of the ones found by Muschel et al. (11); in other words, ones that cannot phagocytose via generalized phagocytosis, but can do so via receptor-mediated phagocytosis. We herein report the isolation and characterization of 10 such lines, isolated after separate mutageneses, from the macrophagelike cell line J774.

MATERIALS AND METHODS

Reagents and Media

Poly-L-lysine hydrobromide (mol wt 330,000), daunomycin hydrochloride, sodium borohydride, and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene-sulfonate (water-soluble carbodiimide) were obtained from the Sigma Chemical Co., St. Louis, MO. Carboxylate-modified latex beads (0.93 μm, carboxylated beads) were a product of the Dow Chemical Co., Indianapolis, IN. Sheep erythrocytes (SRBC)[1] in Alsever's solution were obtained from the animal center of the National Institutes of Health, Bethesda, MD. Rabbit antibody, IgG fraction, against SRBC was from Cappel Laboratories Inc., Cochranville, PA. Alpha-Eagle's minimum essential medium and L-glutamine, 200 mM, were from M.A. Bioproducts, Walkersville, MD. Penicillin (10^4 U/ml)-streptomycin (10^4 U/ml) and kanamycin solution (10^3 μg/ml) were purchased from the Grand Island Biological Co., Grand Island, NY. Fetal bovine serum was from the Reheis Chemical Co., Kankakee, IL. Phosphate-buffered saline (PBS), pH 7.2, contains 0.01 M phosphate and 0.14 M NaCl. [2-^{3}H]Glycine (15 Ci/mmol) was obtained from New England Nuclear, Boston, MA.

Preparation of Modified Latex Beads

Polylysine beads and daunomycin beads were prepared basically according to a method developed by Dr. April Robbins, National Institutes of Health,

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[1] *Abbreviations used in this paper:* PBS, phosphate-buffered saline; SRBC, sheep erythrocytes.

Bethesda, MD, who kindly communicated this unpublished method to us.

POLYLYSINE BEADS: Carboxylated beads, 5 ml, were washed by centrifugation three times with water and once with 1 M pyridine HCl, pH 8.0. The pellet was suspended with gentle stirring in 2 ml of pyridine-HCl, (1.0 M, pH 8.0) containing 50 mg of polylysine, for 2 h. 2 ml of 5.2 M pyridine HCl, pH 5.2, was added, and 0.25 ml of 1 M water-soluble carbodiimide dissolved in 5.2 M pyridine HCl, pH 5.2, was added four times at 1-min intervals. The reaction was continued with stirring at room temperature for 2 d. After that, water-soluble carbodiimide was again added as described above and the reaction was continued for 1 d more. The beads were washed by centrifugation with water three times and with PBS two times.

DAUNOMYCIN BEADS: Daunomycin (20 μmol) was dissolved in 0.4 ml of PBS and oxidized with sodium periodate (20 μmol) at room temperature in the dark for 1 h. Glycerol was added to stop the reaction. The oxidized daunomycin was mixed with 1 ml of polylysine beads (2.2 × 10^{10} particles), which had been washed with 0.15 M KHCO$_3$, pH 9.2, and suspended in the same buffer. The reaction was allowed to proceed for 2 h in the dark at room temperature with occasional mixing. The mixture was then reduced with sodium borohydride (10 μmol) at 37°C for 2 h. The solution was acidified to approximately pH 5.5 with acetic acid, and centrifuged. The product was extensively washed with PBS and PBS containing 10% fetal bovine serum until daunomycin was no longer visible in the supernatant fluid.

Assay for Phagocytosis

General phagocytosis was assayed by measuring uptake of [^{3}H]glycine-coupled beads, which were prepared by a modification (12) of a previously described method (13). Suspended cells (0.5 ml, 5 × 10^5 cells/ml) were incubated with 35 μl of radioactive beads (3,300 cpm, 5 × 10^7 particles) in plastic tubes for varying lengths of time with shaking unless otherwise noted. Cells were separated as follows: 1 ml of 15% sucrose or 20% glycerol in PBS was added carefully along the side of the tube. The sucrose or glycerol solution layers at the bottom of the tube, and centrifugation for 10 min at 500 g yields a pellet of cells at the bottom and free beads at the interface. Cells were washed twice by the same method (addition of 0.5 ml of PBS, mixing, addition of 1 ml of 15% sucrose or 20% glycerol, and centrifugation), solubilized with 0.2 ml of 1% SDS in PBS, and counted.

Fc receptor-mediated phagocytosis was assayed by measuring uptake of antibody-coated SRBC. 1 ml of 5% (vol/vol) SRBC in PBS was incubated with 20 μl of the IgG fraction of rabbit anti-SRBC at 37°C for 20 min essentially as previously described (4). Macrophages (2–5 × 10^4 as a monolayer in 35-mm dishes) were incubated for 1 h with 1.5 ml of medium containing 10% heat-inactivated fetal bovine serum. The IgG-coated SRBC (1.2 × 10^7 in 1.5 ml) were then added, and incubation was continued at 37°C for 1 h. The cells were washed with PBS and rapidly (for 20 s) with water to externally lyse SRBC. PBS was added back and the ingested SRBC were counted under the microscope. At least 100 macrophages in five fields were scored for each point. For the Fc-binding assay, incubation and washing were performed at 4°C and washing with water was omitted.

Assay of Daunomycin Sensitivity

Cells were cultured in bottles with shaking. Logarithmically growing cultures were subdivided and the indicated concentrations of daunomycin added. Aliquots were withdrawn at various times during growth and counted in a Neubauer chamber; viability was assayed by exclusion of Trypan Blue. 20–30 fields (between 500 and 1,000 cells) were counted for each point.

Cell Culture and Cloning

The mouse macrophagelike cell line J774 (14, 15) was kindly provided by Dr. Peter Ralph, Sloan-Kettering Institute for Cancer Research, Rye, NY. The cells were grown as a monolayer in a tissue culture flask or as a suspension in a bottle with shaking at 150 rpm, in α-Eagle's minimum essential medium containing fetal bovine serum (10%), penicillin (100 U/ml), streptomycin (100 μg/ml), and kanamycin (100 μg/ml) at 37°C in a 5% CO$_2$-95% air mixture. The cells were cloned twice by dilution to single colonies. One clone was used for all subsequent work in isolating variants. A similar procedure was used for cloning the variants.

Mutagenesis

J774 cells (10^7 cells of the above clone) were incubated in a T-75 flask in complete medium for 16 h at 37°C, after which N-methyl-N'-nitro-N-nitroso-guanidine was added to a final concentration of 5 μg/ml and incubation continued for another 16 h. The cells were washed with PBS and cultured for

2 more d. After 2-d growth such cultures had 5–10% as many cells as did untreated control cultures.

Selection of Variants

Mutagenized cells (1–2 × 10^6) were incubated with daunomycin beads 1–2 × 10^8 particles) in T-75 flasks at 37°C for 18 h, washed twice with PBS, and cultured for 2 more d. The remaining cells were washed and again incubated with daunomycin beads for 18 h. They were then cultured for 3–7 more d and exposed a third time to daunomycin beads. As a control for the occurrence of spontaneous mutations on intermediate cultivation, flasks containing unmutagenized cells were carried through the entire procedure. In addition, flasks containing normal and mutagenized cells were grown without exposure to daunomycin beads, to provide an estimate for cell growth under nonselective conditions. At several stages during this procedure, the number of viable cells was estimated. Because it was impractical to use Trypan Blue to test viability when we wished to cultivate the cells further, viability was estimated by counting the number of cells per unit area that appeared healthy on phase-contrast microscopy (well-defined nucleus and organelles, well spread, normal in shape). Data on the survival and characteristics of these cultures will be provided in Results.

RESULTS

Toxicity of Daunomycin Beads

The principle of selection is dependent on the ability of cells to ingest the daunomycin beads and of the ingested beads to kill the cells. The effect of daunomycin beads on cell viability was therefore measured.

Cells were incubated with various amounts of daunomycin beads for several hours, extensively washed with PBS to remove free daunomycin beads, and further incubated. Colonies that formed after 7 d of incubation were counted. As shown in Fig. 1, daunomycin beads could kill this clone of J774 completely. The extent of killing was dependent on the time and concentration of beads, and also on the bead-to-cell ratio. Colony forming ability was <0.05% after 4 h of exposure of 2 × 10^4 cells to 3 × 10^7 beads. When polylysine beads not coupled to daunomycin were incubated with cells at these concentrations, the cells retained 80% of their colony-forming ability relative to an untreated control.

Selection of Variants

When cells (10^4 or 10^5) were mutagenized and then exposed to 3 × 10^7 daunomycin beads under the conditions shown in Fig. 1, no viable cells were obtained. The starting cell number was therefore increased to 10^7. Several flasks of cells were
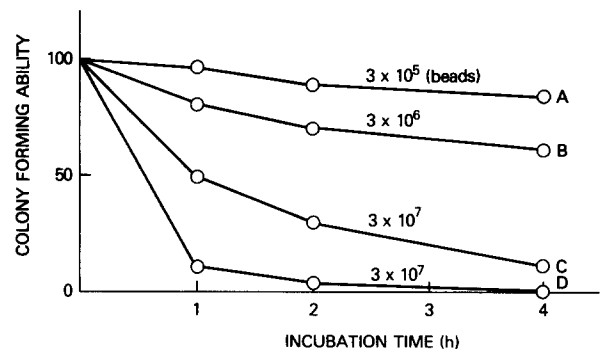

FIGURE 1 Killing of J774 cells by daunomycin beads. Daunomycin beads were added to cells as described at zero time. A, B, and C had 6.5 × 10^4 cells, and D had 2 × 10^4 cells. Numbers of beads (in 2 ml of medium) were shown. Colony-forming ability is given as percentage compared with a culture treated similarly, but with daunomycin beads omitted.

independently subjected to mutagenesis, and selection procedures were applied as described in Materials and Methods.

Inasmuch as the bead-to-cell ratio affects rate and extent of killing (see Fig. 1), and inasmuch as it was technically impractical also to raise the bead concentration 100- to 1,000-fold to compensate for the increased cell number, several cycles of selection were necessary to reduce viability sufficiently for effective selection. 2 d after the first treatment with daunomycin beads, ~90% of the cells were dead. The remaining live cells were subjected to a second cycle of selection, which resulted in reduction of viability to 0.5% of that of a control culture. In contrast, only 0.04% of unmutagenized control cells in five independent flasks survived a second selection. A third cycle of selection completely killed the control cells (<10 viable cells per flask), while $3-7 \times 10^3$ cells/flask of previously mutagenized cells in each of 11 separately mutagenized cultures survived (5×10^{-4} to 1×10^{-3} percent viability compared to the mutagenized cells grown identically but not treated with daunomycin beads). The cells obtained in these experiments showed 50-80% colony-forming ability when tested under the conditions used for Fig. 1, using a bead-to-cell ratio of 1,500, compared to 0.05% colony-forming ability of the parent strain treated similarly.

The isolates have been stable in their characteristics, specifically their efficiency of general phagocytosis (see below), since their initial isolation (~18 mo). However, the variants are apparently labile to freezing and storage using standard conditions (10% glycerol, slow freezing, storage at $-150°C$ [16]), especially those lines most impaired in general phagocytosis. Specifically, after 10 mo of storage, only 10% of vials containing the four most impaired lines contained sufficient viable cells to be recultured, whereas all vials of the parent cell line contained a high percentage of viable cells. We now thaw and reculture vials of variant cells at least once every 6 mo to avoid this problem. The reason for this lability to freezing has not yet been determined.

Determination of Assay Method for General Phagocytosis

Attempts to assay uptake of labeled beads using cells adhering to glass coverslips yielded poor results because the beads adhered to the coverslips. We therefore used the method described in Materials and Methods that permits virtually complete removal of external beads. As shown in Fig. 2, using this method the uptake of [^{3}H]glycine beads at 37°C with or
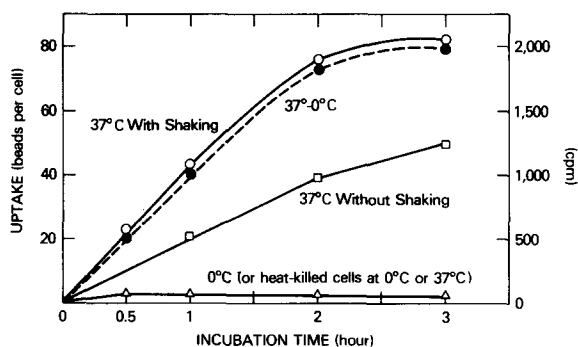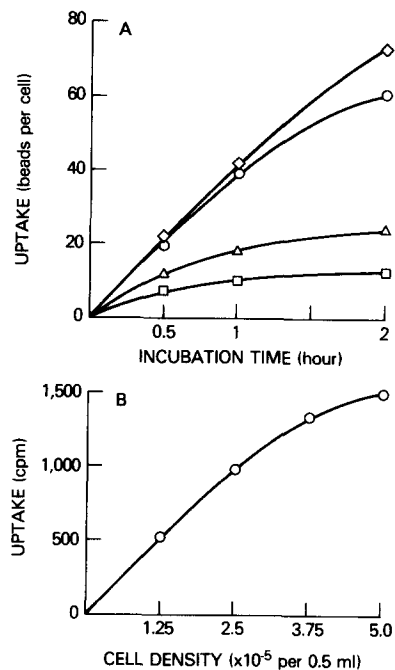
FIGURE 3 Effect of cell and bead concentration on uptake of glycine beads by J774. (A) per 0.5 ml assay mix, 2.5×10^5 cells. Beads/0.5 ml; ◇, 1×10^8; ○, 5×10^7; △, 2.5×10^7; □, 1.25×10^7. (B) 5×10^7 beads per 0.5-ml assay mix. Uptake was measured after 1 h.

without shaking was linear up to almost 2 h of incubation, and shaking increased uptake. Control experiments were performed by incubating the cells at 0°C in an ice water bath or by using heat-killed cells (60°C, 15 min). Controls yielded very low values that were approximately the same as obtained without cells, indicating that no significant binding of the beads to cells was detectable in this assay. The uptake of beads in this and subsequent experiments is defined as the uptake at 37°C (with shaking) minus that at 0°C (closed circles and dashed line, Fig. 2). The beads taken up per cell were calculated from the known specific activity of the beads.

Uptake was dependent on the concentration of beads up to 200 beads per cell. Higher ratios of beads did not increase the rate (Fig. 3A). Uptake was also dependent on cell number, and linear up to 2.5×10^5 cells/assay (5×10^5/ml) (Fig. 3B). Based on these results, subsequent assays used 2.5×10^5 cells and 5×10^7 beads per 0.5-ml assay.

Uptake of Beads by Variants

Phagocytosis of glycine beads by the variants was measured. The time course of uptake of beads by three such variants is shown in Fig. 4. A total of eight variant lines were tested by this means and the results are summarized in Table I and Fig. 5. All except one (3SE5) were significantly lowered in the rate of phagocytosis compared to the parent strain, although none completely deficient in phagocytosis was observed.

Daunomycin Sensitivity of the Variants

The above results indicated that the variants had changed in phagocytosis of beads, but it was necessary to determine whether they might be changed in sensitivity to daunomycin, which would provide a further reason why they show reduced sensitivity to daunomycin beads. The parent line and seven

FIGURE 2 Uptake of glycine beads by J774. Per 0.5 ml assay mix there were 2.5×10^5 cells and 5×10^7 beads. Cells at 0°C, or heat-killed cells at 0°C or 37°C, △; 37°C without shaking, □; 37°C with shaking, ○; uptake at 37°C with shaking minus uptake at 0°C, ●.
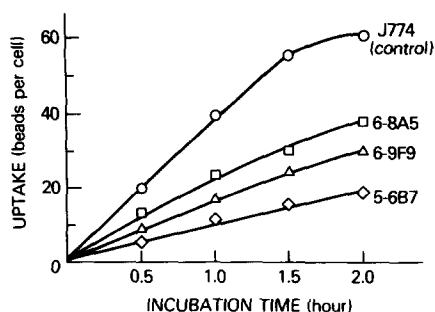
FIGURE 4   Uptake of glycine beads by parent and three variant strains. Conditions as for dotted line on Fig. 2.

TABLE I

General Phagocytosis of Variants

Strain	Uptake
	%
J774 (control)	100*
3-S E-5	90
5-2 E-8	70
4-S A-9	70
6-2 F-12	67
5-1 H-5	62
6-8 A-5	60
6-1 H-5	50
6-8 D-9	52
6-9 F-9	40
5-5 D-2	35
5-6 B-7	32

* Uptake was measured after 1 h of incubation. Control (parental J774) cells took up 40 beads per cell; this value was set as 100%.

variants were tested for growth inhibition, initially in the range of 10^{-9} to 10^{-6} M daunomycin; more detailed growth curves were then performed in the range of 10^{-8} to 10^{-7} M. Both the minimum inhibitory concentration of daunomycin and the concentration required to yield a half-maximal growth rate was calculated. Fig. 6 and Table II show that there were no consistent differences among variants and the parent line. Two variants were slightly more sensitive to daunomycin than the parent and one was slightly less so; all others were identical. Even those differences that exist were very slight (compare Fig. 6A and B). Thus, differences in sensitivity to daunomycin cannot explain the differences between the variant and parent cell lines.

Fc-Mediated Phagocytosis by the Variants

In previous experiments, variants deficient in Fc-mediated phagocytosis have been selected (11); these proved normal in nonspecific phagocytosis. Variants lacking Fc-receptors have also been isolated (3, 17). We therefore tested the ability of our variants to bind and ingest via Fc-receptors. Binding (measured at 4°C) and ingestion (at 37°C) are shown in Table III. The results obtained for receptor-mediated and general phagocytosis are compared in Fig. 5. Most of the variants were comparable to the parent cells in ability to bind and ingest IgG-coated SRBC, and there was no correlation between loss of general phagocytosis and Fc-mediated phagocytic ability.

DISCUSSION

Toxic particles or complexes will kill cells that ingest them, and spare, or select, those that do not. This approach has already been used to kill primary macrophages with immune complexes or ricin (18) and to select variants deficient in Fc receptor-mediated phagocytosis using IgG-coated SRBC containing the toxic drug tubercidin (11). We have now used a
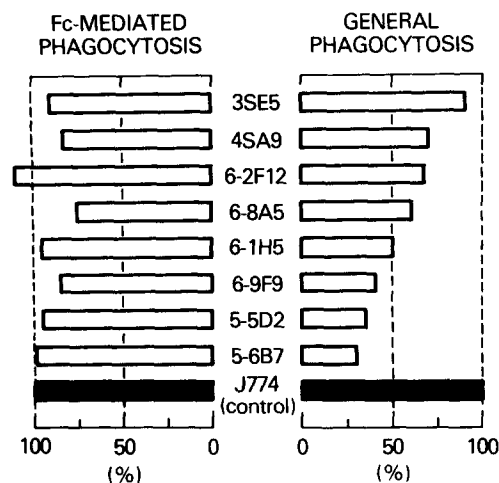

FIGURE 5   Comparison of Fc-mediated and general phagocytosis in variants. The assay for Fc-mediated phagocytosis was as described in Materials and Methods. General phagocytosis was measured by uptake of glycine beads, with incubation for 1 h, with conditions as for the dotted line in Fig. 2. Uptake by the parent strain J774 was set to 100% for both types of phagocytosis; this was equivalent to uptake of 40 glycine beads per cell, and 12 SRBC per cell.
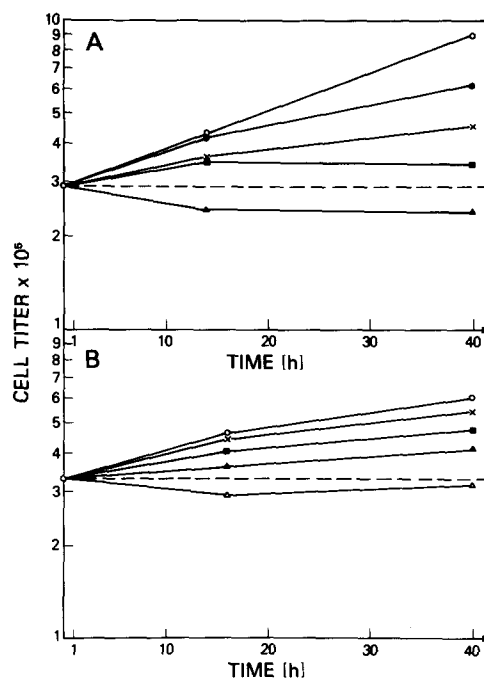

FIGURE 6   Sensitivity to free daunomycin of J774 and one variant. (A) J774. (B) Variant 6-9 F-9, the only strain showing enhanced resistance. Concentration of daunomycin: O, 0; ●, 3.3 × 10^{-8} M; x, 5 × 10^{-8} M; ■, 6.6 × 10^{-8} M; ▲, 8.3 × 10^{-8} M; △, 1.0 × 10^{-7} M.

TABLE II

*Sensitivity of Strains to Daunomycin**

Strain	Inhib$_{50}$[†] Daunomycin concentration $\times 10^{-8}$ M	Minimal inhibitory concentration Daunomycin concentration $\times 10^{-8}$ M
J774	4.5	8.3
4-SA-9	4.65	8.3
6-2F12	4.65	8.3
6-1H5	2.8	6.6
6-8A5	4.6	8.3
6-9F9	6.6	10.0
5-5D2	1.25	6.6
5-5B7	4.5	8.3

* Cells were tested for sensitivity to daunomycin as shown in Fig. 6.
† Inhib$_{50}$, daunomycin concentration yielding half-maximal growth rate.

TABLE III

Fc-Mediated Phagocytosis of Variants

Strain	Ingestion* 37°C	Binding* 4°C
	%	%
3-SE-5	90	70
4-SA-9	83	85
6-8D-9	82	45
6-IH-5	95	90
6-2F-12	110	120
6-9F-9	83	110
6-8A-5	75	103
5-5D-2	95	70
5-6B-7	102	120

* Control cells bound 8 IgG-coated SRBC and ingested 12 IgG-coated SRBC per cell; these values were set as 100%.

conceptually related method devised by Dr. A. Robbins to select variants presumed to be deficient in general phagocytosis, by coupling polystyrene beads to a polylysine spacer and thence to daunomycin. This drug inhibits both transcription and translation, presumably because of its ability to intercalate into DNA (19–22), although a recent report that it can cross-link DNA (23) suggests the possibility of a slightly different mechanism. We presume that daunomycin is hydrolyzed from the bead and exits from the phagolysosome to the cytoplasm, killing the cell.

There are several theoretical methods of acquiring resistance to daunomycin-coupled beads. These include inability of the cell to phagocytose the bead, inability to hydrolyze the daunomycin from the bead, and resistance of enzymes to daunomycin. Of 11 independent isolates selected, we have found that 10 are reduced in rate of phagocytosis; only one shows resistance without lowered ingestion. There is no consistent or significant difference in resistance to daunomycin in the variants. Therefore, we conclude that this method is promising for selection of phagocytosis variants.

However, although it is logical that the observed deficiency in phagocytosis causes, or is directly related to, the resistance to daunomycin beads, this hypothesis has not been proven. Because the partial defect in uptake of beads is associated with complete resistance to the toxin effects of daunomycin beads, the two might be unrelated. However, very small changes in concentration of daunomycin can be associated with large changes in viability of cells (Table II, Fig. 6), so that a small reduction in bead uptake might conceivably have a large effect on survival. Alternatively, it is possible that a single membrane defect might simultaneously reduce bead uptake and reduce transfer of daunomycin from beads to its site of action in the nucleus (for instance, a defect in phagosome fusion, or in hydrolysis of daunomycin from the bead, or in its exit from the phagolysosome). Further experiments will be necessary to establish the events causing this variant phenotype.

We have been able to isolate variants from 11 of 12 separate mutageneses reported here, and 4 out of 5 performed at another time. In contrast, five unmutagenized cultures, tested in parallel to those described here, plus three done at other times, starting with over 10^7 cells/flask, yielded no variants. We therefore postulate that the selection depended on mutation induced at a discrete point in time by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and not a selection of a series of mutational events accumulated in the culture thereafter. These controls were necessary because, for technical reasons, it was necessary to perform three separate exposures to daunomycin beads after mutagenesis, the first and second separated by two doublings, and the second and third by three to eight doublings. However, the control experiments strongly suggest that the number of cells thus generated is sufficiently below the spontaneous mutation rate to exclude the accumulation of several mutations as a mechanism for generation of these variants. In the absence of mapping data or isolation of altered genes this conclusion remains tentative, and so we prefer to designate these lines as variants rather than mutants.

It is striking that the variants thus isolated show no defect in receptor-mediated phagocytosis. This fact provides ample confirmation for the previous postulate that receptor-mediated and general phagocytosis differ in one or more steps. Previous data include the finding of Muschel et al. (11) that variants in Fc receptor-mediated phagocytosis were normal in generalized phagocytosis and the finding of Michl et al. (10) that 2-deoxyglucose selectively inhibited Fc and C3b receptor-mediated phagocytosis without altering either receptor binding or generalized phagocytosis, and our finding the Fc receptor-mediated phagocytosis can be induced by dimethylsulfoxide in a cell line normally lacking it, without affecting general phagocytosis (12). There are, of course, steps in common; for instance, cytochalasin B inhibits both forms of phagocytosis (24, 25), presumably by affecting contractile protein function (26–28). The contractile proteins are involved in pseudopod extension, membrane fusion, and phagosome formation (29, 30) and therefore would be expected to participate in both processes. The variants isolated in this study presumably do not have defects in such processes.

Phagocytosis includes binding followed by ingestion. Specific receptors are involved in receptor-mediated phagocytosis. The nature of the binding structure, if any, in generalized phagocytosis, is unknown. With our method of measuring uptake, we detect virtually no binding of noningested polystyrene beads (see Fig. 2). However, it is possible that binding is so weak that the procedure of separating the beads from the cells by centrifugation removes the beads from the cells, or binding may be too low to detect. It should be noted that these variant lines show approximately normal adhesiveness, as judged subjectively by ease of dislodging the cells from plastic surfaces. This result suggests that there are differences between adhesiveness to plastic and ingestion of plastic beads, as there are between binding to Fc-receptors and subsequent

Fc-mediated ingestion. However, quantitative studies of adhesion are planned to study this hypothesis more thoroughly.

In these and other selections we have never isolated a variant completely lacking generalized phagocytosis. This fact may be accidental or, alternatively, such a complete defect might be lethal. We will attempt to isolate temperature-sensitive variants in the future to distinguish between these possibilities. However, if mutants need only be partially blocked in phagocytosis of daunomycin-derivatized beads to survive (see above), derivatization with another toxic agent might provide a more stringent selection.

These findings and those of other workers suggest that quite separable and independent processes are involved in receptor-mediated and general phagocytosis. The variants described herein, and others isolated in the future by this and other methods, may provide help in defining the membrane-catalyzed steps in general phagocytosis, which so far remain rather obscure.

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