

2-DEOXYGLUCOSE SELECTIVELY INHIBITS Fc AND COMPLEMENT RECEPTOR-MEDIATED PHAGOCYTOSIS IN MOUSE PERITONEAL MACROPHAGES

I. Description of the Inhibitory Effect*

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Before the identification of specific receptors for the Fc portion of immunoglobulin G (1) and the third component of complement (2) on the surface of phagocytic leukocytes, Fenn (3) and others (4) suggested that the phagocytosis-promoting properties of serum opsonins could be accounted for by the capacity of these proteins to alter the physicochemical characteristics of the surfaces of pathogenic microorganisms.

Although immunoglobulins and complement probably do alter the charge and topographic distribution of components on the surface of a particle, it is now evident that it is the specific and coordinated interaction between particle-bound ligands (immunoglobulin G and/or complement) and their corresponding receptors (the Fc and C3 receptors) on the plasma membranes of phagocytic leukocytes which is responsible for the ingestion of opsonized particles by these cells (5). In contrast, a variety of nonpathogenic (rough) strains of bacteria as well as carbon particles, latex, and boiled yeast cell walls (zymosan) are readily ingested by phagocytic leukocytes in the absence of serum opsonins. The phagocytosis of these particles is mediated by unidentified plasma membrane components which appear to be structurally (6), functionally (7), and, as we will show, metabolically independent of the Fc and C3 receptors.

The ingestion of particulate materials requires the expenditure of metabolic energy. In general, mononuclear phagocytes derive the bulk of their metabolic energy from the oxidation of glucose to pyruvate and lactate (8). Inhibitors of glycolysis, such as sodium fluoride, reversibly inhibit the phagocytosis of latex and zymosan and of immunoglobulin G- and complement-coated particles (5, 8). In the course of studying the role of the Fc and complement receptors of mouse mononuclear phagocytes in the ingestion of IgG and complement-coated particles, we noticed that the glucose and mannose analogue 2-deoxy-D-glucose inhibited IgG and complement receptor-mediated phagocytosis of opsonized particles, but not the ingestion of latex or zymosan. Further studies have shown that this sugar has no inhibitory effect on the attachment of IgG- or complement-coated particles to their respective membrane receptors.

The data presented in this and in the accompanying paper (9) document these conclusions and suggest that ingestion of particles mediated by the Fc and C3

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receptors (opsonin dependent) and the phagocytosis of latex and zymosan (opsonin independent) differ fundamentally in their metabolic regulation and requirements. Some of these findings have been reported previously in preliminary form (10).

Materials and Methods

Reagents. Minimum essential medium with Earle's salt solution, containing 5.5 mM glucose (MEM),¹ fetal bovine serum (FBS), and 0.4% trypan blue solution were obtained from Grand Island Biological Co., Grand Island, N. Y. Glucose-free MEM was prepared from amino acids (50 times concentrated) containing glutamine, MEM vitamin solution (100 times concentrated), 7.5% sodium bicarbonate solution, and sodium pyruvate (100 times concentrated, tissue culture grade), all from GIBCO. Glucose came from J. T. Baker Chemical Co., Phillipsburg, N. J. Grade III 2-deoxy-D-glucose (2-dG), crystalline β -D(-) fructose, α -L(-) fucose, D(+) galactose, D(+) mannose, and D(+) glucosamine hydrochloride were obtained from Sigma Chemical Co., St. Louis, Mo. Brewer's thioglycollate medium was purchased in powder form from Difco Laboratories, Detroit, Mich., and prepared as a 4.05% aqueous solution according to the manufacturer's instructions. The solution was autoclaved and kept in the dark at room temperature for at least 3 wk before use.

Animals. Swiss mice, maintained pathogen free at The Rockefeller University, New York, of both sexes and weighing between 25 and 30 g, served as the source of peritoneal macrophages.

Cells. Peritoneal macrophages were harvested and maintained in culture by a modification of the method of Cohn and Benson (11). 2 ml of a suspension of 1.25×10^6 normal peritoneal cells/ml (total yield between 6 and 10×10^6 cells per mouse) in MEM supplemented with 20% heat-inactivated (56°C for 30 min) FBS, and 100 μ g/ml of streptomycin (complete medium) were poured into a 35 mm plastic tissue culture Petri dish (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) containing three glass cover slips 1/2 inch in diameter (Gold Seal Coverglass, no. 550; Ace Scientific Supply Co. Inc., Linden, N. J.). The macrophages were allowed to attach to the cover slips for 4 h at 37°C in a humidified atmosphere containing 5% CO₂-95% air. The cover slip cultures were then washed three times with 2 ml of cold MEM without FBS to remove the nonadherent cells, overlaid with 2 ml of complete medium, and further incubated for 20-44 h at 37°C. In some experiments, mice were injected intraperitoneally (i.p.) with 1 ml of Brewer's thioglycollate medium 4 days before their peritoneal exudate cells ($20-35 \times 10^6$ cells per mouse) were harvested. Differential cell counts of these cells revealed an average of 83% macrophages, 15% small and large lymphocytes, and 2% granulocytes. These cells were resuspended in complete medium at a concentration of 0.75×10^6 cells/ml. 2 ml of this cell suspension were plated and treated as described above.

Particles

SHEEP ERYTHROCYTES (E). E in Alsever's solution (from The Rockefeller University) were washed three times in phosphate-buffered saline without Ca⁺⁺ and Mg⁺⁺ ions (PD) [solution "a" of Dulbecco's phosphate-buffered saline (12)] and suspended as a 5% suspension in PBS or Veronal-buffered glucose containing Ca⁺⁺, Mg⁺⁺, and 0.1% gelatin (VBG) (13).

CHICKEN ERYTHROCYTES (Ch E). Ch E were obtained from outbred white chickens kept at The Rockefeller University. They were collected as a 25% suspension in Alsever's solution, washed three times in PD, suspended as a 5% suspension in PBS, and stored at 4°C until used.

PNEUMOCOCCI. *Diplococcus pneumoniae*, Type 1, strain SV-1, was a gift from Doctors Frank M. Griffin and Maelyn McCarty, Rockefeller University. To insure that all pneumococci were of the smooth encapsulated form, the bacteria were passed through mice. Pneumococci were grown for 18 h at 37°C in brain heart infusion (BHI) broth. 0.5 ml of this suspension were injected i.p. into

¹ *Abbreviations used in this paper:* BHI, brain heart infusion; Ch E, chicken erythrocytes; 2-dG, 2-deoxy-D-glucose or 2-deoxyglucose; E, sheep erythrocytes; E(IgG), E coated with anti-E IgG; E(IgM), E coated with anti-E IgM; E(IgM)C, E(IgM) coated with the first four complement components; FBS, fetal bovine serum; medium, unless otherwise specified contains 5.5 mM glucose; MEM, minimal essential medium with Earle's salt solution, containing 5.5 mM glucose; PBS, phosphate-buffered saline with Ca⁺⁺ and Mg⁺⁺ ions; PD, PBS without Ca⁺⁺ and Mg⁺⁺ ions; VBG, Veronal-buffered glucose with Ca⁺⁺, Mg⁺⁺, and 0.1% gelatin.

Swiss mice weighing ~25 g. When the mice died, they were frozen at -20°C until use. After thawing, the heart was removed aseptically and placed in BHI broth at 37°C in a candle jar for 18 h. The resulting pneumococci were harvested by centrifugation in MEM, counted in a Petrof-Hauser chamber, and resuspended in MEM at a concentration of $\sim 4 \times 10^8$ organisms/ml.

LATEX PARTICLES. Latex particles (Dow Chemical USA, Membrane Systems Div., Midland, Michigan) were washed three times by centrifugation (1,200 g, 30 min) in PD, and stored in PD at 4°C . On the day of an experiment the particles were washed once in MEM and resuspended in MEM at a concentration of 5×10^8 1.07- μm diameter particles/ml or as a 2% (vol/vol) suspension of 5.7- μm diameter particles.

ZYMOBAN. Zymosan from yeast (Sigma Chemical Co.) was suspended in PBS, washed three times at 750 g for 10 min, and stored as a 5% solution in PBS containing 0.02% sodium azide. On the day of an experiment zymosan was washed twice in MEM (750 g, 10 min), and resuspended in MEM at a concentration of 1% (vol/vol).

ANTISERA. Anti-E IgG and IgM, both from rabbits, were the same lot number as used previously by Griffin et al. (5), and were obtained from Cordis Laboratories, Miami, Fla.

Rabbit anti-Ch E antiserum, a gift of Dr. Michel Rabinovitch, New York University Medical Center, New York, was heated at 56°C for 30 min to inactivate complement. It had a protein concentration of 77.5 mg/ml and an agglutination titer of 1:8,192.

Rabbit antiserum against Type 1 pneumococci was a gift of Dr. Robert Austrian of the University of Pennsylvania Medical Center, Philadelphia, Pa. It contained 1.8 mg of protein/ml.

COMPLEMENT. Serum, prepared from Swiss mice and stored in 0.2 ml portions at -70°C , served as a source of complement.

Preparation of Antibody- and Complement-Coated Particles

ANTIBODY-COATED E. 1 ml of 5% (vol/vol) E in PD was incubated with either 125 μg of rabbit anti-E IgG or 13 μg of rabbit anti-E IgM for 20 min at 37°C . The cells were washed twice by centrifugation in PD (10 min, 750 g, $+4^{\circ}\text{C}$), and resuspended in PD [E(IgG)] or VBG [E(IgM)] at a concentration of 1% (vol/vol). These preparations are designated E(IgG) [E coated with anti-E IgG], and E(IgM) [E coated with anti-E IgM].

ANTIBODY-COATED CH E. 1 ml of a 5% (vol/vol) Ch E in PD was incubated with 95 μg of rabbit anti-Ch E antiserum for 20 min at 37°C . The cells were washed twice by centrifugation in PD, and resuspended in PD at a concentration of 1% (vol/vol).

COMPLEMENT-COATED E(IgM). 1 ml of 5% (vol/vol) E(IgM) suspended in VBG were mixed with an equal volume of a 1:5 dilution (in VBG) of freshly thawed mouse serum, incubated for 10 min at 37°C , and washed twice in VBG for 10 min at 750 g and $+4^{\circ}\text{C}$. The pelleted erythrocytes were resuspended in MEM at a concentration of 1% (vol/vol). This preparation [1% complement-coated E(IgM)] is designated E(IgM)C.

Oposonized Pneumococci. $\sim 4 \times 10^8$ pneumococci were incubated with rabbit antiserum (18 μg protein) in 1 ml of MEM with or without 2-dG for 30 min at 0°C . To test for phagocytosis, the suspension was added directly to the test Petri dishes. E(IgG), E(IgM), E(IgM)C, and opsonized pneumococci were prepared fresh on the day of an experiment.

Assay System. Unless otherwise specified, macrophages on glass cover slips were washed twice with MEM (37°C) and incubated for the indicated time interval at 37°C in 2 ml of medium, with or without additional sugars, at the final concentrations given in the Results section. At the end of this preincubation period, the medium was either renewed or exchanged for a second incubation medium as indicated in the Results section.

At the times indicated 0.1 ml of the various erythrocyte preparations, latex, or zymosan suspensions were added to each 35-mm Petri dish, and the incubation was continued for 60 min at 37°C . The cover slip cultures were washed twice with cold MEM, fixed with 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.4) for 20 min at 4°C , and examined by phase-contrast microscopy using a 100 \times oil immersion lens. Experiments were done in duplicates. In some of the experiments one of the duplicate cultures was treated for 2 min at room temperature with 0.85% NH_4Cl as described by Boyle (14) before fixation. This treatment lysed the extracellular E and facilitated the evaluation of the number of phagocytized E.

For each determination at least 100 macrophages were evaluated for the number of attached and/or ingested erythrocytes, latex particles, zymosan, and/or pneumococci. The data obtained are expressed as percent macrophages with erythrocytes or pneumococci attached and/or ingested, or

latex or zymosan particles ingested. Ingestion or attachment index is the percentage of macrophages that ingested or attached erythrocytes multiplied by the average number of erythrocytes ingested or attached per macrophage.

Electron Microscopy. Cells were processed for electron microscopy as described (15, 16), and examined in a Siemens Elmiskop 1A (Siemens Corp., Medical/Industrial Groups, Iselin, N. J.). Since latex is extracted by the propylene oxide used in the final steps of dehydration and embedding, absolute alcohol and epon was used in some experiments instead of propylene oxide to preserve latex inside the phagocytic vacuoles of the macrophages.

Miscellaneous. Macrophage viability was tested for each set of experiments by the trypan blue exclusion method described by Boyse et al. (17). Unless otherwise stated, the viability was more than 90% in all experiments reported. Protein was measured by the method of Lowry et al. (18).

Results

Effect of 2-Deoxy-D-Glucose on Phagocytosis by Resident Mouse Peritoneal Macrophages. Monolayers of 24-h explanted normal mouse peritoneal macrophages were incubated for 2 or 4 h at 37°C in MEM containing 5.5 mM glucose and 50 mM 2-dG. At these times the medium was removed and replaced with 2 ml of fresh medium containing the same concentration of 2-dG and 0.1 ml of a 1% suspension of IgG-coated E or 0.1 ml of 1.07- μ m diameter latex particles (5×10^8 particles/ml). The incubation was continued at 37°C for 60 min at which time the cultures were fixed and the number of particles ingested was counted.

As shown in Table I, attachment of the IgG-coated E to the macrophages was not impaired by 2-dG. However, only 14% of the 2-dG-treated macrophages ingested any E(IgG), with an average of 2.2 E(IgG) per macrophage. 79% of the control macrophages ingested 6.1 E(IgG) each. In contrast, latex particles were ingested equally well by 2-dG-treated and control macrophages (Fig. 1). That the latex particles were indeed ingested by the 2-dG-treated macrophages was confirmed by electron microscopy (Fig. 2).

The inhibitory effect of 2-dG on the phagocytosis of IgG-coated E was propor-

TABLE I
*Effect of 2-dG on Phagocytosis of E(IgG) and Latex Particles by Normal Macrophages**

Treatment of macrophages [‡]	Particle	Attachment		Ingestion	
		Macrophages with erythrocytes attached	Average No. of particles attached per macrophage	Macrophages with particles ingested	Average no. of particles ingested per macrophage
		%		%	
5.5 mM glucose	Latex			99	>10
	E(IgG)	97	14.5	79	6.1
5.5 mM Glucose and 50 mM 2-dG	Latex			93	>10
	E(IgG)	99	12.6	14	2.2

* Each value represents the average of five experiments.

[‡] Monolayers of 24-h explanted macrophages were preincubated in the medium indicated at 37°C for 2-4 h at which time the medium was removed, and the macrophages further incubated for 1 h at 37°C in fresh glucose or 2-dG-containing medium to which the test particle was added.

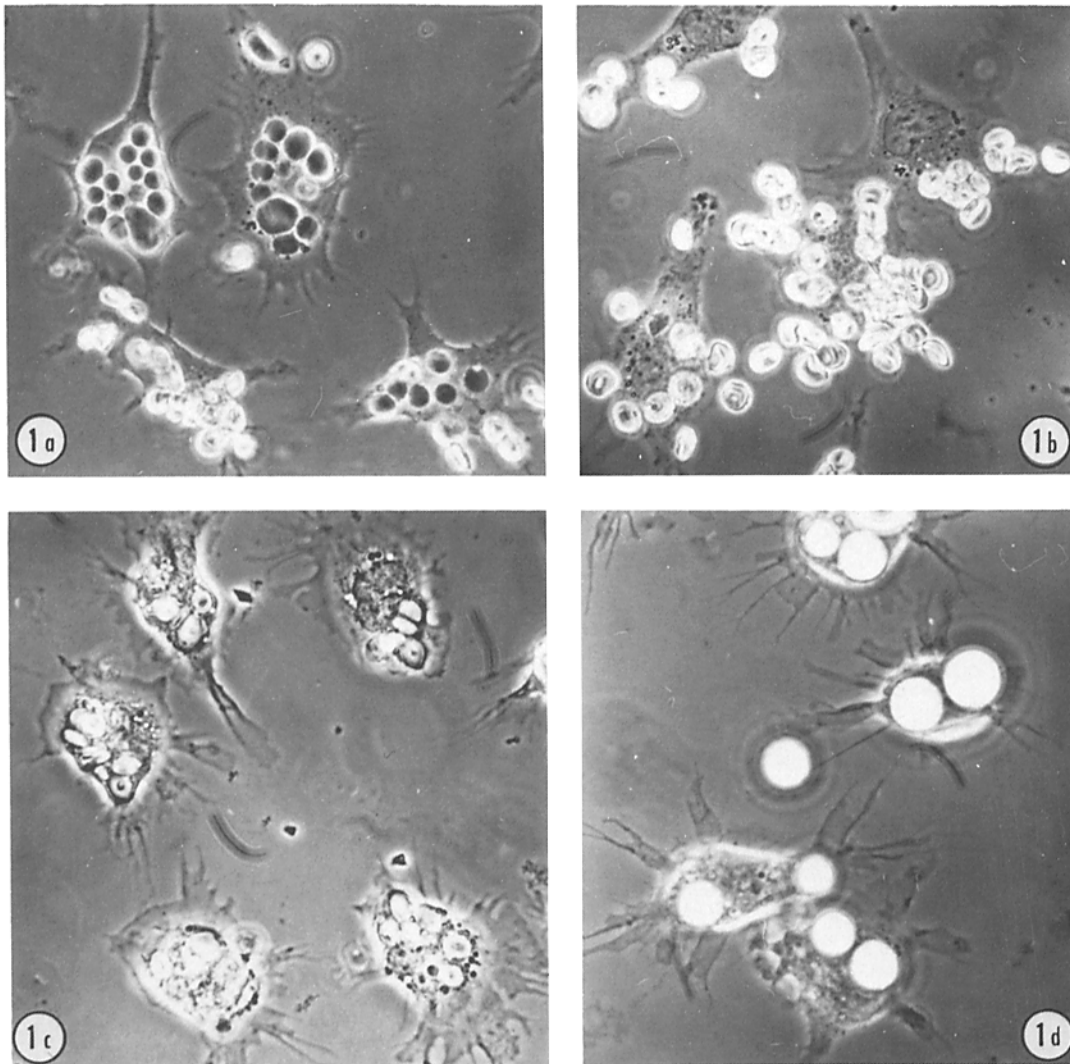


FIG. 1. Normal mouse peritoneal macrophages exposed to different particles in presence or absence of 2-dG. Phase-contrast microscopy ($\times 1,000$). (a) Macrophage incubated in 5.5 mM glucose-containing medium with E(IgG) for 1 h at 37°C. Most of the macrophages have ingested several E(IgG). (b) Macrophages were preincubated in medium containing 5.5 mM glucose and 50 mM 2-dG for 2 h at 37°C, when E(IgG) were added for 1 h at 37°C. There are several erythrocytes bound per macrophage but none is ingested. (c) Zymosan particles were added to macrophages treated as in (b); (d) Latex particles (5.7 μm) were added to macrophages treated as in (b).

tional to the ratio of 2-dG to glucose in the incubation media (Fig. 3). While no inhibition of Fc receptor-mediated phagocytosis was found when equimolar amounts of 2-dG and glucose were present, maximal inhibition of Fc receptor-mediated phagocytosis was observed in the presence of 100 mM 2-dG and 5.5 mM glucose (molar ratio of 2-dG:glucose of 20:1). Inhibition of Fc-mediated phagocytosis in the presence of 50 and 100 mM 2-dG was not a consequence of the

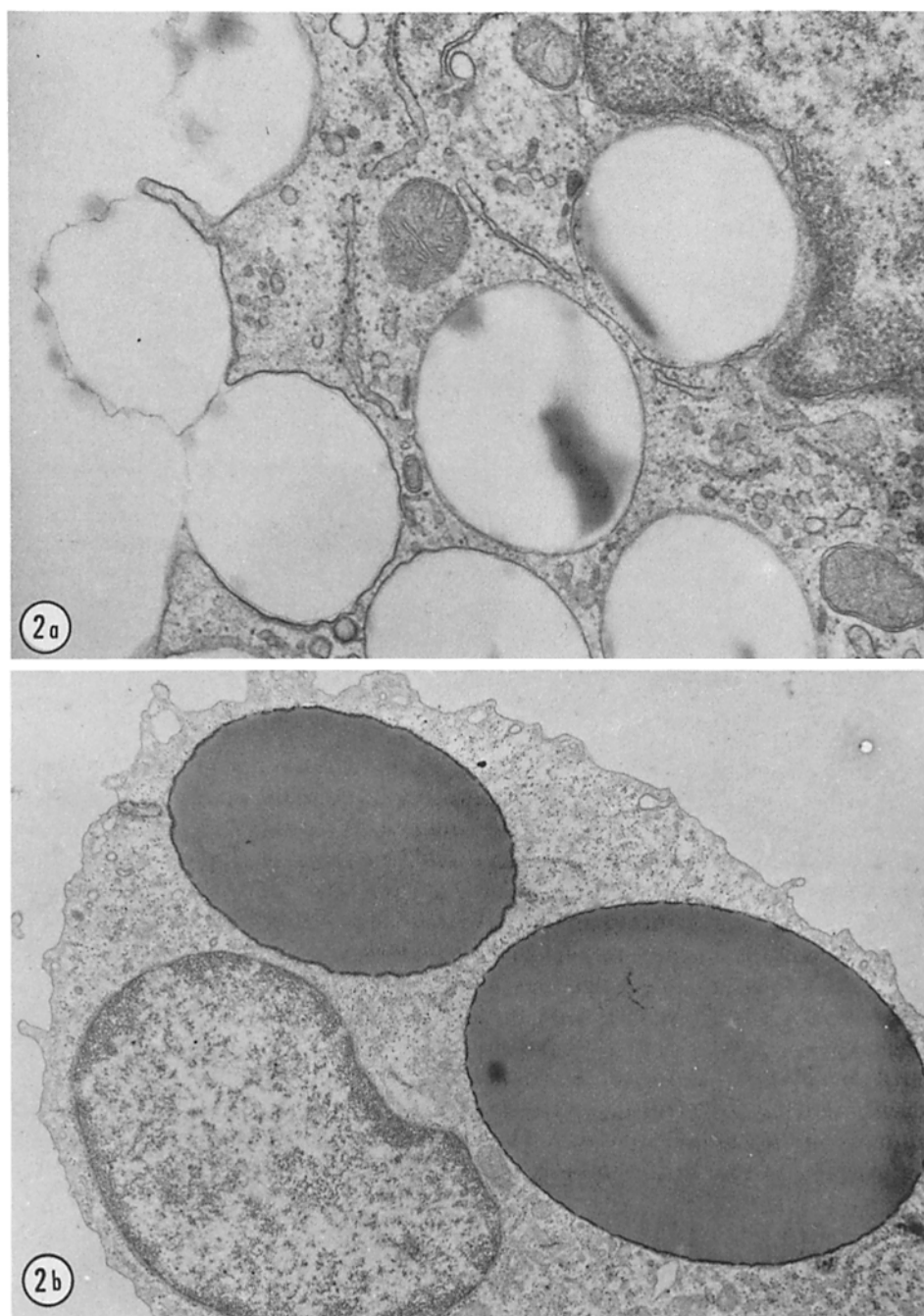


FIG. 2. Electron micrograph of 2-dG-treated normal mouse peritoneal macrophages which have ingested latex particles. Macrophages were incubated for 2 h at 37°C in presence of 50 mM 2-dG at which time 1.07- μ m diameter latex particles (a) or 5.7- μ m diameter latex particles (b) were added to the cultures for an additional hour at 37°C in 2-dG-containing medium. The cells in (a) were dehydrated in propylene oxide, resulting in the extraction of the latex. In (b) absolute ethanol was used instead of propylene oxide; under these conditions the latex was preserved. ((a) \times 28,600; (b) \times 10,000).

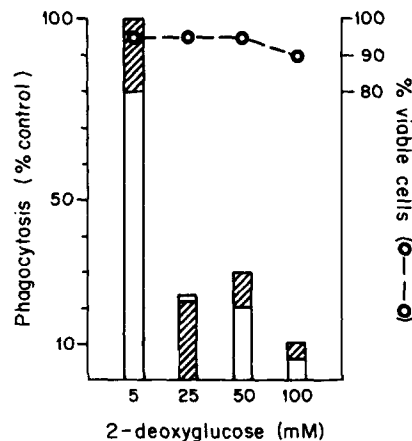


FIG. 3. Inhibition of phagocytosis of E(IgG) by normal macrophages treated with different concentrations of 2-dG. Normal mouse peritoneal macrophages were incubated in medium containing 5.5 mM glucose and varying amounts of 2-dG. (a) After 2 h (open bars) and 4 h (hatched bars) E(IgG) were added for 1 h at 37°C, the preparations were fixed, and ingestion was scored. The ordinate on the left shows percent phagocytosis in relation to the untreated control (see Materials and Methods); (b) the ordinate on the right indicates the percentages of cells excluding trypan blue under the same experimental conditions (○).

increased osmolarity of the medium since addition of 50 or 100 mM glucose to the medium did not inhibit phagocytosis of E(IgG).

Effects of 2-Deoxy-D-Glucose on Macrophage Viability. Macrophage viability was well maintained (>90%) for up to 4 h in serum-free medium containing 5.5 mM glucose and up to 100 mM 2-dG (Fig. 3). However, macrophages incubated under these conditions for more than 4 h showed a marked decrease in viability. Addition of up to 10% (vol/vol) FBS² to these cultures did not improve cell viability. Macrophages incubated in serum-free medium containing 50 mM 2-dG and 5.5 mM glucose for up to 6 h showed no decrease in viability; macrophages incubated for more than 6 h in medium containing 50 mM 2-dG showed decreased viability unless 2-5% FBS was added to the medium. Under the latter circumstances more than 90% of the cells appeared well spread, were capable of ingesting latex particles, and excluded trypan blue for more than 12 h. The presence of FBS in the culture medium, however, did not diminish the 2-dG-mediated inhibition of phagocytosis of E(IgG) by macrophages. Addition of 2-dG in the absence of added glucose caused a marked decrease in cell viability after a 2 h incubation at 37°C. Therefore, in all further experiments we used medium containing 5.5 mM glucose to which sugars were added as noted.

Effect of Pretreatment of E(IgG) with 2-Deoxy-D-Glucose. To determine whether 2-dG inhibited the phagocytosis of E(IgG) by acting on the macrophage or on the erythrocytes, IgG-coated E were incubated for 1 h at 4°C or 37°C in medium containing 50 mM 2-dG; the erythrocytes were then washed, resuspended in 2-dG-free medium, and incubated for 60 min at 37°C with normal macrophages in medium without 2-dG.

The results are shown in Table II. E(IgG) incubated at either 4°C or 37°C in 2-

² In all experiments in which FBS was added to 2-dG-containing media, the FBS was first dialyzed against glucose-free Earle's salt solution.

TABLE II
Phagocytosis of 2-dG Pretreated E(IgG) by Normal Macrophages

Treatment of E(IgG)*	Ingestion of E(IgG)	
	Macrophages with erythrocytes ingested	Average no. of E(IgG) ingested per macrophage
	%	
4°C, 1 h + 50 mM 2-dG → wash → macrophages 37°C, 1 h	92	6.5
37°C, 1 h + 50 mM 2-dG → wash → macrophages 37°C, 1 h	72	6.5

* E(IgG) were incubated in 2-dG-containing medium as shown in the table, washed and resuspended in medium without 2-dG, and added to macrophages that had not been treated with 2-dG.

dG-containing medium were ingested as avidly as those prepared in PD or in medium without 2-dG [compare Table II with data for E(IgG) in Tables I and IV]. This finding clearly indicates that 2-dG exerts its inhibitory effect by acting on the macrophages, and not on the IgG-coated E.

Macrophages Preincubated with 2-Deoxy-D-Glucose at 4°C Retain the Capacity to Ingest E(IgG). At low temperatures the transport and metabolism of a variety of compounds, including sugars, by cells is markedly decreased (19). Attachment of IgG-coated erythrocytes to Fc receptors on the macrophage membrane occurs independently of temperature (5), but the ingestion of these attached erythrocytes is inhibited at low temperatures. Both the decrease in cellular metabolism and the inhibition of phagocytosis are rapidly reversed when the macrophages are warmed to 37°C. We took advantage of the differences in temperature dependence of these processes to determine whether 2-dG has any effect upon the attachment of E(IgG) to the macrophage membrane, and whether this glucose analogue must be transported and/or metabolized to inhibit Fc receptor-mediated phagocytosis.

Normal macrophages were incubated for 15 min at 4°C in either medium alone or in medium containing 50 mM 2-dG. E(IgG) were added, and the incubation at 4°C was continued for an additional hour, at which time the nonattached E(IgG) were removed by washing with cold medium. Some samples were fixed in glutaraldehyde at this point. Other samples were incubated for an additional 60 min at 37°C in medium, or in medium containing 50 mM 2-dG. The presence of 2-dG during the first 60 min at 4°C did not interfere with the attachment of E(IgG) to the macrophages as shown in Table III. Phagocytosis of these attached erythrocytes was not inhibited when the E(IgG)-macrophage complexes were transferred from the cold 2-dG-containing medium into medium without 2-dG at 37°C. In contrast, complete inhibition of phagocytosis was observed when warm 50 mM 2-dG-containing medium was present during the second incubation period at 37°C. These results confirm (Tables I and II) that 2-dG has no effect on the binding of E(IgG) to the macrophage membrane; they further suggest that this sugar must be transported and/or metabolized by the macrophage to inhibit Fc receptor-mediated phagocytosis.

Effect of 2-Deoxy-D-Glucose on the Fate of Preattached E. The experiment described in the previous section suggests that the onset of inhibition of Fc

TABLE III
*2-dG Must be Metabolized by Macrophages to Inhibit Fc Receptor-Mediated Phagocytosis**

Macrophages + E(IgG)	Attachment of E(IgG)		Ingestion of E(IgG)	
	Macro- phages with E(IgG) at- tached	Average no. of E(IgG) at- tached per macro- phage	Macro- phages with E(IgG) in- gested	Average no. of E(IgG) in- gested per macro- phage
	%		%	
4°C, 1 h + glucose medium‡	>99	9.4	—	—
4°C, 1 h + 50 mM 2-dG§	>99	13	—	—
4°C, 1 h + glucose medium‡ → wash → 37°C, 1 h + glucose medium‡	—	—	94	7.1
4°C, 1 h + 50 mM 2-dG§ → wash → 37°C, 1 h + glucose medium‡	—	—	98	7.2
4°C, 1 h + 50 mM 2-dG§ → wash → 37°C, 1 h + 50 mM 2-dG§	>99	9.5	0	0

* Each value represents the average of three experiments.

‡ Medium containing 5.5 mM glucose.

§ Medium containing 5.5 mM glucose and 50 mM 2-dG.

receptor-mediated phagocytosis by 2-dG is very rapid at 37°C. To examine this point more closely IgG-coated E were attached to normal macrophages in medium without 2-dG at 4°C. The nonattached erythrocytes were removed, and the cover slip cultures were incubated at 37°C in medium with or without 50 mM 2-dG. At appropriate time intervals samples were removed, fixed in glutaraldehyde, and the number of erythrocytes attached or phagocytized was counted. By this method the time required for attachment of erythrocytes to the macrophage surface can be discounted, and the rate of ingestion is the only parameter being measured.

As shown in Fig. 4, normal macrophages incubated in glucose-containing medium begin to phagocytize the preattached E(IgG) immediately after transfer to 37°C, and by 15–30 min the major proportion of the erythrocytes have been ingested. In contrast, virtually no phagocytosis was observed when the E(IgG)-macrophage complexes were incubated at 37°C in medium containing 50 mM 2-dG for up to 2 h. Thus, when used at an optimal concentration and at 37°C, 2-dG exerts its inhibitory effect upon Fc receptor-mediated phagocytosis within 15–30 min of its addition to the medium.

Previous experiments showed that the blockade of Fc-receptor function mediated by anti-macrophage IgG is relative, not absolute. That is, anti-macrophage IgG-treated macrophages could be induced to ingest preattached E(IgG) by addition of a supra-agglutinating amount of anti-E IgG (5). Using similar methods we sought to determine whether the capacity of 2-dG to inhibit Fc receptor-mediated phagocytosis is absolute or merely reflects a quantitative difference in Fc receptor sensitivity of the 2-dG-treated macrophage. E(IgM)C were incubated with normal macrophages in the presence of 2-dG. These complement-coated erythrocytes attach to resident peritoneal macrophages but are

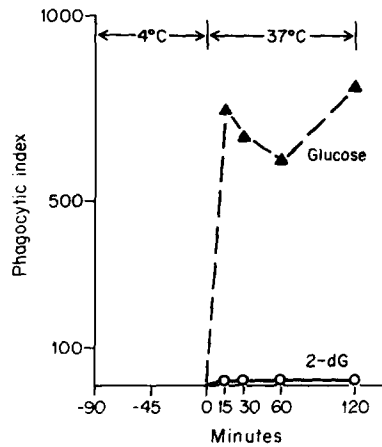


FIG. 4. Phagocytosis of preattached E(IgG) is inhibited by addition of 2-dG. E(IgG) were attached to 48-h explanted normal mouse peritoneal macrophages for 90 min at 4°C in medium without 2-dG. The preparations were washed with cold medium to remove nonattached erythrocytes, and cultures were incubated at 37°C with (○) or without (▲) 50 mM 2-dG added. At the indicated time points samples were fixed, and the phagocytic index determined.

not ingested by them (20, 21). The nonattached erythrocytes were removed and the E(IgM)C-macrophage complexes further incubated in 2-dG-containing medium to which supra-agglutinating amounts³ of anti-E IgG were added. Regardless of the concentration of anti-E IgG used, the inhibitory effect of 2-dG could not be overcome (Table IV). Thus, the capacity of 2-dG to inhibit Fc receptor-mediated phagocytosis reflects a qualitative alteration in macrophage physiology.

Does the Size of the Test Particle Influence its Uptake by 2-Deoxy-D-Glucose-Treated Normal Macrophages? Since the initial observations on the inhibition of Fc receptor-mediated phagocytosis by 2-dG were made by comparing the ingestion of IgG-coated E of about 4.5- μ m diameter with latex particles of only 1.07- μ m diameter, we questioned whether the size of the particle influenced the result obtained. We therefore performed experiments in which the phagocytosis of IgG-opsonized particles of different sizes was compared with the phagocytosis of similar sized latex and zymosan particles in presence and absence of 50 mM 2-dG.

Antibody opsonized smooth encapsulated pneumococci ($\sim 1 \mu$ m in diameter), IgG-coated E ($\sim 4.5 \mu$ m in diameter), and IgG-coated Ch E ($\sim 6 \times 9 \mu$ m in diameter) were used as representatives of particles whose attachment and ingestion by mouse macrophages requires the presence of specific opsonins. Latex particles (1.07 μ m in diameter and $5.7 \pm 1.5 \mu$ m in diameter) and zymosan (4–6 μ m in diameter) were used as representatives of particles that are ingested in the absence of specific opsonins and independently of the functional integrity of the Fc and complement receptors (7, 22). As shown in Table V and Fig. 1, 2-dG-treated normal macrophages ingest 1.07 μ m and $5.7 \pm 1.9 \mu$ m

³ The amount of anti-E IgG used was 50- to 100-fold higher per erythrocyte than the amount needed to opsonize a 5% suspension of E.

TABLE IV
Effect of Excess Anti-E IgG on the Ingestion of Preattached E(IgM)C by 2-dG-Treated Normal Macrophages

Macrophage-erythrocyte complexes*	Anti-E IgG ‡ µg protein per ml added	Attachment by macrophages				Ingestion by macrophages			
		2-dG		Control		2-dG		Control	
		%	Average no. per macrophage	%	Average no. per macrophage	%	Average no. per macrophage	%	Average no. per macrophage
Macrophages + E(IgG)	—	99	8.1	89	5.8	9	1	94	6.2
Macrophages + E(IgM)	—	ND§	ND	6	3	ND	ND	1	1
Macrophages + E(IgM)C	—	99	20	99	21	0	0	1	0
Macrophages + E(IgM)C	65	—	—	—	—	6	2	81	4.4
Macrophages + E(IgM)C	130	—	—	—	—	3	2.3	90	5

* Macrophages were preincubated at 37°C for 2½ h in medium with or without 50 mM 2-dG. Then fresh medium with or without 2-dG, and opsonized particles were added. Incubation was continued at 37°C for 30 min.

‡ Macrophage-E(IgM)C complexes were incubated in presence of anti-E IgG at 37°C for 30 min.

§ ND, not done.

diameter latex particles, and zymosan to about the same extent as glucose-treated control macrophages. In contrast, the ingestion of opsonized pneumococci and IgG-coated E and Ch E was completely inhibited in 2-dG-treated macrophages but proceeded normally in control cultures. Thus, the size of the particles employed clearly does not determine whether or not they are ingested by 2-dG-treated macrophages.

Inhibition of Fc Receptor-Mediated Phagocytosis by 2-Deoxy-D-Glucose is Reversible. Although cell viability is well maintained in the presence of 2-dG, it seemed possible that this glucose analogue inhibits Fc receptor function by causing irreversible damage to those cell structures directly involved in the phagocytosis of IgG-coated particles. To examine this possibility, 2-dG-treated normal macrophages were placed into fresh 5.5 mM glucose-containing medium and challenged with E(IgG) at various intervals after removal of 2-dG. The results of this experiment are illustrated in Fig. 5. Macrophages preincubated for 2 h in 2-dG, and then incubated with E(IgG) in 2-dG-containing medium for an additional hour, were markedly inhibited (11% of control) in their phagocytosis of E(IgG). On the other hand, macrophages similarly pretreated with this glucose analogue and then incubated with E(IgG) in glucose-containing medium showed complete restoration of their capacity to ingest E(IgG) within 1 h of removal of 2-dG. Thus the effects of 2-dG on Fc receptor-mediated phagocytosis are fully and rapidly reversed by glucose.

Effect of Different Hexoses Upon 2-Deoxy-D-Glucose-Mediated Inhibition of Fc Receptor Function. The rapid reversal in glucose-containing medium of 2-dG-mediated inhibition of phagocytosis of E(IgG), and the necessity to use at least 5–10 times higher concentrations of 2-dG in presence of 5.5 mM glucose to obtain a significant inhibitory effect (see Fig. 3) prompted us to examine whether the addition of glucose or other hexoses to 2-dG-containing medium could reverse the 2-dG effect. Normal macrophages were incubated at 37°C in presence or absence of 50 mM 2-dG. After 2 h the medium of the control and the 2-dG-treated

TABLE V
*The Effect of 2-dG is not Influenced by the Size of the Test Particle**

Particles‡	Size	Ingestion of particles by macrophages			
		2-dG		Control	
		Macro- phages with particles in- gested	Average no. of particles ingested per macrophage	Macro- phages with particles in- gested	Average no. of particles ingested per macrophage
	μm	%		%	
Latex	1.07	95	>20	>99	>20
Pneumococci op- sonized with IgG	~1.1	4	>5	>99	>10
Zymosan	~4-6	85	6	98	9
E opsonized with IgG	~4.5	4	2	72	5.6
Latex	5.7 ± 1.5	73	2-3	94	3
Ch E opsonized with IgG	~6 × 9	3	1	80	4

* Each value represents the average of two experiments. Conditions as for Table I.

‡ Approximately equal numbers of particles were added within each particle size class examined.

cultures were changed. Some of the cultures were further incubated for 2 h in 2-dG or control medium to which the hexose to be tested was added at a final concentration of 50 mM. Other cultures were maintained for the same length of time in 2-dG- or glucose-containing medium. E(IgG) were added during the last hour of incubation after which the cultures were fixed and the number of erythrocytes ingested was enumerated. The results are shown in Fig. 6.

74% of the macrophages incubated throughout in glucose-containing medium phagocytized an average of 6.4 erythrocytes. As expected, of the macrophages maintained throughout in 2-dG-containing medium, only 25% ingested an average of 2.8 erythrocytes each, giving a phagocytic index which was 15% of the control. Macrophages incubated in 2-dG to which an equimolar amount of glucose or mannose had been added showed a marked resumption in their capacity to ingest E(IgG), exhibiting phagocytic indices of 80 and 62% of control values, respectively. In contrast, addition of equimolar amounts of fucose, galactose, glucosamine, or fructose did not reverse the inhibitory effects of 2-dG. Macrophages incubated in medium containing 10^{-1} M glucose showed levels of ingestion of E(IgG) similar to control values (phagocytic index 83%), confirming that the increased osmolarity of medium containing equimolar amounts of 2-dG and another hexose did not contribute significantly to the observed inhibition of phagocytosis of E(IgG). These results clearly show that only glucose and mannose can reverse the 2-dG-mediated inhibition of Fc receptor function.

The effect of mannose was further investigated. Normal macrophages were incubated for 2.75 h in 2-dG-containing medium to which mannose, in concentrations varying from 5×10^{-4} to 5×10^{-2} M, was added. E(IgG) were added to the culture for the last 45 min of the experiment, after which the number of erythrocytes phagocytized was enumerated. The presence of mannose reversed

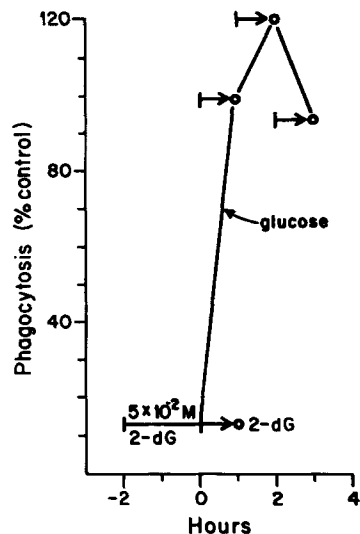


FIG. 5. 2-dG reversibly inhibits phagocytosis of E(IgG) by normal macrophages. Normal mouse peritoneal macrophages were incubated in presence or absence of 50 mM 2-dG for 2 h at 37°C. After this time, indicated as 0 h, some of the 2-dG containing cultures were washed twice and placed into 5.5 mM glucose-containing medium without 2-dG at 37°C. E(IgG) were added for the 1-h periods indicated by the arrows. Ordinate represents percent of the phagocytic index obtained in the experimental as compared with the control values.

the 2-dG-mediated inhibition of phagocytosis of E(IgG) in a dose-dependent fashion (Table VI). Mannose, in a molar concentration 1/100 that of 2-dG, caused a more than twofold reversal of the 2-dG effect (phagocytic index increased from 17 to 39% of control), while macrophages incubated in equimolar concentrations of mannose and 2-dG exhibited a phagocytic index 64% of control values. These findings contrast strikingly with those obtained when glucose was used to reverse the effect of 2-dG. Partial reversal was found using mannose at concentrations as low as 5×10^{-4} M, while a more than 10-fold higher concentration of glucose was required to achieve a similar effect.

Additional experiments showed that the inhibitory effect of 2-dG on the phagocytosis of E(IgG) was reversed by the addition of mannose or glucose to the medium after a 6 h preincubation in 2-dG (data not given). The capacity of mannose or glucose to reverse the effect of 2-dG after 6 h confirms that the macrophages are viable and can regain their functional integrity after prolonged culture in 2-dG-containing medium.

2-Deoxy-D-Glucose Inhibits Phagocytosis Mediated by the Complement Receptor of Thioglycollate-Elicited Macrophages. Macrophages derived from the peritoneal cavities of normal mice bind but do not ingest E(IgM)C (20). In contrast, macrophages elicited by the prior injection of thioglycollate medium into the peritoneal cavity bind and ingest E(IgM)C via their complement receptors (20). Thioglycollate-elicited macrophages were incubated in control and 2-dG-containing medium and tested for their capacity to ingest E(IgM)C, E(IgG), latex particles, and zymosan. The ingestion of E(IgM)C and E(IgG) was markedly inhibited in 2-dG-containing medium (phagocytic indices of 1 and 10%, respectively, of control values), while the ingestion of latex and zymosan re-

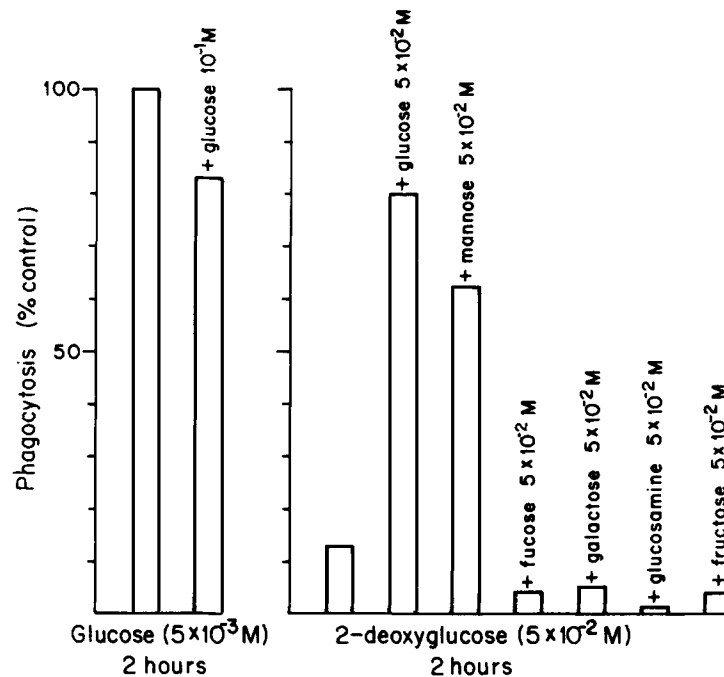


FIG. 6. Effect of several hexoses on 2-dG inhibition of phagocytosis of E(IgG) by normal macrophages. Normal mouse peritoneal macrophages were incubated in 50 mM 2-dG-containing medium. After 2 h at 37°C, fresh medium containing 50 mM 2-dG plus equimolar amounts of various hexoses was added. The incubation proceeded for 2 h at 37°C. E(IgG) were added during the last hour of incubation, and ingestion was scored. Control cultures (left portion of the graph) had no 2-dG added. Phagocytosis is expressed as percent of the phagocytic index obtained in the absence of 2-dG.

maintained at control levels in the presence of this glucose analogue (Table VII). Thus phagocytosis mediated by both the Fc and complement receptors are inhibited by 2-dG.

Discussion

Several lines of evidence indicate that the Fc and C3 receptors of mouse macrophages are chemically distinct and reside on separate molecules (2, 22, 23), and that they function independently of one another in the attachment and ingestion phases of phagocytosis (7, 20). The capacity of 2-dG to inhibit phagocytosis, mediated by the Fc and C3 receptors of mouse mononuclear phagocytes while leaving phagocytosis mediated via the opsonin-independent pathway unperturbed, demonstrates a previously unsuspected relationship between the Fc and C3 receptor systems, and indicates that there is a qualitative difference between opsonin-dependent and opsonin-independent pathways of phagocytosis.

Effect of 2-Deoxy-D-Glucose on Cell Viability. Incubation of macrophages in glucose-free medium containing 2-dG causes rapid (within 2 h) and irreversible cellular damage. For this reason we have used media containing 5.5 mM glucose and 50 mM 2-dG. Under these conditions the inhibitory effect of 2-dG on immune ligand-specific phagocytosis occurs rapidly. Similar inhibitory effects are ob-

TABLE VI
*Mannose Reverses the Inhibition of Phagocytosis of E(IgG) by 2-dG**

Treatment of macrophages, 37°C, 2 h	Presence of E(IgG), 37°C, 45 min	Ingestion of E(IgG) by macrophages			
		Macrophages with E(IgG) in- gested	Average no. of E(IgG) ingested per macrophage	Phagocytic in- dex	Control phag- ocytic index
		%			%
5.5 mM glucose + 50 mM mannose	5.5 mM glucose + 50 mM mannose	86	8	688	100
50 mM 2-dG + 5 mM glu- cose	50 mM 2-dG + 5 mM glu- cose	43	2.8	120	17
50 mM 2-dG + 0.5 mM mannose	50 mM 2-dG + 0.5 mM mannose	66	4	264	39
50 mM 2-dG + 5 mM man- nose	50 mM 2-dG + 5 mM man- nose	70	5	350	51
50 mM 2-dG + 50 mM man- nose	50 mM 2-dG + 50 mM mannose	82	5.3	425	64

* Each value represents the average of two experiments.

TABLE VII
*2-dG Inhibits Phagocytosis of E(IgG) and E(IgM)C by Thioglycollate-Elicited
 Macrophages**

Particle	2-dG			Control		
	Macro- phages with parti- cles in- gested	Average no. of par- ticles in- gested per macro- phage	Phago- cytic in- dex	Macro- phages with parti- cles in- gested	Average no. of par- ticles in- gested per macro- phage	Phago- cytic in- dex
	%			%		
Latex (5.7 μ m)	70	2-3		86	3	
Zymosan	96	8		98	>10	
E(IgG)	10	3	30	98	11	1,080
E(IgM)C	6	1	6	75	8	600

* Each value represents the average of three experiments. Conditions as follows: Monolayers of 24- to 48-h explanted macrophages were preincubated for 4 h in medium containing 5.5 mM glucose (control) or medium containing 5.5 mM glucose and 50 mM 2-dG. At this time the medium was removed and the macrophages were further incubated for 1 h at 37°C in fresh glucose or 2-dG-containing medium to which the particle to be tested was added.

served when macrophages are incubated in medium containing 5.5 mM 2-dG and 5.5 mM glucose, but their onset requires a longer period of incubation. In both cases the effect is completely reversible and the capacity of the macrophages to ingest via the opsonin-independent pathway remains unperturbed. These findings confirm that the inhibitory effects of 2-dG on Fc- and C3 receptor-mediated phagocytosis are not consequences of premorbid changes in cellular function.

Several reports (24-26) indicate that phagocytosis is reduced in hyperosmolar medium. Since the addition of 50 mM glucose or 50 mM mannose to medium containing 50 mM 2-dG reverses the inhibitory effect of this sugar, it is evident

that the osmolarity of the medium is not a significant factor in the capacity of 2-dG to inhibit immune ligand-specific phagocytosis.

2-Deoxy-D-Glucose Qualitatively Alters Fc and C3 Receptor Function. Previous studies of Fc and C3 receptor function have used agents that altered ligands on the surface of the particle (5, 23, 27) or blocked the interaction of these ligands with their corresponding receptors on the macrophage surface (2, 5, 20, 23). The blockade of Fc receptor function achieved in the latter case is only quantitative; it can be overcome by increasing the amount of IgG on the test particle (see Fig. 1 of reference 5). In contrast, 2-dG has no apparent effect on IgG-coated erythrocytes (Table II) and does not inhibit the binding of IgG- or C3-coated erythrocytes to the macrophage's Fc (Table III) and C3 (data not shown) receptors. Moreover, 2-dG causes a qualitative change in the response of the macrophage's Fc receptors to a phagocytic stimulus. Addition of 50- to 100-fold more anti-erythrocyte IgG than the amount needed to opsonize E does not overcome the inhibitory effect of 2-dG on phagocytosis mediated by the Fc receptor (Table IV).

The experiments of Askenase and Hayden (28) and of Unkeless and Eisen (29) suggest that mouse macrophages contain two distinct Fc receptors: (a) A trypsin-sensitive structure which mediates the binding of cytophilic antibody (IgG 2a) to the macrophage surface. (b) A trypsin-resistant structure which mediates ingestion of IgG-coated erythrocytes. We have examined the effects of trypsin on 2-dG-treated macrophages and find that the Fc receptor whose function is inhibited by 2-dG is resistant to digestion with this protease.

Metabolism of 2-Deoxy-D-Glucose is Required to Inhibit Fc Receptor-Mediated Phagocytosis. The inhibitory effect of 2-dG is evident within 15 min of rewarming E(IgG)-macrophage complexes from 4°C to 37°C in medium containing 2-dG. However, this sugar has no inhibitory effect when added to a similar culture maintained at 4°C, and removed from the medium before rewarming the culture to 37°C. Both the transport and the phosphorylation of 2-dG to 2-dG-6-phosphate (30-32) and other phosphorylated metabolites (33) are temperature-dependent processes. For this reason we assume that 2-dG must accumulate intracellularly and be metabolized to inhibit Fc receptor- and C3 receptor-mediated phagocytosis, and that in the short period of warming the cells from 4°C to 37°C, uptake and metabolism of 2-dG occur more rapidly than the reactions leading to the ingestion of E(IgG).

Mode of Action of 2-Deoxy-D-Glucose. Although we do not know the mechanism by which 2-dG exerts its selective inhibitory effects on Fc and complement receptor phagocytosis, we can exclude at least two of the many possible targets of this sugar. As we show in the accompanying paper (9), the inhibitory effect of 2-dG on receptor-mediated phagocytosis can be dissociated from the effects of 2-dG on cellular ATP generation.

A second possible target of 2-dG are the contractile proteins, actin and myosin, and their regulatory cofactors, which are found in a variety of nonmuscle cells including macrophages (34). These proteins are believed to generate the locomotive forces necessary to move the advancing membrane pseudopods around a particle. Griffin et al. (5) have suggested that the interactions of immune ligands (IgG and complement) with their corresponding receptors on the macrophage membrane initiate the signals that determine the spatial

orientation and direction of movement of these contractile elements. Since the functional integrity of these contractile elements presumably is required for the phagocytosis of latex and zymosan particles, and since 2-dG has no inhibitory effect on the ingestion of these particles, it seems unlikely that the inhibitory effect of 2-dG on Fc receptor- and complement receptor-mediated phagocytosis is determined by an effect of 2-dG on these contractile proteins. We assume it is not. Rather, we suggest that 2-dG interferes with one or more of the reactions which link these receptors with the intracellular contractile proteins.

Summary

Incubation of normal or thioglycollate-elicited mouse peritoneal macrophages with 2-deoxy-D-glucose (2-dG) inhibits the capacity of these macrophages to phagocytize IgG- or complement-coated particles via their Fc and C3 receptors. 2-dG has no inhibitory effect on the capacity of these macrophages to phagocytize latex or zymosan particles, which are ingested in the absence of specific opsonins, and it does not inhibit binding of IgG- or C3-coated particles to their respective receptors on the macrophage's plasma membrane. 2-dG exerts its inhibitory effect on the macrophage and not on the opsonized particle. The inhibition is independent of particle size, occurs within 15–30 min of addition of this glucose analogue to the medium at 37°C, cannot be overcome by supra-agglutinating amounts of opsonizing antibody, and is completely reversible by substitution of 5.5 mM glucose for 50 mM 2-dG in the medium. Addition of equimolar amounts of glucose or mannose, but not of fructose, galactose, fucose, or glucosamine, to medium containing 50 mM 2-dG results in substantial reversal of the inhibitory effect of 2-dG on Fc and C3 receptor mediated phagocytosis.

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