A THERMOCHEMICAL STUDY OF THE PRODUCTION OF LACTATE BY GLUTAMINOLYSIS AND GLYCOLYSIS IN MOUSE MACROPHAGE HYBRIDOMA CELLS

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INTRODUCTION

The macrophage is one of the non-specific effectors of immunity. It is characterized by its phagocytic and microbicidal properties in which engulfment is accompanied by a respiratory burst to produce potentially lethal oxygen metabolites: hydrogen peroxide, superoxide anion, hydroxyl radicals and singlet oxygen [1]. Both phagocytosis which requires ATP and the burst which needs NADPH for the reduction of oxygen [2] are highly energetic processes which require study. Fresh macrophages, however, are extremely heterogeneous in type and do not grow in culture without mitogens. Therefore, it was decided to immortalize them by hybridization with lymphosarcoma cells, selecting clones which retained the immunological properties of phagocytosis and cytotoxicity [3]. As well as studying these functions in the hybridoma cells, it was essential to know more of their catabolic pathways, especially with regard to the recruited ability of growth.

The enthalpy balance method [2] was used in this investigation. The heat flux of the cultured hybridoma cells was compared with the overall reaction enthalpy flux, J_H, expressed as $J_{B,r}\ \Delta_r H_B$, where $J_{B,r}$ is the reaction flux [$mol \cdot s^{-1} \cdot m^{-3}$] of the substance B in each individual catabolic reaction or pathway (r) and $\Delta_r H_B$ is its molar reaction enthalpy in a stoichiometric form with the modulus of the reaction r of unity $|\nu_B| = 1$ [4]. When the heat flux/enthalpy flux ratio, $Y_{Q/H} = J_Q/J_H$ [4] equals 1 then all the reactions and side reactions in a complex reaction system such as a cell, which does not exchange energy in the form of work, have been brought into account. This method ensures that reactions are not missed when investigating metabolic pathways.

The most powerful accompaniment to calorimetry in constructing an enthalpy budget is the measurement of oxygen consumption to give the so called calorimetric/respirometric (CR) ratio [5] which is a heat flux/reaction flux ratio, $Y_{Q/B} = J_Q/J_B$, with O_2 inserted for substance B [4]. All potential respiratory substrates (amino acids and fatty acids as well as carbohydrates) obey Thornton's Rule to give similar theoretical oxycaloric equivalents, $\Delta_k H_{O_2}$ of -450 $kJ \cdot mol^{-1}$ O_2 ± 5% [5] (k for catabolic reaction). The existence and intensity of anaerobic processes can be detected in cells by the calorimetric/respirometric flux ratio Y_{Q/O_2} in relation to $\Delta_k H_{O_2}$ calculated for purely oxidative catabolism.

The general aim of our studies is to construct enthalpy balances for a clone of mouse macrophage hybridoma cells, 2C11-12 [6], grown under resting conditions and after stimulation to produce a respiratory burst. Cells activated with the modulating agents, lipopolysaccharides and interferon-γ, selectively utilized considerable amounts of glutamine, producing ammonia and some glutamate [7]. Both resting [8] and activated [7] cells excreted significant quantities of lactate even though the culture conditions allowed no limitation in oxygen. McKeehan [9]

highlighted the possibility that glutamine partly could be oxidized to lactate and coined the phrase 'glutaminolysis' to describe the pathway, which is prominent in rat lymphocytes [10], human fibroblasts and several types of cancer cell [11]. The specific aim of the current study, therefore, was to report progress in constructing an enthalpy balance for activated macrophage hybridoma cells in the context of the different pathways for lactate production.

METHODS

The 2C11-12 mouse macrophage cells were cultured as previously described [3,6,8] in plastic T-flasks. They were harvested by mechanical agitation and counted by Coulter particle counter. Viability was determined by a fluorescent dye method [12]. For short-term experimentation, cells were prepared as previously [8] and finally resuspended in Dulbecco PBS with 5.5 $mmol \cdot dm^{-3}$ glucose, 2 $mmol \cdot dm^{-3}$ glutamine, 2 $g \cdot dm^{-3}$ bovine serum albumin and 20 $mmol \cdot dm^{-3}$ HEPES, pH 7.2±0.1, 272 mOsm. After incubating for 60 min with an air headspace, experiments were terminated by adding 25% w/v $HClO_4$ and removing protein by centrifugation [12]. After neutralization, glucose was determined using the O-toluidine method (Sigma), amino acids [12] and catabolic enzymes [13] spectrophotometrically and pyruvate, lactate and succinate by standard Sigma UV test kits [7]. Dissolved gases were estimated in a parallel culture without an air headspace. Oxygen was determined with a Clarke-type

TABLE 1

Flux of major metabolites in the simple incubation medium of 2C11-12 mouse macrophage hybridoma cells activated with 1 $ng \cdot cm^{-3}$ bacterial lipopolysaccharide and 10 $U \cdot cm^{-3}$ recombinant interferon-γ (average of 5 experiments)

Metabolite B	J_B ± SEM[a] [$pmol \cdot s^{-1} \cdot 10^{-6}$ cells]
Glucose	-37 ± 4
Glutamine	-23 ± 2
Glutamate	6 ± 0.5
Aspartate	1 ± 0.1
Serine	2 ± 0.3
Alanine	3 ± 0.2
Pyruvate	2 ± 0.2
Lactate	61 ± 6
Succinate	0.5 ± 0.03
Oxygen	-57 ± 4
Carbon dioxide	53 ± 9
Ammonia	22 ± 2
CO_2/O_2 ratio	0.9 ± 0.2
Lac/O_2 ratio	1.1 ± 0.1
$\frac{1 - {}^{14}CO_2}{6 - {}^{14}CO_2}$ 1	1.2 ± 0.2

[a] For statistical analysis of the ratio, standard errors SE (*Y/Z*), were calculated from the index of variation (*IV* = SE/*X*) of the two measurements (*Y* and *Z*, generically *X*),

$$SE(Y/Z) = X(Y/Z)\sqrt{IV_Y^2 + IV_Z^2} \quad 2$$

sensor [8], carbon dioxide using a Metrohm 692 ion meter with an Orion CO_2 electrode [7] and ammonia by the indophenol method [14]. Radioisotope studies were carried out as described previously [15], with the additional substrate of L-(U-^{14}C) glutamine (specific activity 168 MBq). Condensed ^{14}C-labelled metabolites were separated using an isocratic Pharmacia HPLC-system. A Bio-Rad Aminex HPX-87H column was used for the samples containing ^{14}C-glucose and a Bio-Rad Aminex HPX-87C column for ^{14}C-glutamine-containing samples. Fraction-collected eluant was analyzed on a Wallac RackBeta 1215 counter.

TABLE 2
Heat flux of 2C11-12 mouse macrophage hybridoma cells, J_Q [$\mu W \cdot 10^{-6}$ cells]*, experimental heat/O_2 flux ratio or calorimetric/respirometric ratio, Y_{Q/O_2}, and calculated enthalpy/O_2 flux ratio or molar enthalpy of catabolic reaction, $\Delta_k H_{O_2,(ox+Lac)}$, both in units* [$kJ \cdot mol^{-1}$ O_2] *(see Table 1)*.

	X ± SE
Heat flux, J_Q	-34 ± 2
CR ratio, Y_{Q/O_2}	-592 ± 91
$\Delta_k H_{O_2,(ox+Lac)}$	-553 ± 5

RESULTS

Activated macrophage hybridoma cells consumed glutamine to produce *(1)* glutamate by deamination with evolution of ammonia, and *(2)* aspartate, serine and alanine by transamination (Table 1). The remainder of the glutamine could have been completely oxidized to CO_2 and NH_3 or partially oxidized to pyruvate and lactate. Glucose could have been oxidized fully but at least some of it may have participated in glycolytic reactions to produce lactate (Table 1). The respiratory exchange ratio, CO_2/O_2 of 0.92 indicates that carbon dioxide production is lower than would be expected for the oxidation of these two substrates and the highly exothermic CR ratio of heat flux and oxygen flux indicated that the cells undertook simultaneous aerobic and anaerobic processes (Table 2).

Table 3
Metabolic flux of [U-^{14}C] glucose, J_{Glc} [$pmol \cdot s^{-1} \cdot 10^{-6}$ cells]*, and [U-^{14}C] glutamine, J_{Gln}* [$pmol \cdot s^{-1} \cdot 10^{-6}$ cells]*, by activated 2C11-12 mouse macrophage hybridoma cells in standard incubation medium (average of 4 experiments).*

	J_{Glc}	[%][a]	J_{Gln}	[%][a]
Lactate	55± 5	73	9± 1	38
Pyruvate	0.5± 0.1	0.7	1± 0.1	5
Carbon dioxide	23± 2	10	10± 1	9
Glutamate			7± 0.5	29
Totals		83.7		81

[a]Calculated from specific activity of relevant substrate.

	Enzyme activity [pmol·s^{-1}·10^{-6} cells]
Acetoacetyl-CoA thiolase	2.6
Citrate synthetase	15.6
Glutaminase	11.7
Hexokinase	10.4
Lactate dehydrogenase (LDH)	94.6
NAD^+ - linked malate dehydrogenase (MDH)	56.1
Phosphoenolpyruvate carboxykinase (PEPCK)	1.3
Pyruvate kinase (PK)	39.6
Pyruvate dehydrogenase (PDH)	0.87

The C1/C6 ratio is evidence that the pentose phosphate pathway was active only weakly in these cells (Table 1). Uncertainty over the catabolic routes for carbon led to the radioactive experiments summarized in Table 3. Although ^{14}C tracing has interpretative problems in this context, the data does indicate that the majority of glucose was converted to lactate and the same fate occurred to the large part of glutamine. The activities of some key enzymes in glycolysis and the tricarboxylic acid cycle are shown in Table 4. Of particular interest to the fate of glutamine are the relatively high activities of glutaminase, MDH, PEPCK, PK and LDH. A clue to the reason for the pyruvate-lactate shunt is provided by regarding the LDH/PDH ratio.

CONCLUSIONS

Stoichiometric calculations of data for glutamine and oxygen consumption and for ammonia production by cells (Table 1) showed that not all the glutamine was fully oxidized to CO_2 [7], and that there was no scope for glucose oxidation. The CR ratio (Table 2) was more negative than would be expected from the complete oxidation of carbohydrates and amino acids (-450 kJ·mol^{-1} [5]) reinforcing the possibility that glucose may form lactate by glycolysis. The catabolic heat change per mol O_2 for these reactions can be calculated from the equation,

$$\Delta_k H_{O_2,(ox+Lac)} = \Delta_k H_{O_2} + Lac/O_2 \cdot \Delta_k H_{Lac} \quad (1)$$

The value of the oxycaloric equivalent, $\Delta_k H_{O_2}$, for the complete oxidation of glucose in 20 mmol·dm^{-3} HEPES is -503 kJ·mol^{-1} O_2 [8], for glutamine is -452 kJ·mol^{-1} O_2, so an average was used, -478 kJ·mol^{-1} O_2 [7]. The lactate was excreted into a buffer consisting of 20 mmol·dm^{-3} HEPES, and 9.3 mmol·dm^{-3} $NaHPO_4$ and 1.5 mmol·dm^{-3} KH_2PO_4, which give molar enthalpy changes for the production of lactate, $\Delta_k H_{Lac}$, of -77 and -59 kJ·mol^{-1} lactate, respectively, so

Table 5
Theoretical heat flux of activated 2C11-12 mouse macrophage hybridoma cells calculated from the reaction flux (see Table 1) and molar reaction enthalpy for each reaction pathway.

		Molar reaction enthalpy $\Delta_r H_B$ [kJ·mol^{-1}][a]	Heat flux J_Q [μW·10^{-6} cells]
1.	**Actual**		**-34**
2.a	**Calculated** from Table 1		
	1. Oxidation of 1 Gln to CO_2	-2084	-47.9
	2. Oxidation of 1 Glc to CO_2	-3018	-111.7
	3. Glycolysis of 1 Glc to Lac	-153	-4.7
	Total		**-164.3**
2.b	**Calculated** from Table 3		
	1. Oxidation of 1 Gln to CO_2	-2084	-4.6
	2. Oxidation of 1 Gln to Lac	-695	-3.1
	3. Oxidation of 1 Gln to Pyr	-890	-0.2
	4. Oxidation of 1 Glc to CO_2	-3018	-11.6
	5. Glycolysis of 1 Glc to Lac	-153	-4.2
	Total		**-23.7**

[a]Calculated from [16], see [8].

a 1:1 figure was used, -68 kJ·mol^{-1} Lac. Inserting these in Eq.(1) gave a value for the overall enthalpy change per oxygen consumed, $\Delta_k H_{O_2,(ox+Lac)}$ of -553 kJ·mol^{-1} O_2, statistically similar to the experimental CR ratio (Table 2).

It could be supposed from Table 1 that the major elements in the catabolic process were complete oxidation of glutamine and glycolytic conversion of glucose to lactate but, according to calculations given in Table 5, these would give a heat flux of -(47.9 + 4.7) = -52.6 μW·10^{-6} cells compared with the actual value of 34 μW, an enthalpy recovery of only 0.65, which clearly overestimates oxidation. Oxygen consumption and ammonia production figures (Table 1) gave rise to the idea that not all the glutamine was fully oxidized and some may have been oxidized partly in glutaminolysis [9-11,17,18] to lactate. Despite pitfalls in interpreting carbon trace studies, the data in Table 3 provides evidence of glutaminolysis. Using the enthalpy balance method (Table 5) on these results, (Table 3) gave an enthalpy flux of -23.7 μW·10^{-6} cells and a recovery of 1.43.

The data in Table 3 indicates that lactate was produced by glycolytic conversion of glucose and glutaminolytic oxidation of glutamine. Further evidence for the latter can be derived from the high activities of the relevant enzymes, as seen in Table 4. It is unlikely that the pyruvate to lactate shunt was required for the

production of NAD^+ to oxidize glyceraldehyde-3-phosphate because energy producing processes are plenty. It is more likely to be simply due to the high LDH/PDH ratio (see Table 4) and low capacity of the malate-aspartate shunt.

By using the data in Table 3, the production of lactate by partial oxidation of glutamine reduces the calculated $\Delta_k H_{O_2,(ox+Lac)}$ to -526 $kJ \cdot mol^{-1}$, close to the oxycaloric equivalents. Irrespective of this, it is probable that the major contributor to the deficit in enthalpy recovery was fatty acid oxidation because the BSA used in the incubation medium was standard and not free of fatty acids. It seems likely that cells growing under aerobic conditions produce lactate by partial oxidation of glutamine (glutaminolysis) and glycolytic conversion of glucose not to satisfy an energy demand but to provide carbon and nitrogen precursors for biosynthetic processes [10,17]. This poses an interesting problem in the regulation of branched pathways [18].

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