

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

II. Dissociation of the Inhibitory Effects of 2-Deoxyglucose on
Phagocytosis and ATP Generation*

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In the preceding paper (1) we have shown that incubation of normal and thioglycollate-elicited mouse macrophages in 2-deoxy-D-glucose (2-dG)-containing medium leads to rapid inhibition of Fc and complement receptor (opsonin dependent)-mediated phagocytosis by these cells. However, this sugar has no inhibitory effects on the phagocytosis of latex and zymosan particles (opsonin independent) by either normal or thioglycollate-elicited macrophages. The inhibitory effects of 2-dG on Fc- and complement receptor-mediated phagocytosis are rapidly reversed by removing this sugar from the incubation medium or by adding an equimolar amount of glucose or mannose to the 2-dG-containing medium.

Phagocytosis of particulate materials by macrophages, whether it occurs by the opsonin-dependent or opsonin-independent pathways is an energy requiring process (2). The failure of 2-dG to block ingestion via the opsonin-independent pathway, and the rapidity with which mannose reversed the inhibitory effect of 2-dG on phagocytosis via the opsonin-dependent pathway (1), suggested to us that the capacity of this sugar to inhibit Fc- and complement receptor-mediated phagocytosis is unrelated to the well known inhibitory effect of this sugar upon cellular ATP generation (3, 4). The data reported in this paper confirm this hypothesis. Some of these findings have been reported previously in preliminary form (5).

Materials and Methods

Reagents. Desiccated firefly lantern extract, 0.1 M sodium arsenate buffer, pH 7.4, containing 0.04 M magnesium sulfate, and adenosine triphosphate (ATP) sodium salt were obtained from

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¹ *Abbreviations used in this paper:* 2-dG, 2-deoxy-D-glucose or 2-deoxyglucose; E, sheep erythrocytes; E(IgG), E coated with anti-E IgG; 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.

Sigma Chemical Co., St. Louis, Mo. All other reagents were purchased and prepared as described previously (1).

Macrophage Cultures for ATP Determinations. Peritoneal macrophages were harvested and maintained in culture as described (1, 6). Since large numbers of macrophages were required for ATP determinations, the protocol described previously was modified as follows: 4 ml of a cell suspension containing 2.5×10^6 normal cells/ml or 1.25×10^6 thioglycollate-elicited cells/ml, respectively, were plated in minimum essential medium with Earle's salt solution, containing 5.5 mM glucose (MEM) supplemented with 20% heat-inactivated (56°C for 30 min) fetal bovine serum (FBS) and 100 μ g/ml of streptomycin (complete medium) in 60 mm plastic tissue culture dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) for 4 h; at this time the medium was removed, the cells were washed three times with 5 ml of cold medium, and subsequently incubated in 5 ml of complete medium for an additional 20 or 44 h at 37°C.

Particles. Latex particles (1.07- μ m diameter), zymosan from yeast, and sheep erythrocytes (E) coated with rabbit anti-E IgG [E(IgG)] or with rabbit anti-E IgM and mouse complement [E(IgM)C] were prepared and used as described (1).

Determination of Cellular ATP Content. Cellular ATP content in macrophages was assayed by the firefly-luciferase method as described by Stanley and Williams (7). The buffered and desiccated firefly lantern extract was reconstituted with 5 ml cold distilled water. To degrade any endogenous ATP the extract was kept in ice for 20 h before use. For cellular ATP assays, macrophage monolayers were rinsed with 4 ml of cold phosphate-buffered saline with Ca^{++} and Mg^{++} ions, the cells were scraped into 0.5 ml of cold 0.04 M Tris-borate buffer (pH 9.2) (8), placed immediately into a boiling water bath for 5 min, cooled, and centrifuged for 10 min at 1,200 g at 4°C. 25 and 50 μ l of the resulting supernatant fluids were assayed in duplicates for ATP on the same day. 50 μ l of firefly extract were added to plastic scintillation vials containing 3 ml of phosphate and arsenate buffer and 25 or 50 μ l of the sample to be measured. The light emission of each sample was counted immediately $2 \times$ for 0.1 min each in a Mark II Nuclear-Chicago liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Standard ATP solutions were prepared in Tris-borate buffer from a 10^{-6} M frozen stock solution. The intensity of the emitted light was directly related to the amount of ATP present in the sample, resulting in a linear decrease of the amount of measurable light from 10^{-9} to 10^{-12} mol of ATP. Each sample was counted twice in succession. Because of the lower background luminescence, the second set of counts was used to calculate the amount of ATP in each sample. The results are expressed as moles ATP per milligram protein. The presence of 2-dG or other sugars in control standard preparations did not influence the ATP determinations. Total cell protein was measured by the method of Lowry et al. (9).

Macrophage viability was tested for each set of experiments by the trypan blue exclusion method described by Boyse et al. (10). Unless otherwise stated, the viability was found more than 90% in all experiments reported. All other experimental methods were as described in the preceding paper (1).

Results

Effect of 2-Deoxy-D-Glucose on the ATP Content of Normal Macrophages. Unstimulated mouse peritoneal macrophages generate energy mainly by anaerobic glycolysis (2). Since 2-dG is a known inhibitor of the key glycolytic enzyme phosphohexose-isomerase, the presence of this glucose analogue leads to a decrease in cellular ATP levels (3, 4). It was therefore important to determine: (a) the extent to which macrophage ATP levels are decreased in presence of 2-dG; (b) if this decrease is reversed and to what extent by removal of 2-dG; (c) if cellular ATP levels are altered by addition of other hexoses to 2-dG-containing medium; and (d) whether the decrement in macrophage ATP levels induced by 2-dG is responsible for the inhibitory effect of this glucose analogue on Fc receptor-mediated phagocytosis. This last question was of special importance since the phagocytosis of latex and zymosan particles is not inhibited by 2-dG, a finding that suggested that lowered cellular ATP levels are not a sufficient

explanation for the inhibitory effect of 2-dG on Fc receptor-mediated phagocytosis.

Since in the experiments designed to answer these questions large numbers of macrophages were required, the cells used for ATP determinations were cultured in 60-mm Petri dishes as described in the Materials and Methods. Macrophages used to monitor phagocytosis of latex particles and of E(IgG) were cultured on glass cover slips and treated in parallel under the same conditions as those used for ATP determinations.

Normal macrophages were incubated in 2-dG-containing medium, and at appropriate intervals the cells were harvested, and the ATP content was assayed (Fig. 1a). The cellular ATP content decreased by about 50% during a 2 h exposure to 2-dG. Removal of 2-dG at this time followed by a 2 h incubation in glucose-containing medium led to an almost complete restoration of control ATP levels. However, addition of an equimolar concentration of glucose, mannose, fructose, fucose, or galactose to the 2-dG-containing medium did not increase cellular ATP content; indeed, all of these hexoses in combination with 2-dG allowed an even further diminution in cellular ATP content. In contrast to their inability to increase cellular ATP levels, addition of glucose and mannose restored the capacity of these 2-dG-treated macrophages to ingest E(IgG) (Fig. 1b). The phagocytic index² increased from 10% of control levels to 79% in the presence of glucose and to 60% in the presence of mannose. Phagocytosis of latex particles in the different sugar mixtures was not significantly different from control values (data not shown).

These findings confirm that 2-dG causes a marked decrease in the ATP content of normal mouse peritoneal macrophages. They show that this decrease can only be reversed by removing 2-dG from the incubation medium, and that the capacity of mannose or glucose to reverse the 2-dG-mediated inhibition of Fc receptor function occurs without a concomitant increase in cellular ATP levels.

Effect of 2-Deoxy-D-Glucose on ATP Content and Fc Receptor Function of Thioglycollate-Elicited Macrophages. Thioglycollate-elicited macrophages incubated in 5.5 mM glucose-containing medium have about five times more ATP/mg cell protein than their unstimulated counterparts (Table I). Incubation of thioglycollate-elicited macrophages in medium containing 50 mM 2-dG and 5.5 mM glucose for 4 h at 37°C reduced the cellular ATP content to 27% of the amount found in thioglycollate-elicited macrophages maintained in medium with 5.5 mM glucose. The inhibitory effect of 2-dG on cellular ATP generation was reversed by removing the 2-dG-containing medium after 4 h, and incubating the cells in medium with 5.5 mM glucose (Fig. 2); under these conditions cellular ATP levels rose to 73% of control levels within 2 h and returned to normal within 5-7 h. 2 h after removal of the 2-dG-containing medium there was also a marked recovery in the capacity of thioglycollate-elicited macrophages to ingest E(IgG). Thus, despite the increased ATP content and phagocytic capacity (11) of thioglycollate-elicited macrophages, the effects of 2-dG upon these cells are similar to the effects of this sugar on unstimulated macrophages.

Effect of other Hexoses on 2-Deoxy-D-Glucose-Mediated Inhibition of ATP

² Phagocytic index is the percentage of macrophages that ingested erythrocytes multiplied by the average number of erythrocytes ingested per macrophage.

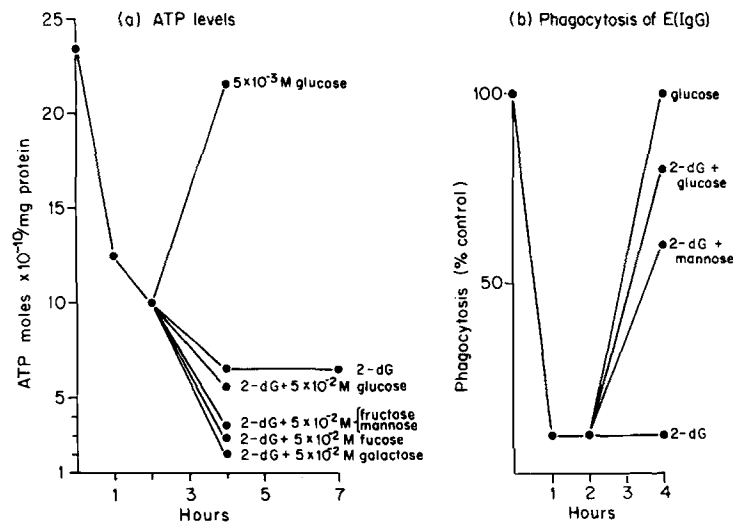


FIG. 1. Effect of 2-dG on normal mouse peritoneal macrophages is altered by other hexoses. (a) Macrophages were incubated in 50 mM 2-dG-containing medium, and after appropriate time intervals samples were assayed for ATP. Additional hexoses in equimolar amounts were added at $t = 2$ h, and incubation was continued for 2 h at 37°C. (b) Treatment of macrophages was the same as described for (a), but E(IgG) were added to the cultures 1 h before the times indicated by the closed circles.

TABLE I
Effect of 2-dG on the ATP content of Mouse peritoneal Macrophages*

	ATP	No. of experiments
	<i>nmol/mg protein</i> \pm <i>SEM</i> †	
Normal macrophages‡		
5.5 mM glucose	3.9 \pm 2.4	8
5.5 mM glucose + 50 mM 2-dG	0.8 \pm 0.3	5
Thioglycollate macrophages‡		
5.5 mM glucose	20.3 \pm 0.7	8
5.5 mM glucose + 50 mM 2-dG	3.1 \pm 1.3	5

* Experimental conditions: The cells were washed three times with warm medium without FBS. 4 ml of fresh warm 5.5 mM glucose-containing medium with or without 50 mM 2-dG were added per Petri dish, and the cultures were incubated for another 4 h at 37°C.

† SEM, standard error of mean.

‡ 10^7 normal peritoneal cells and 5×10^6 thioglycollate-elicited peritoneal cells, respectively, were plated in 60-mm Falcon plastic Petri dishes in 4 ml MEM containing 5.5 mM glucose and 20% FBS, washed, and incubated in the same medium in a 5% CO₂-95% air atmosphere at 37°C. Normal macrophages and thioglycollate-elicited macrophages were maintained in culture for 20-44 h and 20-26 h, respectively, before use.

Generation and Complement Receptor Function in Thioglycollate-Elicited Macrophages. As shown previously (Figs. 1a and b, and reference 1), the inhibitory effect of 2-dG on Fc receptor-mediated phagocytosis by normal macrophages is reversed when equimolar amounts of mannose or glucose are added to the

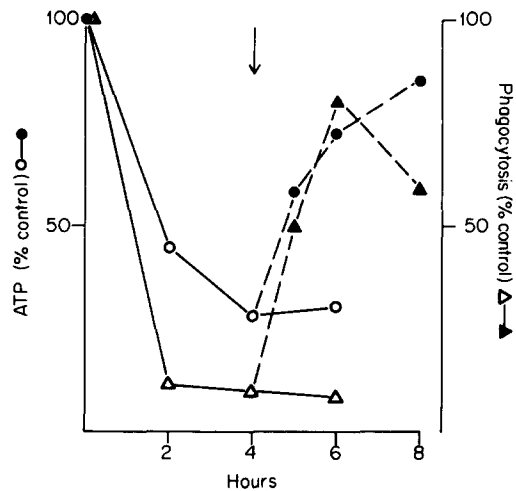


FIG. 2. 2-dG reversibly inhibits the phagocytosis of E(IgG) and the generation of ATP in thioglycollate-elicited macrophages. Thioglycollate-elicited mouse peritoneal macrophages were incubated in presence of 5.5 mM glucose and 50 mM 2-dG for 4 h at 37°C. At this time (indicated by the arrow) some of the 2-dG-containing cultures were washed twice, and placed into 5.5 mM glucose-containing medium without 2-dG at 37°C. ATP measurements were done at the times indicated by open (with 2-dG) and closed (2-dG removed) circles using cells maintained in 60-mm Petri dishes. To evaluate phagocytosis, E(IgG) were added to parallel cultures of macrophages on cover slips (see Materials and Methods) 1 h before the times indicated by open (with 2-dG) and closed (2-dG removed) triangles. The results are expressed as percent of the ATP values (left ordinate) and percent of the phagocytic index (right ordinate), respectively, obtained in control incubations without 2-dG.

medium. However, in the presence of 2-dG these hexoses do not stimulate ATP generation in normal macrophages. To examine the effects of these and other hexoses on 2-dG-mediated inhibition of complement receptor function and cellular ATP generation in thioglycollate-elicited macrophages, these cells were incubated for 2 h at 37°C in medium containing 50 mM 2-dG. E(IgM)C were present during the 2nd h of incubation. Macrophages treated with 2-dG ingested only 10% as many E(IgM)C as untreated macrophages (Fig. 3b). Under these conditions binding of E(IgM)C to the macrophage membrane was not inhibited by 2-dG (data not shown).

The inhibitory effect of 2-dG on complement receptor-mediated phagocytosis was promptly and completely reversed when the macrophages were washed and incubated for an additional 1 or 2 h in glucose-containing medium (Fig. 3b). Addition of 50 mM glucose or 50 mM mannose to the 2-dG-treated cultures also reversed the inhibitory effect of 2-dG on complement receptor-mediated phagocytosis. Under these conditions the reversal occurred more slowly and to a lesser extent than when 2-dG was removed from the culture medium entirely. Addition of fucose, fructose, galactose, or pyruvate to the incubation medium did not alter the inhibitory effect of 2-dG on complement receptor-mediated phagocytosis (Fig. 3b).

The effect of these sugars on the ATP content of 2-dG-treated thioglycollate-elicited macrophages is described in Fig. 3a. After a 2 h incubation in 2-dG-containing medium cellular ATP content fell to 23% of the control and remained

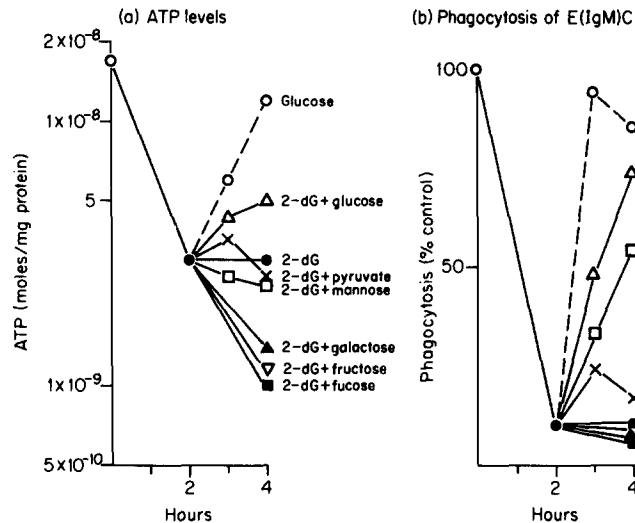


FIG. 3. The addition of other hexoses alters the effects of 2-dG on thioglycollate-elicited mouse peritoneal macrophages. (a) Macrophages were incubated in 50 mM 2-dG-containing medium for 2 h at 37°C. At this time additional hexoses were added in equimolar amounts and the incubation was continued for 2 h at 37°C; after appropriate time intervals samples were assayed for ATP. (b) Treatment of macrophages was the same as described for (a), but E(IgM)C were added 1 h before the times indicated: 5.5 mM glucose only (○); 5.5 mM glucose plus 50 mM 2-dG (●); and 5.5 mM glucose plus 50 mM 2-dG for 2 h at 37°C, at which time the following sugars were added: 50 mM glucose (△), or 50 mM mannose (□), or 50 mM galactose (▲), or 50 mM fructose (▽), or 50 mM fucose (■), or 5 mM pyruvate (*).

at this level for the next 2 h despite continued incubation of the cells in medium containing 2-dG. Addition of mannose, galactose, fructose, fucose, or pyruvate to the 2-dG-containing medium from the 2nd to the 4th h of incubation led at most to a 10% further decrease in cellular ATP content, while addition of glucose to the 2-dG-containing medium during this same interval led to a small (~9%) increase in cellular ATP content. Removal of 2-dG and incubation of the cells in glucose-containing medium restored ATP levels to 71% of control in 2 h. With the exception of the modest increase in ATP content when the thioglycollate-elicited macrophages were incubated in equimolar amounts of glucose and 2-dG (compare Fig. 1a and Fig. 3a), these findings are similar to those obtained when we examined the effect of these hexoses on unstimulated mouse macrophages treated with 2-dG.

Discussion

Effects of 2-Deoxy-D-Glucose on Cellular ATP Levels. Although the attachment of IgG- and complement-coated particles to receptors on the macrophage plasma membrane is independent of metabolic energy, their phagocytosis is an energy consuming process (2). 2-dG is a potent inhibitor of glycolysis (3). The first reports of its effects on cellular physiology were concerned with its inhibition of glucose phosphorylation by hexokinase, with the inhibition of phosphohexose-isomerase and thereby of the glycolytic pathway by the phosphorylated product of the hexokinase reaction, 2-dG-6-phosphate, and with the capacity of

2-dG-6-phosphate to consume high energy phosphates (4, 12, 13). Since the mouse peritoneal macrophage generates most of its energy via the glycolytic pathway (2), addition of 2-dG to the incubation medium results in a steady reduction in cellular ATP content (Figs. 1-3) during the first 4 h of incubation. It is evident that this relatively slow reduction in cellular ATP content is not responsible for the inhibitory effects of 2-dG on Fc and complement receptor-mediated phagocytosis since paralysis of these receptors occurs within 15 min of addition of 2-dG to the incubation medium at 37°C (Fig. 4 in reference 1).

Normal macrophages contain approximately 3.9×10^{-9} mol of ATP/mg cell protein, while thioglycollate-elicited macrophages contain nearly fivefold as much ATP (2.0×10^{-8} mol)/mg cell protein (Table I). (Calculated per 10^6 cells thioglycollate-elicited macrophages contain about twice as much ATP as normal macrophages.) Incubation of normal or thioglycollate-elicited macrophages in 2-dG-containing medium for 4 h at 37°C reduces the cellular ATP content to 21 and 15% of control levels, respectively. (Even at this level of inhibition, 2-dG-treated thioglycollate-elicited macrophages contain nearly as much ATP per cell as normal macrophages cultured in the absence of 2-dG). Despite these changes in cellular ATP levels in the presence of 2-dG, phagocytosis of latex and zymosan particles by these cells continues at control levels. In contrast, Fc- and C3 receptor-mediated phagocytosis is almost completely inhibited. Addition of mannose or glucose restores the capacity of these cells to phagocytize via their Fc and/or C3 receptors without a concomitant increase in cellular ATP levels. These findings clearly dissociate the inhibitory effects of 2-dG on cellular ATP generation from the inhibitory effects of this glucose analogue on Fc receptor-mediated phagocytosis. Finally, these data indicate that the amount of ATP required to fuel the ingestion process must be rather small compared to the total cell content since latex and zymosan particles and IgG- and complement-coated erythrocytes are phagocytized when only 15-25% of the original ATP levels are available.

Sodium fluoride, a potent inhibitor of enolase, is frequently employed as a reversible glycolytic inhibitor in cellular studies (14-16). Incubation of unstimulated macrophages in medium containing 10^{-3} M NaF for 1 h at 37°C causes an ~40% reduction in cellular ATP levels but inhibits the ingestion of latex particles and E(IgG) by 76 and 79%, respectively (reference 2, and J. Michl, unpublished observations). 2-dG causes a greater reduction in cellular ATP levels than does NaF in this time interval but unlike NaF does not inhibit opsonin-independent phagocytosis. These findings further emphasize that reduced cellular ATP generation or content is not a sufficient explanation for the inhibitory effects of either 2-dG or NaF on phagocytosis; they further suggest that the mechanisms by which these two glycolytic inhibitors alter membrane functions are unrelated to one another and to the glycolytic enzymes which have been identified as one of their targets.

2-Deoxy-D-Glucose Inhibits Transglycosylation Reactions. If the inhibitory effects of 2-dG on Fc- and C3 receptor-mediated phagocytosis are unrelated to the capacity of 2-dG to lower cellular ATP levels, by what mechanism does this sugar inhibit membrane functions? Although we do not yet know the answer to this question we can suggest one possible mechanism consistent with another known effect of 2-dG.

Many membrane proteins are glycoproteins; 2-dG is an inhibitor of cell wall formation in yeast (17-19), and of viral envelope glycoprotein biosynthesis in mammalian and avian cells infected with enveloped animal viruses (20-23). In the case of cells infected with enveloped viruses, nonglycosylated or aberrantly glycosylated protein precursors of the envelope glycoproteins accumulate intracellularly in the presence of 2-dG. Removal of 2-dG from the culture medium, or addition of mannose or glucose to the culture medium, permits resumption of glycoprotein synthesis, viral envelope biogenesis, and viral maturation (20). Sugars other than glucose and mannose are unable to reverse these inhibitory effects of 2-dG (23, 24). We have obtained similar results in our studies of the effects of 2-dG on macrophages (Figs. 1*b* and 3*b*; and Table VI and Fig. 6 of reference 1); only mannose and glucose reverse the inhibitory effect of 2-dG on Fc- and C3 receptor-mediated phagocytosis. In the case of both the enveloped viruses and of Fc- and C3 receptor-mediated phagocytosis the inhibitory effects of 2-dG are reversed by much lower concentrations of mannose than glucose. At a molar ratio of mannose:2-dG of 1:100 phagocytosis of E(IgG) was increased to about twice the amount found in the absence of mannose (Table VI of reference 1), while glucose was ineffective at a glucose:2-dG molar ratio of 1:10 (Fig. 3 of reference 1). Although the capacity of mannose to reverse the inhibitory effect of 2-dG was more pronounced at low mannose concentrations, the addition of equimolar amounts of glucose and 2-dG resulted in a more complete reversal of Fc- and C3 receptor-mediated phagocytosis than did equimolar amounts of mannose and 2-dG. Whether the increased effectiveness of mannose at low concentrations is related to its entry into the cytoplasm via a transport system which is not inhibited by 2-dG or to the enhanced capacity of mannose to compete with the inhibitory metabolite of 2-dG at the latter's site of action is unresolved.

The formation of 2-dG-containing oligosaccharides has been reported in plants (25, 26), bacteria (27), yeast (28), and mammalian cells (21); and 2-dG-containing glycolipids have been identified in yeast (29) and in hamster fibroblasts (30). UDP-2-dG and GDP-2-dG have been identified as glycoprotein (? glycolipid) precursors in a variety of cells (31, 32). The capacity of 2-dG, as the nucleotide sugar, to participate in transglycosylation reactions suggests that 2-dG may be incorporated into one or more macrophage glycoproteins and/or glycolipids instead of mannose, or instead of the metabolites of mannose or glucose, or that the presence of 2-dG-containing sugar nucleotides inhibits the glycosylation of the precursor proteins and/or lipids. According to this view, the capacity of mannose or glucose to reverse the inhibitory effects of 2-dG on Fc- and C3 receptor-mediated phagocytosis might be explained by the capacity of mannose- and glucose-containing nucleotides to compete with 2-dG-containing nucleotides for key glycosyl transferases. The net effect of such competition would be the resumption of normal glycoprotein and/or glycolipid formation and the repair of defects in cellular physiology caused by aberrant or defective glycosylation.

Summary

Macrophages incubated in 2-deoxy-D-glucose (2-dG)-containing medium showed a marked decrease in cellular ATP content, and were unable to ingest IgG- and complement-coated erythrocytes via the corresponding membrane

receptors for these ligands. However, the inhibitory effects of 2-dG on Fc- and C3 receptor-mediated phagocytosis were not a consequence of lowered macrophage ATP levels since addition of glucose or mannose to the culture medium restored the capacity of the macrophages to ingest IgG- and C3-coated particles without increasing ATP levels. These results indicate that Fc- and C3 receptor-mediated phagocytosis (opsonin dependent) differs qualitatively from the ingestion of latex and zymosan particles (opsonin independent); they suggest that the same regulatory molecules govern the responses of phagocytic cells to signals initiated by both the Fc and C3 receptors. The possibility that these molecules are regulated by glycosylation is discussed.

Note Added in Proof. Subsequent to our report on the effect of 2-deoxy-D-glucose on receptor-mediated phagocytosis in mouse peritoneal macrophages (*J. Cell Biol.*, 1975, 67:281 a), L. A. Boxer et al. (*Clin. Res.*, 1976, 24:304 A) and R. L. Baehner et al. (*J. Cell Biol.*, 1976, 70:1059) showed similar inhibitory effects of 2-deoxy-D-glucose on receptor-mediated phagocytosis in guinea pig polymorphonuclear leukocytes. Their observations are in press in the "Journal of Cellular Physiology."

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