REQUIREMENT FOR HEXOSE, UNRELATED TO ENERGY PROVISION, IN T-CELL-MEDIATED CYTOLYSIS AT THE LETHAL HIT STAGE*

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Although much information has been accumulated on the process of cytolysis by thymus-dependent (T)[1] lymphocytes, the precise biochemical basis for the lytic mechanism is still unclear. The present knowledge of the subject has recently received extensive review (1–3). Three approaches have been adopted for the study of the metabolic processes essential for T-cell-mediated cytolysis. First, experiments have been carried out using support media which have been deprived of primary metabolic substrates. For example, assessment of cytolysis has been performed in glucose-free medium, under anaerobic conditions (4), or in the absence of divalent cations (5). Second, drugs have been used to block cytolysis and the findings have been interpreted in relation to the reported major effects of these drugs on metabolic processes. However, in general no control has been provided to indicate that the observed effect on cytolysis was causally linked to the reported metabolic effects. This approach has been fully reviewed by Martz (2). Finally, competitive inhibitors have been used, with a check on the specificity of their action through reversal of their effect with excess normal substrate. Probably the best example of such an approach has been the recent work of MacDonald (6) using 2-deoxy-D-glucose as an inhibitor of cytolysis, and D-glucose or D-glucose analogs to achieve reversal. These results appear to have formally established a role for glucose or a glucose analog in T-cell-mediated cytolysis. Interestingly, some of MacDonald's results indicated that glucose might not be acting simply as an energy source.

Most previous investigations with drugs, including 2-deoxy-D-glucose, have failed to establish at which stage(s) of cytolysis the agents were acting. T-cell-mediated cytolysis can now be considered as a complex phenomenon including a number of discrete stages (7, 8). The first recognition stage is followed by the "lethal hit" stage during which an irreversible lesion is inflicted upon the target

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[1] *Abbreviations used in this paper:* βNAD, β-nicotinamide adenine dinucleotide; DFBS, dialysed fetal bovine serum; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; FBS, fetal bovine serum; LDH, lactate-dehydrogenase; LPBS, low phosphate-buffered saline; T lymphocyte, thymus-dependent lymphocyte.

cell by the effector cell. This is in turn followed by the target cell disintegration stage. Our approach to an analysis of these various stages makes use of a "Ca^{++} pulse" method (3, 9). This method is based on the observation that although the cytolytic process as a whole requires Ca^{++} (5, 9–13), the recognition stage can proceed in the presence of Mg^{++} only (9, 13, 14), and neither Ca^{++} nor Mg^{++} are required at the disintegration stage (5, 8, 10, 15, 16). Ca^{++}, therefore, is necessary only at the lethal hit stage (9, 13). Consequently, the successive addition of Mg^{++}, Ca^{++}, and finally EDTA, provides a simple and convenient way of isolating and studying the lethal hit stage (9). It can be used to investigate the impact of drugs at various stages of the lytic process (3).

In this study we investigated the carbohydrate requirement of T-cell-mediated cytolysis and showed: (*a*) using chemically defined media or the competitive inhibitor 2-deoxy-D-glucose, that glucose or certain glucose analogs are required for the cytolytic process; (*b*) using the Ca^{++} pulse technique and cytochalasin A, which is a selective inhibitor of recognition, that the carbohydrate requirement for cytolysis can be localized at the lethal hit stage; (*c*) that cytolysis can also take place in phosphate-buffered saline plus dialyzed fetal bovine serum without added glucose but even in this support medium cytolysis is blocked by 2-deoxy-D-glucose; (*d*) by studying the three main energy-producing pathways of carbohydrate catabolism, that a major requirement for carbohydrate in T-cell-mediated cytolysis is independent of its capacity to act as an energy source.

Materials and Methods

Media. RPMI-1640 (Gibco Bio-Cult, Glasgow, Scotland) was used as the standard tissue culture medium and as a diluent. Low phosphate-buffered saline (LPBS) was made up as an aqueous solution containing 137 mM NaCl, 2.7 mM KCl, 1.5 mM K_2HPO_4, 3.2 mM Na_2HPO_4 (12 H_2O), 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ (6 H_2O), and 10 mg/l of phenol red. Heat-inactivated (56°C, for 30 min) fetal bovine serum (FBS) (batch 802.42; Eurobio, Paris, France) was used as such or (after dialysis against 100 vol of LPBS with two changes over three 24-h periods) as dialyzed FBS (DFBS).

Chemicals. Chemicals used were D-glucose (Prolabo, Paris, France), 2-deoxy-D-glucose and cytochalasin A (Aldrich Chemical Co., Inc., Milwaukee, Wis.), lactate-dehydrogenase (LDH) from pig heart, and β-nicotinamide adenine dinucleotide (βNAD) grade 1, both from C. F. Boehringer and Sons (Mannheim, W. Germany), and hydrazine hydrate (98% pure; Merck AG, Darmstadt, W. Germany). D-[1-^{14}C]glucose (sp act 4–15 mCi/mM), D-[6-^{14}C]glucose (sp act 3.0 mCi/mM), and 2-deoxy-D-[1-^{14}C]glucose (sp act 59 mCi/mM) were obtained from the Radiochemical Centre (Amersham, England). Sodium ^{51}Cr-chromate (sp act >50 mCi/mg) was obtained from the Commissariat à l'Energie Atomique (Gif-sur-Yvette, France).

Preparation of Sensitized Spleen Cell Populations Containing Cytolytic T Cells. C57BL/6 anti-DBA/2 (*H-2^b* anti-*H-2^d*; *b* anti-*d* for short) cell populations containing cytolytic T cells, and the reciprocally sensitized *d* anti-*b* cells were obtained by mixed lymphocyte culture as follows. Mouse splenic responder cells (2×10^7) and irradiated (2,000 rads) allogeneic splenic stimulating cells (2×10^6) were coincubated (37°C in 5% CO_2 atmosphere, for 4–5 days) in an upright plastic flask (Falcon 3013; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in a 20-ml vol of RPMI-1640 with 10% FBS, 50 U/ml of penicillin, 50 μg/ml of streptomycin, 2 mM L-glutamine, and 2×10^{-5} M 2-mercaptoethanol. The sensitized cell populations were used for cytolysis and metabolic tests. It is amply documented that the cytolysis obtained under these sensitization conditions and using a short-term assay (see below) is due to T cells (17, 18). It should be stressed that these populations sensitized in vitro contain only a small minority of cytolytic T cells (19) with other T cells, and 30–40% of non-Thy 1-bearing cells (unpublished observations).

Cytolysis Tests. All the assays were done in Cooke microtiter plates with V-shaped wells

(Cooke Laboratory Products, Div. Dynatech Laboratories Inc., Alexandria, Va.), each well receiving 2×10^5 washed, sensitized cells and 10^4 twice-washed P 815 (*d* mastocytoma) cells which had been ^{51}Cr-labeled overnight (20). Upon addition of both types of cells, the microplates were briefly centrifuged (200 *g*, 2 min) to hasten cell-to-cell contacts (20, 21). Incubation was for 4 h at 37°C unless stated otherwise.

In standard assays, target and effector cell populations were washed and resuspended in LPBS and mixed in the cold. Each V-shaped well received successively (*a*) 50 μl of drug and/or nutrient solution, (*b*) 50 μl of the cell mixture (followed by centrifugation), (*c*) after 1 h of incubation at 37°C, 50 μl of 12 mM EDTA to stop further cytolysis (16), (*d*) 5 min afterwards, 50 μl of RPMI-1640 with 10% FBS. The plates were incubated for 3 h at 37°C after the addition of EDTA to allow ^{51}Cr to be released from injured cells. Cell feeding under EDTA cover, after the initial cytolytic stages, allowed exacting media to be tested while the level of spontaneous ^{51}Cr release was kept low.

In Ca^{++} pulse assays (3, 9), target and effector cell populations were washed and resuspended in RPMI-1640 with 1% FBS, 1 mM Mg^{++} (as $MgCl_2$), and 1 mM of ethylene glycol bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA; Sigma Chemical Co., St. Louis, Mo.). The presence of EGTA ensured neutralization of the Ca^{++} contained in RPMI-1640, while Mg^{++} was still available in excess to the cells. The cell populations were mixed in the cold. Each V-shaped well received successively (*a*) 50 μl of the cell mixture (followed by centrifugation), (*b*) after a 40-min incubation at 37°C, 50 μl of Ca^{++} as $CaCl_2$ solution to a final concentration of 1 mM, (*c*) after a further 20-min incubation at 37°C, 50 μl of 12 mM EDTA. Glucose solutions at various concentrations were added as a 50-μl fraction per well either ab initio, or 10 min before the addition of Ca^{++} (prepulse), or just after the addition of EDTA (postpulse). Protocols of individual experiments differing from this general outline are described in Results. The plates were incubated for 3 h at 37°C after the addition of EDTA.

At the end of the incubation period, the plates were centrifuged again, the radioactivity of a 100-μl aliquot from each well was compared with the initial radioactivity of 5×10^3 target cells, and cytolysis was expressed as the averaged percentage of ^{51}Cr released from target cells in triplicate wells.

Measurement of Lactate Production. Sensitized spleen cells were incubated in V-shaped wells of microplates (per well, 4×10^5 cells for 3 h, or 10^6 cells for 1 h, at 37°C in a total volume of 100 μl of LPBS containing the nutrients mentioned in Results). At the end of the incubation, perchloric acid was added to achieve 2% vol/vol in a total volume of 200 μl/well. The plates were centrifuged, and 150-μl supernatant aliquots were transferred to new microplate wells. These aliquots were brought to pH between 6 and 8 (estimated by phenol red in medium) with 20% KOH, and then stored at 4°C for 15 min. The plates were then centrifuged, and 100-μl aliquots were added to tubes containing 100 μl 2% vol/vol βNAD, 1 ml of Tris-hydrazine buffer, pH 9.5, and water to 2.97 ml. The optical density of these solutions was read at 340 nm in 1-cm cuvettes (E^{340} 1 cm). A 30-μl volume of the LDH solution was then added, and the E^{340} 1 cm of solutions was read after 30 min and again after 40 min to check stability. The difference between the initial and final spectrophotometer readings was then subtracted from the medium only blank reading to give ΔE. The lactate present was calculated using the molar extinction coefficient of NADH. Lactate standards were included in experiments. The averaged results for duplicate wells were expressed as nanomoles of lactate produced per 10^6 cells per h.

Measurement of $^{14}CO_2$ Production from D-[1-^{14}C]Glucose, D-[6-^{14}C]Glucose and 2-Deoxy-[1-^{14}C]Glucose. Sensitized spleen cells were incubated in 6 × 40-mm polystyrene tubes (RT 15; Sterilin, Teddington, Middlesex, England) placed in rubber cap-sealed scintillation vials (Intertechnique, Plaisir, France). Conditions for incubation were the same as for the lactate assay except that 0.025 μCi of [^{14}C]glucose was added to each RT 15 tube. In most experiments, cold glucose was included in excess as specified in Results. At the end of the incubation period, a 200-μl volume of 0.5 M KOH was injected through the rubber seal into each vial outside the RT 15 tube. A 100-μl volume of 30% perchloric acid was then injected into each RT 15 tube. The vials were incubated in a shaking water bath for 1 h at 37°C to allow all CO_2 to be absorbed by the KOH. The RT 15 tubes were then removed and scintillation fluid (5 ml) was added directly to each vial. The amount of CO_2 produced from carbon 1 or 6 of D-glucose, termed 1-C.CO_2 and 6-C.CO_2 respectively, was estimated as:

$$\frac{\text{counts per minute measured}}{\text{counts per minute added}} \times \text{nanomoles D-glucose added.}$$

(This made no allowance for glucose recycled from the pentose cycle and diluting D-[1-^{14}C]glucose, but in the experiments quoted, the total glucose added to cultures was sufficiently large to make this a minor error.) The main purpose of the metabolic measurements in this study was to gain information about the doses of drugs which effectively stop and the levels of nutrients which effectively support energy production by the three major glycolytic pathways. Only rough estimates of absolute traffic could be derived from the data presented. Methods of calculating the relative activity of the main glucose utilization pathways were discussed in detail by Katz and Wood (22). Measurements of 1-C.CO_2, 6-C.CO_2, and lactate production were expressed as nmol/10^6 cells/h.

Repetition of Experiments. All cytolysis and metabolic experiments reported were carried out at least three times yielding similar results. All nutrient and drug solutions were prepared fresh for each assay.

Results

The Relative Contribution of Different Metabolic Pathways to Energy Production in Sensitized Spleen Cells at Different Glucose Concentrations. To set a basis for the studies of carbohydrate utilization during cytolysis, the relative contribution of the main metabolic pathways of energy production in sensitized spleen cells was examined. Although the metabolic activity of cytolytic T cells is not necessarily reflected by the sum activity of 5-day one-way mixed lymphocyte cultures, it was considered that these measurements would provide a better indication of the active dose range of metabolic inhibitors than could be obtained from reports of previous studies on totally different cell populations. Sensitized spleen cells (10^6) were incubated for 3 h in a total volume of 100 μl of LPBS in the presence of different concentrations of D-glucose, after which the amount of metabolites produced was assessed (Table I).

Lactate production of >4 mM glucose (800 nmol/culture) was constant, and the relative contribution of the pentose cycle to lactate production was well under 5%. Lactate production was markedly less when limiting glucose concentrations were used, with a corresponding increase of the relative importance of the pentose cycle and Krebs cycle. Experiments using lower concentrations of glucose in 100-μl cultures showed that lactate production was below the level of sensitivity of the assay used at $\cong$0.05 mM glucose. This value is useful for interpreting experiments (described below) on the supportive role of DFBS in cytolysis.

The Carbohydrate Requirement of T-Cell-Mediated Cytolysis. Two lines of evidence strongly suggested that D-glucose (or one of its metabolites or analogs) was required for T-cell-mediated cytolysis. First, good levels of specific cytolysis could be produced in LPBS plus D-glucose, but there was no detectable cytolysis in LPBS alone (Fig. 1). Second, 2-deoxy-D-glucose was able to competitively inhibit cytolysis when in excess over glucose (Fig. 2). It was possible to delay the addition of glucose for 40 min in cultures initiated in LPBS and still get cytolysis (Table II, group 1). This showed that glucose deprivation was not immediately lethal for the cytolytic T cells. Furthermore, the blocking effect of 2-deoxy-D-glucose added ab initio could be reversed by the addition of excess glucose 40 min later (Fig. 3). This indicated a complete reversibility by glucose of the 2-deoxy-D-glucose block of cytolysis. Taken together, these results

TABLE I

Assessment of 1-C.CO_2, 6-C.CO_2, and Net Lactate Production by Sensitized Spleen Cells Cultured in LPBS Plus Varying Concentrations of Glucose

Production of*	Glucose concentration (mM)					
	16	8	4	2	1	0.5
Lactate	30	30	29	25	18	7
1-C.CO_2	3.9	4.5	6.6	6.2	5.9	4.6
6-C.CO_2	0.24	0.24	0.31	0.31	0.26	0.24

* Results are expressed in nmol/10^6 cells/h.

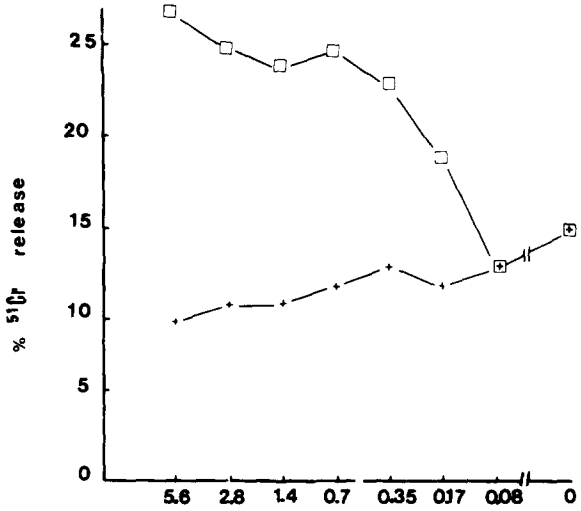

FIG. 1. Concentration of D-glucose able to support T-cell-mediated cytolysis in LPBS. Each culture contained 10^4 target cells (P 815 of *H-2^d* origin) and 2×10^5 effector cells. Cytolysis mediated by *b* anti-*d* (□) and *d* anti-*b* (+) effector cells.

showed that glucose was required to support cytolysis and not just to ensure cytolytic cell survival.

The Stage of the Cytolytic Process Where Glucose is Required. Calcium pulse experiments were carried out to see if glucose was required for the Ca^{++}-dependent lethal hit stage of cytolysis. 2-deoxy-D-glucose was added to glucose-containing effector cell-target cell mixtures, either ab initio, 10 min before the Ca^{++} pulse, or just after the Ca^{++} pulse. Block occurred with prepulse but not with postpulse addition of 2-deoxy-D-glucose (Fig. 4). This type of experiment indicated that glucose was required at the lethal hit stage but did not give information about possible glucose requirements at the recognition stage. To investigate this, the drug cytochalasin A was used. At appropriate concentrations, this agent inhibits recognition but not the Ca^{++}-dependent lethal hit stage (23).[2] Cell mixtures were set up in LPBS only, and glucose was added either before or after the addition of cytochalasin A. The detailed protocol of the

[2] P. Golstein, C. Foa, and I. C. M. MacLennan. 1978. Mechanism of T-cell-mediated cytolysis: the differential impact of cytochalasins at the recognition and lethal hit stages. *Eur. J. Immunol.* In press.

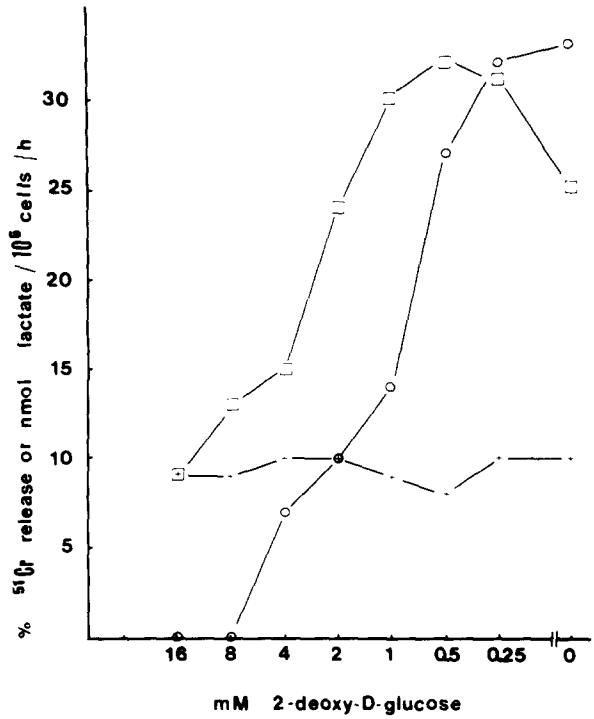

FIG. 2. Concentration of 2-deoxy-D-glucose required to inhibit (*a*) specific T-cell-mediated cytolysis; (*b*) lactate production by sensitized T cells. Cytolysis mediated by *b* anti-*d* (□) and *d* anti-*b* (+) effector cells. (○), Lactate production by *b* anti-*d* cells. The basic culture medium was LPBS + 3 mM D-glucose.

TABLE II

Lack of Glucose Requirement for the Cytochalasin A-Dependent Stage of Recognition

Group	Time (min) of culture addition*						Cytolysis of *d* target cells by the following effector cells‡	
	Pre 0	0	30	40	60	65	*b* anti-*d*	*d* anti-*b*
1	LPBS	Cells in LPBS	LPBS	Glucose	EDTA	RPMI 10% FBS	28.8	16.2
2	LPBS	"	Cytochalasin A	"	"	"	25.3	14.9
3	Cytochalasin A	"	LPBS	"	"	"	18.2	15.3
4	LPBS	"	LPBS	LPBS	"	"	17.1	16.7
5	Glucose	"	Cytochalasin A	"	"	"	26.2	16.1

* Additions at time pre 0, 0, 30, and 40 min in 25 μl. Additions at 60 and 65 min in 50 μl. No serum additive was used before 65 min. Cultures were harvested at 4 h.

‡ Cytolysis expressed as percent ^{51}Cr release (means of quadruplicate cultures).

experiment and its results are shown in Table II. A comparison of groups 2 and 5 in particular indicated that the cytochalasin A-inhibitable step did not require glucose. Taken together, these experiments showed that D-glucose was required during the lethal hit stage of cytolysis, but was not required at either the cytochalasin A-inhibitable recognition stage, nor the ^{51}Cr release stage.

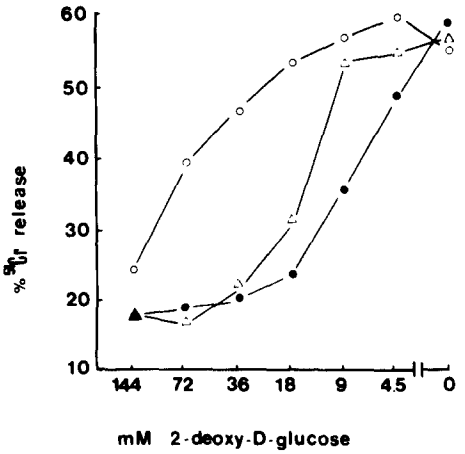

FIG. 3. Ability of D-glucose to reverse the inhibition of cytotoxicity induced by 2-deoxy-D-glucose. All cultures were set up with 10^4 P 815 and 2×10^5 *b* anti-*d* cells in RPMI-1640 (11.5 mM D-glucose) with 1% FBS plus the dilutions of 2-deoxy-D-glucose shown. The cultures were then centrifuged and incubated for 40 min at 37°C before glucose supplements were added to the culture: no supplementary D-glucose (●), 18 mM D-glucose (△), 72 mM glucose (○). Cultures were harvested 3 h later.

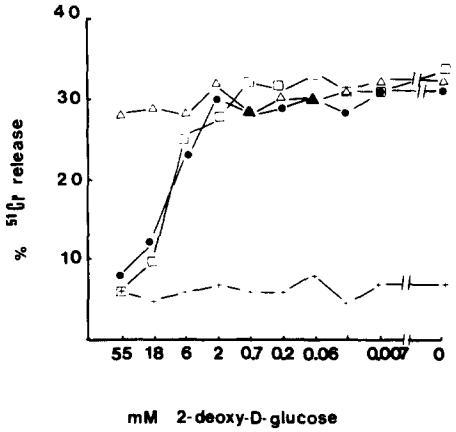

FIG. 4. The stage of T-cell-mediated cytolysis inhibited by 2-deoxy-D-glucose. Cultures of 10^4 P 815 and 2×10^5 effector cells were set up in RPMI-1640 + 1% FBS, 1 mM EGTA, and 1.5 mM Mg^{++} to allow binding without lysis. After 40 min, Ca^{++} was added (final concentration 1 mM) to initiate the lethal hit. 20 min later, EDTA was added (final concentration 3 mM). Cultures were harvested 3 h later. (△), *b* anti-*d* effector cells, 2-deoxy-D-glucose added after EDTA; (□), *b* anti-*d* effector cells, 2-deoxy-D-glucose added ab initio; (●), *b* anti-*d* effector cells, 2-deoxy-D-glucose added before Ca^{++} pulse; (+), *d* anti-*b* effector cells, 2-deoxy-D-glucose added ab initio.

Three Lines of Evidence Suggesting that the Role of D-Glucose in T-Cell-Mediated Cytolysis Is Unrelated to Its Capacity to Provide Energy

2-DEOXY-D-GLUCOSE, WHICH BLOCKS CYTOLYSIS, CAN ACT AS AN ENERGY SOURCE. The experiments exemplified in Fig. 2 clearly showed the efficiency of 2-deoxy-

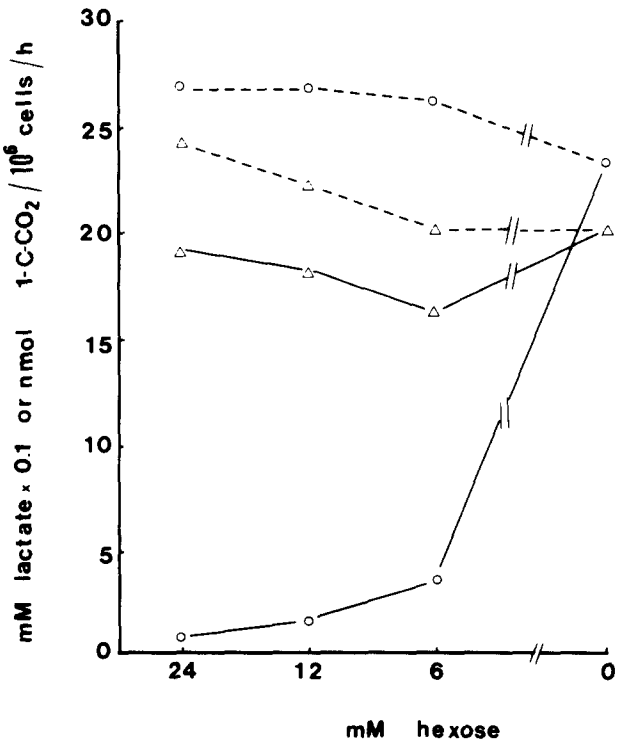

FIG. 5. Failure of 2-deoxy-D-glucose to inhibit 1-C.CO_2 production from the 1-C atom of glucose by *b* anti-*d* effector cells. Medium used was 5 mM D-glucose in LPBS. 1-C.CO_2 production (△——△), net lactate production (○——○) with added 2-deoxy-D-glucose; 1-C.CO_2 production (△- - - △), net lactate production (○- - -○) with added D-glucose.

D-glucose at blocking lactate production. However, as this agent does not block the cytochrome system (24), it could be a source of energy via the pentose cycle, provided that it did not prevent the reduction of NADP in the first or second enzymatic steps of this cycle. This was investigated in two ways. First, the effect of 2-deoxy-D-glucose on $^{14}CO_2$ production from D-[1-^{14}C]glucose by sensitized spleen cells was assessed. Fig. 5 shows that 2-deoxy-D-glucose, even at concentrations which inhibited lactate formation, did not significantly inhibit 1-C.CO_2 production from glucose. The second set of experiments tested the relative amounts of $^{14}CO_2$ produced from D-[1-^{14}C]glucose and 2-deoxy-D-[1-^{14}C]-glucose. These confirmed the previous experiments in that equivalent amounts of $^{14}CO_2$ were produced from both radiochemicals (Table III). This shows that 2-deoxy-D-glucose could be processed through the first two enzymatic steps of the pentose cycle and thus provide a potential energy source in the form of reduced NADP, which if transported to mitochondria, could be oxidized to yield ATP.

OTHER SUBSTRATES FOR ENERGY METABOLISM DO NOT SUPPORT CYTOLYSIS. A number of known substrates for energy metabolism were tested for their capacity to support T-cell-mediated cytolysis. These were glycerol, pyruvate, and the following glycogenic amino acids: alanine, arginine, asparagine, glycine, histidine, phenylalanine, serine, and valine. They were assessed at

TABLE III
Ability of Sensitized Spleen Cells to Produce 1-C.CO_2 from 2-Deoxy-D-Glucose in the pentose cycle

Culture conditions*			Production of 1-C.$^{14}CO_2$‡
2-Deoxy-[1-^{14}C]glucose	[1-^{14}C]Glucose	D-Glucose	
μM	*μM*	*mM*	
0.5	–	5.6	0.96
–	7.5	5.6	0.74

* Sensitized cells are cultured in RPMI-1640 with either [1-^{14}C]D-glucose or 2-deoxy-[1-^{14}C]D-glucose added.
‡ Expressed as nmol $CO_2/10^6$ cells/h.

TABLE IV
Ability of Exogenous Pyruvate to Compete with Pyruvate Produced by Glycolysis

Release of*	Pyruvate concentration (mM)			
	10	3.3	1.1	0
		cpm		
6-C.$^{14}CO_2$	65	126	210	626
1-C.$^{14}CO_2$	4,100	5,782	6,930	3,811

* Cultures were set up in LPBS plus 0.5 mM glucose including either [1-^{14}C]D-glucose or [6-^{14}C]D-glucose. The radioactive CO_2 released by the cultures was measured and is given here as counts per min (cpm). The rise in 1-C.CO_2 production associated with pyruvate is considered in the discussion.

final concentrations of 10 mM in LPBS and three twofold dilutions from this. Positive control groups with D-glucose were included. None of these substrates permitted significant T-cell-mediated cytolysis, whereas D-glucose supported significant cytolysis in each experiment (not shown). Experiments were set up with pyruvate or a mixture of glycogenic amino acids to see if the agents could be used as substrates for aerobic metabolism. Pyruvate strongly inhibited 6-$C^{14}CO_2$ production from glucose (Table IV), indicating that exogenous pyruvate could compete with cell-derived pyruvate as a substrate for energy metabolism. The ability of the glycogenic amino acids to act in this way was not clear from these experiments (not shown). An overall conclusion from these and the 2-deoxy-D-glucose experiments is that provision of energy without glucose or DFBS (see below) is not sufficient to support T-cell-mediated cytolysis.

DIALYZED FBS CAN SUPPORT T-CELL-MEDIATED CYTOLYSIS IN THE ABSENCE OF DETECTABLE LACTATE PRODUCTION. Experiments were set up to see if LPBS enriched with DFBS only, without added glucose, could support cytolysis. It was found (Fig. 6) that as little as 0.03% DFBS was able to do so. This result immediately posed serious questions in relation to the glucose dependence of cytolysis, for (*a*) thoroughly dialyzed FBS should contain no free glucose, and (*b*) there is insufficient nondialyzable carbohydrate in FBS for the agent to be

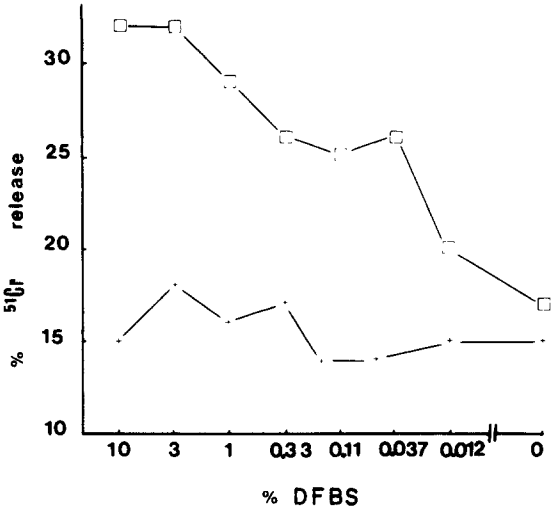

FIG. 6. Concentration of DFBS able to support T-cell-mediated cytolysis in LPBS. Each culture contained 10^4 P 815 target cells and 2×10^5 *b* anti-*d* (□), or *d* anti-*b* (+) effector cells.

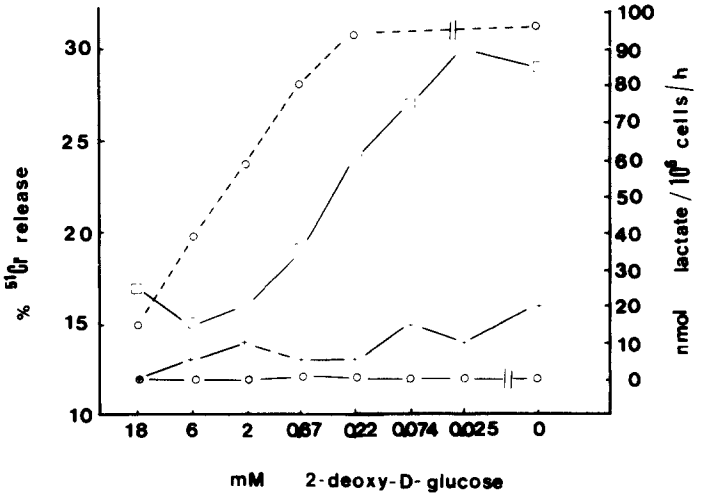

FIG. 7. Ability of 2-deoxy-D-glucose to inhibit T-cell-mediated cytolysis in LPBS + 5% DFBS and failure of 5% DFBS to support net lactate accumulation by effectors. Cytolysis by *b* anti-*d* (□), or *d* anti-*b* (+) effector cells. Lactate production by *b* anti-*d* cells in LPBS + 5% DFBS (○——○), or in LPBS + 3 mM glucose (○- - -○).

acting simply as a glucose source. Even if the mean carbohydrate content were 10% of serum proteins (100 g/liter) and all were available hexose, there would be only 0.017 mM hexose in 0.03% DFBS. This is well below the minimum glucose concentration able to support cytolysis in LPBS (Fig. 2). However, carbohydrates do appear to be essential in DFBS-supported cytolysis, since this was blocked by 2-deoxy-D-glucose (Fig. 7). Perhaps even more interestingly, when incubated in LPBS plus 5% DFBS (an excess of >100-fold over the

TABLE V
Level of 2-Deoxy-D-Glucose Required to Inhibit Cytolysis in LPBS Plus Various Amounts of DFBS

2-Deoxy-D-glucose	DFBS (%)			
	25	8	2	0.7
mM				
10	17*	15	16	18
3.3	17	16	17	17
1.1	22	22	21	20
0	30	33	33	33

* Cytolysis expressed as percent ^{51}Cr release from 10^4 *d* target cells in the presence of 2×10^5 *b* anti-*d*-sensitized spleen cells. Percent ^{51}Cr release in presence of *d* anti-*b* cells is not shown but was in the range of that seen with *b* anti-*d* cells plus 10 mM 2-deoxy-D-glucose.

TABLE VI
Synergism between Glucose and DFBS in Overcoming the Block on Cytolysis Induced by 2-Deoxy-D-Glucose

2-Deoxy-D-glucose (mM)	LPBS + 10% DFBS				LPBS + 6 mM glucose			
	16	8	4	0	16	8	4	0
D-Glucose supplement *mM*								
0	4/4*	5/4	7/4	20/4	7/6	8/7	10/7	22/8
8	17/5	22/5	24/6	20/4	10/6	14/7	17/8	22/6
16	23/5	24/4	25/5	22/5	14/7	16/7	20/7	24/10
32	24/5	23/5	23/5	24/6	19/9	21/8	21/8	26/12

* Cytolysis expressed as percent ^{51}Cr release from 10^4 *d* target cells in presence of 2×10^5 *b* anti-*d*/*d* anti-*b* effector cells.

minimum DFBS concentration required to support cytolysis), sensitized spleen cells did not produce measurable levels of lactate (Fig. 7). These experiments led us to two conclusions. First, whereas glucose did not seem to be involved in DFBS-supported cytolysis, as demonstrated by the quantitative considerations above, and by the absence of lactate production, clearly something closely related to glucose must be involved because of the inhibitory effect of 2-deoxy-D-glucose. Second, T-cell-mediated cytolysis could take place in the absence of detectable lactate production.

Does DFBS Directly Provide a Necessary Substrate for Cytolysis? To test this, the dose of 2-deoxy-D-glucose required to block cytolysis in LPBS plus dilutions of DFBS was assessed. The results of such an experiment are shown in Table V. The concentration of 2-deoxy-D-glucose required to inhibit cytolysis was found to be independent of the amount of DFBS added to cultures. Such a result would seem to indicate that 2-deoxy-D-glucose is not competing directly with a substrate in DFBS.

Synergism between D-Glucose and DFBS. A consistent observation has been that it was far more difficult to inhibit cytolysis with 2-deoxy-D-glucose when both DFBS and D-glucose were present than when either supportive agent

was present alone. A typical experiment is illustrated in Table VI. This synergism is marked at DFBS concentrations >5%, but is not obvious with 1% DFBS in LPBS. This observation reinforces the conclusions drawn above that DFBS is acting neither as a direct glucose source nor simply as an agent for mobilizing glucose.

Discussion

Now that it is possible to isolate different stages of T-cell-mediated cytolysis, the task of unravelling the biochemical basis of the process has become more approachable. This is exemplified in our study, which suggests an absolute requirement for glucose or a glucose derivative during cytolysis, and also indicates that this requirement is confined to the calcium-dependent lethal hit stage of cytolysis.

First, is D-glucose really necessary for cytolysis? It seems from the experiments showing specific T-cell-mediated cytotoxicity in LPBS plus D-glucose, but not in LPBS alone, that D-glucose is able to satisfy some molecular requirement for cytolysis. But these experiments do not tell us whether D-glucose is obligatory, or simply a substrate for synthesis which could be replaced by other molecules. At first the experiments showing lysis in LPBS with <0.1% DFBS appeared to indicate that D-glucose was not obligatory. But the finding that 2-deoxy-D-glucose blocked cytolysis in this support medium returned the balance in favor of glucose involvement. However, we know that cytolysis only proceeds in LPBS plus D-glucose when the D-glucose concentration is >0.1 mM, and that there is insufficient carbohydrate in 0.04% dialysed FBS in LPBS to provide this level of D-glucose. Also, the experiments showing no lysis in LPBS plus amino acids or pyruvate make it improbable that hexose could be synthesized from DFBS protein catabolism. This seems to leave three possible explanations for the mechanism of action of DFBS: (*a*) that it mobilizes carbohydrate from the cells, (*b*) that DFBS provides a carbohydrate molecule which is more efficient than glucose, (*c*) that DFBS increases the efficiency of cytolysis in some way, so as to reduce the requirement for glucose, for instance by bringing about increased cell-cell binding. The second of these possibilities seems unlikely because the concentration of 2-deoxy-D-glucose needed to block cytolysis in LPBS plus dialyzed FBS is independent of the concentration of dialyzed FBS (Table V). This observation is, however, compatible with explanation (*a*), (*c*), or a combination of both explanations.

Another indirect line of evidence compatible with D-glucose requirement at the lethal hit stage comes from studies with cytochalasins. Both cytochalasin A and B will block cytolysis when added to cultures ab initio, but only cytochalasin B inhibits when added after recognition and before the calcium pulse.[2] It is interesting that cytochalasin B, but not cytochalasin A blocks glucose utilization by sensitized spleen cells (25).[2] Perhaps the most convincing argument in favor of an absolute requirement for D-glucose or a D-glucose analog in cytolysis is the fact that 2-deoxy-D-glucose reversibly blocks cytolysis in complete culture medium (ref. 6 and Fig. 3). The selectivity of the reaction is further emphasized by MacDonald's observations (6) that many other hexoses and pyruvate fail to reverse the 2-deoxy-D-glucose block of cytolysis. Galactosamine, galactose,

fucose, and fructose all failed to reverse the block. However, it is of great interest that 5-thio-D-glucose and mannose could reverse this inhibition. The efficiency of 5-thio-D-glucose in competing with 2-deoxy-D-glucose, combined with the failure of trioses to support cytolysis, seems to indicate that D-glucose is used in the cytolytic process either as intact hexose or an early modification of hexose.

This argument implies that the role of D-glucose at the lethal hit stage is unrelated to the capacity of this compound to act as an energy source. The experiments shown in Table IV indicate that pyruvate is a perfectly good source of energy (ATP generation) in sensitized spleen cells as a whole. There is no good reason to suppose that the cytolytic T cells in this cell mixture should not also be able to derive energy from pyruvate by aerobic metabolism. However, pyruvate does not support cytolysis. Also, the aerobic metabolic pathways are not blocked by 2-deoxy-D-glucose (24) which is able to inhibit cytolysis. These results indicate that energy provision alone is not sufficient for cytolysis to proceed in simple salt solutions.

Is energy required at all for T-cell-mediated cytolysis? Most published work on this question involves the use of metabolic inhibitors. Iodoacetate has been shown to block T-cell-mediated cytolysis at $\cong 10^{-4}$ M (3, 26). This concentration of drug does block lactate production in sensitized spleen cells, but fails to inhibit aerobic metabolism.[3] Table I clearly indicates that Krebs cycle activity becomes the major source of energy at low glucose concentrations, so it is hard to conclude that iodoacetate is acting by blocking energy supplies necessary for cytolysis. This conclusion is reinforced by the observation that pyruvate fails to reverse the effect of this drug (3). These experiments as well as those with DFBS (Fig. 7) indicate that cytolysis can occur where net lactate accumulation is unmeasurable. Sodium azide in normal culture medium causes 50% block of cytolysis at $\cong 30$ mM (3, 26–28). This concentration of azide is approximately ten times that required to cause an equivalent inhibition of 6-C CO_2 production from glucose, but it is insufficient to inhibit glycolysis.[3] However, in the absence of exogenous glucose but in the presence of DFBS, i.e. under conditions that we found led to no detectable accumulation of lactate, cytolysis was blocked by concentrations of azide equivalent to those required to inhibit the terminal cytochrome system (4), which seems to indicate a minimal energy requirement for cytolysis.

The finding that 2-deoxy-D-glucose does not inhibit the formation of reduced NADP by the first two stages of the pentose cycle is of interest. Granulocytes undergo a burst of pentose-cycle activity during phagocytosis (29). It has been suggested that reduced NADP plays a vital role in the production of bactericidal products, particularly hydrogen peroxide (30). However, Michl et al. (31, 32) have shown that phagocytosis, but not binding of C_3 and IgG-coated erythrocytes by mouse peritoneal macrophages, is blocked by 2-deoxy-D-glucose. This effect appeared to be independent of ATP production. These authors showed that 2-deoxy-D-glucose did not inhibit opsonin-independent phagocytosis of latex particles, which suggests that this block may be interfering with a specific recognition process. It will be interesting to see if comparative studies show any

[3] I. C. M. MacLennan and P. Golstein. 1978. Manuscript in preparation.

close similarities between this block on phagocytes compared with that seen on T-cell-mediated cytolysis. Our data do not exclude a role for NADPH, but they do show that the 2-deoxy-D-glucose inhibition is not due to prevention of reduction of this coenzyme. NADPH could potentially act as an energy source if it were transported to mitochondria. However, there is no evidence that this occurs in cytotoxic T cells. Indeed, the experiment shown in Table IV suggests that NADPH may be accumulating in the cytoplasm. This experiment shows that pyruvate potentiates the production of 1-C.CO_2 from glucose, and this may be because pyruvate can oxidize NADPH either by combination with CO_2 to produce malate (33) or by reduction of pyruvate to lactate (34). The removal of NADPH is important, as this substance acts as a feedback inhibitor of glucose-6-phosphate oxidation by glucose-6-phosphate dehydrogenase (35).

How then is D-glucose acting in the cytolytic process, and how is 2-deoxy-D-glucose interfering with it? One of the actions of 2-deoxy-D-glucose which has recently received much attention has been its effect on the glycosylation of proteins. Eagon and Heath (36) showed that a K light chain made by BALB/c plasmacytoma cells cultured in 2-deoxy-D-glucose was synthesised and secreted without its carbohydrate side chain. These authors showed that 2-deoxy-D-glucose inhibited the incorporation of glucosamine, mannose, and galactose, which seems to indicate that this inhibitor has a broad range of action on hexose incorporation with glycoproteins. Hughes et al. (37) demonstrated a similar broad specificity of action of 2-deoxy-D-glucose in inhibiting protein glycosylation by hamster kidney cells. However, Meager et al. (38), using the same system, showed less inhibition of mannose incorporation than glucosamine and galactose. Mannose, as well as glucose, was found by MacDonald to reverse the 2-deoxy-D-glucose block of T-cell-mediated cytolysis (6). It has been shown that 2-deoxy-D-glucose interferes with virus-induced cell fusion (39). This effect is also reversed by mannose and glucose. Thus, the processes involved in these cases seem to exhibit similar specificity for glucose, mannose, and 2-deoxy-D-glucose.

It has been suggested that the impairment of cell fusion mentioned above may be due to a lack of replacement of shed surface glycoproteins, which is in line with a glycoprotein requirement for virus-induced cell fusion. For T-cell-mediated cytolysis at the lethal hit stage, a glycoprotein may also be required, synthesized by glycosylation of a preformed protein either in permanence or only upon a post-recognition activation process. This glycosylation step could be the one which is inhibited by 2-deoxy-D-glucose. Alternatively, this agent could inhibit another hexose-specific molecular recognition step occurring during the lethal hit. Whatever the proposed mechanism, it would have to fit in with the alterations of hexose concentration requirement observed in the presence of FBS.

Models such as these are of some help in designing further experiments to probe the mechanism of the lethal hit in T-cell-mediated cytolysis. These models must now take into account not only the fact that this stage of the lytic process is dependent on calcium ions and is sensitive to agents such as azide and iodoacetate, but also that it has a requirement for hexose which is independent of its capacity to act as an energy source.

Summary

The requirement for D-glucose in T-cell-mediated cytolysis was studied using mouse spleen cells sensitized against alloantigens in vitro. Glucose was required for cytolysis: (*a*) cytolysis proceeded in a simple buffered salt solution containing Ca^{++} and Mg^{++} (low phosphate-buffered saline, LPBS) in the presence but not in the absence of added glucose; (*b*) 2-deoxy-D-glucose blocked cytolysis. The block by this agent was overcome by excess glucose added as late as 40 min after the inhibitor. This block was not due to inhibition of NADP reduction, since 2-deoxy-D-glucose failed to interfere with the rate of CO_2 production by the pentose cycle which we found to be of significant activity in sensitized spleen cells; (*c*) dialyzed fetal bovine serum (DFBS) in LPBS supported cytolysis in the absence of added glucose. However, 2-deoxy-D-glucose was also inhibitory under these conditions, suggesting that carbohydrate was required here as well. Further results supported the conclusion that DFBS was not acting as a direct source of the required carbohydrate.

The relationship between cytolysis, glucose requirement, and provision of energy was studied. As little as 0.1 mM D-glucose in LPBS supported cytolysis. At this glucose concentration, there was no measurable accumulation of lactate in sensitized spleen cells, but Krebs cycle activity was detectable. In 3 mM glucose or above, the range covered by standard tissue culture media, anaerobic glycolysis became a major source of energy in sensitized spleen cells. Consequently, it appears that in standard tissue culture medium, effector cells can generate sufficient energy for cytolysis either by aerobic or anaerobic metabolism. However, the addition of an energy source alone in the absence of glucose was insufficient to support cytolysis in LPBS. Pyruvate in LPBS did not support cytolysis but was shown to be a good substrate for aerobic metabolism in sensitized spleen cells. Glycogenic amino acids and glycerol also failed to support cytolysis.

The stage of cytolysis at which glucose is required was investigated. Glucose was necessary for the calcium-dependent lethal hit phase, but not for the cytochalasin A-blockable recognition stage, nor for ^{51}Cr release from injured target cells. Models for the lethal hit process are discussed, which are compatible with the observed requirement for certain hexoses unrelated to their capacity to serve as sources of energy.

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