ENERGY METABOLISM
AND T-CELL-MEDIATED CYTOLYSIS

II. Selective Inhibition of Cytolysis by 2-Deoxy-D-Glucose*

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The destruction of appropriate target cells by cytolytic thymus-derived lymphocytes (CTL)[1] is an active energy-requiring phenomenon (1–3). In the accompanying paper (4), we presented evidence that T-cell-mediated cytolysis could be supported by either oxidative or glycolytic energy pathways and was in general strongly inhibited only in those situations in which both respiration and glycolysis were simultaneously blocked. A particular example of this synergistic inhibition of CTL function was the ability of the glucose analogues 2-deoxy-D-glucose (2-DG) and 5-thio-D-glucose (5-SH-G) to reversibly inhibit cytolysis when oxygen was absent or when its utilization was metabolically blocked. In the course of studying these glucose analogues, however, we made the unexpected observation that cytolysis could also be inhibited under aerobic conditions if 2-DG was present in sufficient excess over glucose. This somewhat anomolous finding prompted a more complete investigation of the effect of 2-DG and other glucose analogues on cytolysis. The present communication demonstrates that 2-DG inhibits cytolysis in a reversible fashion by competing quantitatively with glucose, whereas other glucose analogues such as 5-SH-G and 3-O-methylglucose fail to inhibit cytolysis under comparable conditions. Furthermore, data are presented to suggest that the inhibition of cytolysis by 2-DG is not related to energy production per se. Other possible mechanisms for the inhibitory effect of 2-DG are discussed.

Materials and Methods

Mice. Adult mice of the inbred strains C57BL/6 and DBA/2 were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Reagents. 2-DG, D-glucose, α-D(+)fucose, 2-deoxy-D-galactose, D(+)mannose, D(+)galactose, D(+)glucosamine HCl, sodium pyruvate, β-D(−)fructose, and 3-O-methyl-α-D-glucopyranoside (3-O-methylglucose) were obtained from Sigma Chemical Co., St. Louis, Mo. 5-SH-G was obtained from Pfanstiehl Labs., Inc., Waukegan, Ill. All reagents were dissolved in phosphate-buffered saline and diluted to six times the desired final concentration immediately before use.

Generation of CTL. CTL were generated in vitro in mixed leukocyte cultures (MLC) as described in the accompanying paper (4). After 4–6 days of culture, recovered cells were washed

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[1] *Abbreviations used in this paper:* BME, Eagle's basal medium; CTL, cytolytic thymus-derived lymphocytes; 2-DG, 2-deoxy-D-glucose; MLC, mixed leukocyte culture; 5-SH-G, 5-thio-D-glucose.

three times in glucose-free Eagle's basal medium (BME) supplemented with 2% (vol/vol) dialyzed fetal calf serum and 10 mM HEPES buffer. The glucose concentration in this medium (measured by the glucose oxidase technique) was $<5 \times 10^{-6}$ M. Viability was assessed by trypan blue exclusion, and the number of viable MLC cells was adjusted to 5×10^5/ml.

Target Cells. P-815 mastocytoma cells were maintained in vitro and labeled with $Na_2{}^{51}CrO_4$ as described previously (5). Labeled cells were washed three times in glucose-free BME supplemented as described above and adjusted to a final concentration of 5×10^4 cells/ml.

Cytolytic Assay. The technical details of the assay and method of calculation of results were described in detail in the accompanying paper (4). Spontaneous release in the presence of all reagents tested did not exceed control spontaneous release by more than 3% under the experimental conditions employed.

Results

Selective Inhibition of T-Cell-Mediated Cytolysis by 2-DG. In the accompanying communication (4), we presented evidence that the expression of T-cell-mediated cytolysis was not critically dependent upon glycolytic energy production. In particular, cytolysis was not significantly inhibited when glucose was absent or when its utilization was blocked by the presence of equimolar concentrations of the glucose analogues 2-DG and 5-SH-G. In the course of these experiments, however, we made the unexpected observation that cytolysis was completely inhibited by 2-DG in the absence of exogenous glucose (Table I). Quantitative analysis of this phenomenon (Fig. 1) indicated that the inhibition was dose dependent. The concentration of 2-DG required to inhibit cytolysis by 50% under these conditions was approximately 0.5 mM and complete inhibition was observed in the presence of 5 mM 2-DG.

The effect of other glucose analogues on cytolysis in the absence of exogenous glucose was also investigated. As can be seen from Fig. 1, neither 5-SH-G nor 3-O-methylglucose inhibited cytolysis when present at the same molar concentrations as 2-DG. Since 5-SH-G was shown in the accompanying paper (4) to be quantitatively as potent an inhibitor of cytolysis as 2-DG when cellular respiration was blocked, these results raised the possibility that the inhibitory effect of 2-DG under aerobic conditions may not be related to energy production.

Reversibility of Effect of 2-DG on Cytolysis. In view of the observed inhibition of cytolysis by 2-DG in low glucose medium, it was important to determine whether or not this effect was reversible. To this end, populations of MLC cells or labeled mastocytoma target cells in low glucose medium were incubated with 5 mM 2-DG for 90 min and subsequently washed and tested in a cytotoxicity assay in glucose-supplemented medium. It can be seen that the inhibitory effect of 2-DG on cytolysis was completely reversible upon washing (Table II). These studies indicated that 2-DG was not toxic under the assay conditions employed; however, it was not possible to determine whether the inhibitory action of 2-DG was directed at the CTL or target cell (or both).

Competition between 2-DG and Glucose. The failure to observe any significant inhibitory effect of 2-DG on cytolysis in medium containing equimolar concentrations of 2-DG and glucose (Table I and accompanying paper) suggested a possible competition between the two hexoses. This possibility was investigated further by assessing cytolysis at three different concentrations of 2-DG in the presence or absence of various concentrations of glucose. The results of such an experiment (Fig. 2) indicated that inhibition of cytolysis was dependent on

TABLE I

Effect of 2-DG on Cytolysis in the Presence or Absence of Glucose*

2-DG concentration	Glucose concentration	Specific lysis
M	*M*	%
None	5×10^{-3}	44
5×10^{-3}	5×10^{-3}	48
None	$<5 \times 10^{-6}$	50
5×10^{-3}	$<5 \times 10^{-6}$	0

* MLC cells (10^5) were assayed for cytotoxicity in low glucose ($<5 \times 10^{-6}$ M) or glucose-supplemented medium in the presence or absence of 5×10^{-3} M 2-DG.
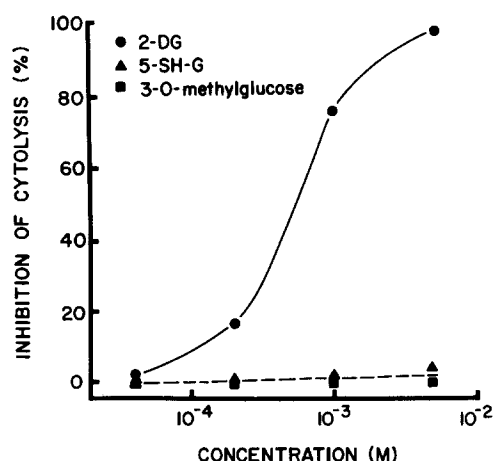

FIG. 1.   Selective inhibition of cytolysis by 2-DG. MLC cells in low glucose medium were assayed for cytotoxicity on ^{51}Cr-labeled mastocytoma cells in the presence of various concentrations of the glucose analogues 2-DG (●—●), 5-SH-G (▲---▲), or 3-O-methylglucose (■---■). Data are expressed as percent inhibition relative to control in the absence of analogue (absolute cytolysis 45%).

the ratio of 2-DG:glucose present and was not related to the absolute concentration of either hexose. Interestingly, these findings are formally analogous to studies of the competitive inhibition of certain reversible enzymatic reactions. In fact, if cytolysis is taken to be the end result of some form of enzymatic activity depending upon glucose as a substrate (and therefore a quantitative measure of its reaction velocity), the data of such a competition experiment can be plotted according to the Lineweaver-Burk formulation (Fig. 3). Although formal justification for this analogy is lacking, the results obtained are nevertheless consistent with the possibility that 2-DG inhibits cytolysis via competitive inhibition of some enzymatic reaction which normally utilizes glucose (or metabolites of glucose) as its substrate. Several possible reactions which may be inhibited by 2-DG will be discussed later.

Effect of Other Sugars on the Inhibition of Cytolysis by 2-DG.   In view of the ability of glucose to competitively reverse the inhibition of cytolysis induced by

TABLE II

Reversibility of Effect of 2-DG on CTL and/or Target Cells*

Pretreatment of cells		Percent specific ^{51}Cr release		
MLC	Mastocytoma	10:1‡	3:1	1:1
–	–	74	47	20
2-DG	–	82	56	30
–	2-DG	79	54	25
2-DG	2-DG	82	52	25

* MLC cells (10^6/ml) or ^{51}Cr-labeled mastocytoma cells (5×10^5/ml) in low glucose medium were incubated in the presence or absence of 5 mM 2-DG for 90 min. All cells were then washed and assayed for cytotoxicity in the combinations indicated.
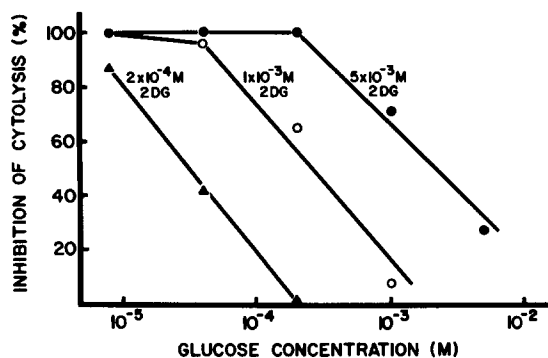
‡ Lymphocyte:target cell ratio.

FIG. 2. Competition between 2-DG and glucose for inhibition of cytolysis. Mixtures of MLC cells and ^{51}Cr-labeled target cells in low glucose medium were assayed for cytolysis in the presence of 5×10^{-3} M (●—●), 1×10^{-3} M (○—○), or 2×10^{-4} M (▲—▲) 2-DG and varying concentrations of glucose as indicated. Data are expressed as percent inhibition relative to control without 2-DG (absolute cytolysis 48%).

2-DG, it was decided to compare the effect of other sugars under these conditions. To this end, sodium pyruvate and several hexoses were added at a final concentration of 5 mM to mixtures of MLC cells and labeled target cells in the presence of 5 mM 2-DG. As can be seen from Table III, the addition of mannose completely reversed the inhibitory effect of 2-DG. On the contrary, equimolar quantities of pyruvate, fructose, fucose, galactose, and glucosamine did not significantly alter the degree of inhibition observed in the presence of the analogue.

The competition between 2-DG and mannose was further investigated by varying the concentration of mannose or glucose added to cytolytic interactions inhibited by 5 mM 2-DG. The results of these studies demonstrated a dose-dependent reversal of inhibition that was quantitatively comparable for the two hexoses (Table III).

Competition between Other Glucose Analogues and 2-DG. Although 3-*O*-methylglucose and 5-SH-G were unable to inhibit cytolysis in the absence of glucose, it was nonetheless of interest to determine whether or not these other
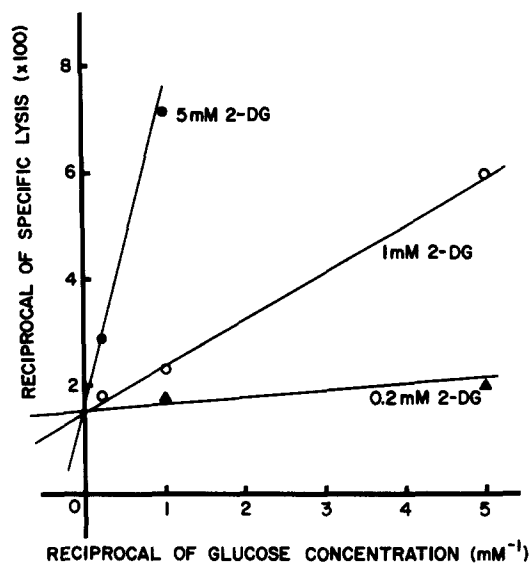
FIG. 3.   Lineweaver-Burk plot demonstrating competitive nature of inhibition of cytolysis by 2-DG. Data from the same experiment as in Fig. 2 have been used to plot reciprocal of reaction velocity (specific lysis) vs. reciprocal concentration of substrate (glucose) for various concentrations of inhibitor (2-DG). Symbols are as for Fig. 2.

TABLE III

*Effect of Pyruvate and Various Hexoses on the Inhibition of Cytolysis by 2-DG**

2-DG concentration	Hexose added (concentration)	Specific lysis
M	*M*	%
None	None	55
5×10^{-3}	None	0
5×10^{-3}	Pyruvate (5×10^{-3})	0
5×10^{-3}	Glucose (5×10^{-3})	49
5×10^{-3}	Glucose (1×10^{-3})	29
5×10^{-3}	Glucose (2×10^{-4})	6
5×10^{-3}	Mannose (5×10^{-3})	58
5×10^{-3}	Mannose (1×10^{-3})	28
5×10^{-3}	Mannose (2×10^{-4})	4
5×10^{-3}	Fructose (5×10^{-3})	0
5×10^{-3}	Galactose (5×10^{-3})	1
5×10^{-3}	Fucose (5×10^{-3})	1
5×10^{-3}	Glucosamine (5×10^{-3})	2

* MLC cells (10^5) in low glucose medium were assayed for cytotoxicity on ^{51}Cr-labeled mastocytoma cells in the presence of 2-DG and the indicated concentration of hexose.

analogues, like glucose, could competitively reverse the inhibitory effect of 2-DG on cytolysis. To test this possibility, MLC cells and target cells were mixed in low glucose medium in the presence of 1 mM 2-DG, and various concentrations of the other analogues were added. As in the previous competition experiments, various concentrations of glucose were added to the inhibited mixtures in
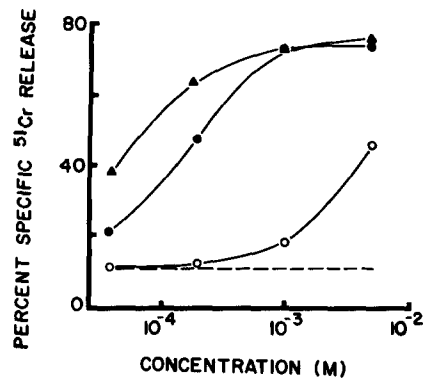
FIG. 4. Effect of other glucose analogues on the inhibition of cytolysis by 2-DG. MLC cells in low glucose medium were assayed for cytotoxicity in the presence of 1×10^{-3} M 2-DG alone (---) or in addition to various concentrations of glucose (▲—▲), 5-SH-G (●—●), or 3-O-methylglucose (O—O). Cytolysis in the absence of inhibitor was 67%.

parallel in each experiment as a positive control. The results of a typical experiment (Fig. 4) demonstrated that 3-O-methylglucose was completely unable to compete with 2-DG under these conditions, whereas 5-SH-G was able to reverse the inhibition induced by 2-DG at concentrations comparable to those of glucose.

Discussion

The experiments described in this report demonstrate that the glucose and mannose analogue 2-DG is a potent reversible inhibitor of T-cell-mediated cytolysis when low concentrations of glucose are present in the assay medium. In contradistinction to the results presented in the accompanying paper (4), this inhibition was not dependent on the simultaneous inhibition of aerobic energy metabolism and was highly selective in the sense that other analogues of glucose such as 5-SH-G and 3-O-methylglucose and deoxysugar analogues such as 2-deoxy-D-galactose (H. R. MacDonald, unpublished data) were not effective inhibitors under similar experimental conditions. Furthermore, inhibition of cytolysis by 2-DG could be competitively overcome by addition of either glucose or mannose, and by the glucose analogue 5-SH-G, but not by sodium pyruvate, other hexoses, or 3-O-methylglucose. These studies thus raise the possibility that mechanisms other than simple inhibition of energy metabolism may be responsible for the inhibitory effect of 2-DG observed in the present study.

The well-documented inhibitory effect of 2-DG on glycolytic energy production in mammalian cells was discussed in detail in the accompanying paper (4), and operational evidence for an energy-related anaerobic inhibition of cytolysis by the analogue was presented. Despite these results, however, several observations suggest that the competitive inhibition of cytolysis by 2-DG observed under aerobic conditions cannot be explained in this manner. Firstly, the concentration of 2-DG (relative to glucose) required to inhibit cytolysis under aerobic conditions differs substantially from that required for inhibition when cellular respiration is blocked. Whereas equimolar concentrations of 2-DG and glucose were sufficient to block cytolysis completely in the presence of sodium azide (Table IV of reference 4) or under conditions of extreme hypoxia (Table III of

reference 4), a molar ratio of 25:1 was required for complete inhibition of cytolysis by 2-DG under aerobic conditions (Fig. 2). Since the inhibition of cytolysis by 2-DG in the absence of respiration is probably a quantitative measure of the capacity of the analogue to inhibit glycolytic energy production, the fact that a 25-fold molar excess was required to inhibit cytolysis under aerobic conditions is consistent with the possibility that the inhibition is not directly related to energy depletion.

A stronger argument that the inhibitory effect of 2-DG on cytolysis may be unrelated to energy production comes from the parallel studies of the effects of 2-DG and 5-SH-G on cytolysis. Although the metabolism of 5-SH-G has not been extensively studied, it is known that the thiosugar, like 2-DG, is phosphorylated and accumulation of its phosphorylated derivatives leads to inhibition of phosphoglucomutase and hexokinase (6). In the accompanying paper (4), we have shown that 5-SH-G inhibits cytolysis as well (if not better) than 2-DG under conditions where aerobic energy production was either absent or metabolically blocked (Fig. 2 and Table III of reference 4). These findings strongly suggest that 5-SH-G interferes with glycolysis in CTL as effectively as 2-DG, and hence the failure of the thiosugar to block cytolysis under aerobic conditions provides strong operational evidence that the inhibitory action of 2-DG on CTL is not related to glycolytic energy production. Furthermore, the ability of 5-SH-G, itself a potent inhibitor of glycolysis, to competitively reverse the inhibition of cytolysis caused by 2-DG (Fig. 4) is inconsistent with the hypothesis that glycolytic energy production is involved.

Additional evidence that inhibition of cytolysis by 2-DG is probably not directly related to energy production in CTL can be inferred by comparing the current results with the recent data of Michl et al. (7, 8). These workers demonstrated that the inhibitory effect of 2-DG on Fc and complement receptor-mediated phagocytosis mediated by mouse peritoneal macrophages could be readily dissociated from its inhibitory effect on cellular ATP generation. In particular, the addition of glucose or mannose to 2-DG-inhibited macrophages resulted in a restoration of their phagocytic function with no concomitant increase in ATP generation. The operational similarity between these findings and the restoration of cytolysis caused by the addition of glucose or mannose to 2-DG-inhibited CTL suggests strongly that ATP generation is likewise not involved in the latter phenomenon. Lack of availability of purified populations of CTL for ATP measurements precludes a more direct approach to this question.

The failure of 3-O-methylglucose to inhibit cytolysis or to competitively reverse the inhibition induced by 2-DG is probably related to a failure of CTL to metabolize this analogue. In this context, it is known that 3-O-methylglucose competitively inhibits glucose transport but is not phosphorylated or further metabolized in mammalian cells (9, 10). In the accompanying paper (4), we showed that 3-O-methylglucose, in contrast to 5-SH-G or 2-DG, failed to inhibit cytolysis under conditions where respiration was blocked, suggesting that the analogue was not effective in interfering with glycolytic energy metabolism in CTL under these conditions. The conclusion that T-cell-mediated cytolysis can be dissociated from glucose transport was reached independently by Bubbers

and Henney (11), who observed a differential effect of cytochalasin A on cytolysis and glucose transport.

If the inhibition of T-cell-mediated cytolysis by 2-DG is not related to its effect on CTL energy production, other possible mechanisms of action of 2-DG must be considered. In this regard, it is noteworthy that the incorporation of 2-DG into glycoproteins and glycolipids has been demonstrated in mammalian cells (12, 13), and nucleoside-diphosphate derivatives of 2-DG have been reported (14). Furthermore, incorporation of 2-DG has been shown to lead to defective cell wall formation in yeast (15) and aberrant viral envelope glycoprotein synthesis in certain virus-infected mammalian cells (16, 17). In view of these findings, it seems possible that 2-DG is being incorporated into glycoproteins and/or glyco-lipids in CTL, or alternatively, as suggested by the competitive inhibition studies, metabolites of 2-DG might compete with glucose metabolites for key enzymes (such as glycosyl transferases) involved in normal glycoprotein biosyn-thesis or assembly. In either case, the net result might be a reversible change in CTL membrane conformation which could be manifested as a failure of the CTL to recognize and/or damage its target. Consistent with this view that inhibition of cytolysis is related to defective glycoprotein biosynthesis is the observation that the inhibitory effects of 2-DG on CTL could be reversed by addition of glucose or mannose, but not by other hexoses (Table III). A similar result has been obtained with the reversal of 2-DG-mediated inhibition of viral envelope glycoprotein synthesis (16, 17). In either case, the reversal of inhibition by these sugars could be interpreted as a competition between their metabolites and the metabolites of 2-DG as glycoprotein precursors or alternatively a competition for important glycosyl transferases. The ability of 5-SH-G to reverse the inhibitory effect of 2-DG on cytolysis could be interpreted in this context to mean that metabolites of the thiosugar are formed which compete with metabolites of 2-DG in a similar fashion. In fact, competition between nucleoside-diphosphate deriv-atives of 5-SH-G and glucose has been reported (18), although the effect of this competition on membrane glycolipids and/or glycoproteins is not known. Clearly, more detailed studies of the metabolism of 5-SH-G are required before this hypothesis can be critically evaluated.

Although the foregoing discussion has concentrated on the possible conse-quences of 2-DG metabolism in CTL, it should be noted that effects on the target cells cannot be excluded due to the complete reversibility of the phenomenon (Table II). In this context, however, we have recently demonstrated that lysis of the same mastocytoma target cells by alloantibody and complement or by antibody-dependent cell-mediated cytotoxicity (using human peripheral blood lymphocytes as effector cells) is not inhibited by 2-DG under conditions where murine T-cell-mediated cytolysis is completely blocked (H. R. MacDonald, unpublished data). The latter observations are consistent with (but do not prove) the hypothesis that the inhibitory action of 2-DG in the present system can be attributed to a direct effect on CTL.

Whatever the molecular basis of the inhibition of T-cell-mediated cytolysis by 2-DG may be, it is interesting to note that the release of histamine in immediate hypersensitivity reactions is also inhibited by comparable concentrations of 2-DG in rat mast cells (19) and in human basophils (20). Furthermore, as men-

tioned above, recent reports by Michl and his colleagues (7, 8) have demonstrated a similar inhibitory effect of 2-DG on Fc and complement receptor-mediated phagocytosis by mouse peritoneal macrophages. The latter results are strikingly similar to the current study in that inhibition of phagocytosis was dependent upon the presence of 2-DG in sufficient molar excess over glucose, and could be competitively reversed by the addition of glucose or mannose, but not by other hexoses. The striking similarities between their results and ours raise the possibility of previously unsuspected functional (or structural) relationships between T-cell-mediated cytolysis and other receptor-mediated phenomena. More detailed biochemical characterization of the mechanisms involved and of the receptors upon which they depend is required to critically test such a possibility.

Summary

The effect of the hexose analogue 2-deoxy-D-glucose (2-DG) on T-cell-mediated cytolysis has been investigated. 2-DG inhibited cytolysis in glucose-free medium but not in medium containing equimolar concentrations of glucose. This inhibition was reversible and quantitatively competitive with glucose. Among other natural sugars examined, only mannose competed effectively with 2-DG and reversed the inhibition of cytolysis, whereas sodium pyruvate, fructose, galactose, fucose, and glucosamine were without effect. Mannose and glucose were equally effective in competing with 2-DG on a molar basis. When other glucose analogues such as 5-thio-D-glucose (5-SH-G) and 3-O-methylglucose were investigated under the same conditions, no inhibition of cytolysis was observed; however, 5-SH-G (but not 3-O-methylglucose) was able to reverse the inhibitory effect of 2-DG in a competitive fashion. Taken together with the data presented in the accompanying paper, these findings provide strong evidence that 2-DG inhibits T-cell-mediated cytolysis by a mechanism that is unrelated to energy production. The possibility that inhibition is related to interference with membrane glycoprotein synthesis is discussed.

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