The Lactate Issue Revisited: Novel Feeding Protocols To Examine Inhibition of Cell Proliferation and Glucose Metabolism in Hematopoietic Cell Cultures

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It is well established that cell proliferation in batch (unfed) hematopoietic cell cultures is greatly inhibited relative to that in cultures with feeding. What is not known, however, is the nature of this inhibition. On the basis of our observations in hematopoietic cultures that cell proliferation ceases when the lactate concentration ([lactate]) exceeds 20 mM (accompanied by a decrease in culture pH), we investigated the effect of lactate accumulation on cell proliferation, metabolism, and differentiation. We differ in our approach from previous efforts in that we have tried to more accurately recreate the manner in which lactate accumulates in culture by employing a daily feeding protocol in which [lactate] and/or pH in the fresh medium was adjusted to match the conditions prior to feeding. We conclude that the decrease in pH associated with lactate accumulation significantly inhibits both cell proliferation and metabolism. Although inhibition in cultures with high [lactate] and low pH is similar to that in unfed cultures, pH control in unfed cultures does not alleviate the inhibition, indicating that other inhibitory factors are also present. Thus, pH control is necessary, but not sufficient, to eliminate inhibition of cell growth and metabolism in unfed hematopoietic cell cultures. We also conclude that high [lactate] and low pH have little effect on cell differentiation in fed cultures, although there is evidence to suggest that low pH may play a role in monocyte differentiation in unfed cultures.

Introduction

It has been hypothesized (and shown in recent clinical trials) that transplantation of large numbers of granulocytic post-progenitor cells ($>10^7$ cells/kg patient) will be necessary to eliminate the 8−14 day period of neutropenia that follows high-dose chemotherapy and blood stem cell transplantation (*17*, *22*, *24*). Most technologies recently employed for cell production in clinical trials have relied on batch cultures, using many tissue-culture flasks or gas-permeable bags inoculated at relatively low cell densities ($\sim 10^4$ $CD34^+$ cells/mL) (*1*, *2*, *6*, *22*, *27*). While low-density cultures minimize the need for medium exchange, the handling and subsequent processing of many culture devices (typically 10 or more) is undesirable from a clinical standpoint. To reduce the culture volume by increasing the cell density represents a process development challenge that will require a more detailed understanding of both the nutritional requirements of hematopoietic cells and their sensitivity to the toxic effects of metabolite accumulation.

Observations in our laboratory show that cell proliferation ceases—in cultures of varying cell densities and feeding frequencies—when the lactate concentration approaches 20 mM (*21*). It was unclear, however, if the observed growth inhibition was due to the presence of the lactate ion itself or the accompanying decrease in medium pH. Previous studies in a variety of other cell lines have shown medium acidification to be the primary cause of growth inhibition (*4*, *7*−*9*, *15*, *18*, *20*, *23*, *26*). These studies were performed by initially adding different concentrations of a lactate salt (with or without pH adjustment of the culture) and observing the corresponding growth kinetics. However, there is reason to believe that this approach does not represent a realistic portrayal of what actually happens in culture. Large step increases in lactate concentration (and decreases in pH) are not observed in practice. Instead, lactate gradually accumulates, allowing for the possibility of adaptation, a feature that has been noted with ammonia in hybridoma cultures (*11*, *15*, *16*). Furthermore, it has been suggested that exogenous addition of an inhibitor is different from the inhibitor being endogenously produced and released into the medium (*10*). For these two reasons, experiments performed by initially adding exogenous lactate may yield results different from those in which lactate is endogenously produced in a more gradual fashion.

We have designed a set of experiments in an effort to more accurately assess inhibition due to lactate accumulation in hematopoietic cell cultures of peripheral blood mononuclear cells (PB MNCs). We employed a daily feeding protocol in which the concentration of lactate ion

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([lactate]) and/or pH in the fresh medium was adjusted to match the conditions prior to feeding. This strategy accomplishes two goals that previous efforts did not. First, the changes in [lactate] and pH occur more gradually, as would typically happen in batch cultures. Second, because we are replacing only the lactate that was produced by the cells, we have circumvented the contention that exogenous addition of an inhibitor is not the same as if it were endogenously produced. Because PB MNCs are a heterogeneous population of cells from various cell lineages and stages of differentiation, we studied the effects of lactate accumulation on cell differentiation as well as proliferation. We also studied the effect on glucose consumption and lactate production rates.

Materials and Methods

Cell Samples and Liquid Cultures. Samples of PB MNCs were obtained from cancer patients following chemotherapy-induced mobilization with or without G-CSF (Response Oncology, Memphis, TN). Samples were collected after written consent under protocols approved by the various Institutional Review Boards. Approximately 48 h after collection, samples were suspended at $(3-4) \times 10^5$ cells/mL in a commercially available serum-free medium (XVIVO-20; BioWhittaker, Walkersville, MD) supplemented with 50 ng/mL stem cell factor (SCF; Amgen, Thousand Oaks, CA), 5 ng/mL IL-3 (R&D Systems, Minneapolis, MN and Novartis, East Hanover, NJ), 10 ng/mL IL-6 (R&D Systems and Novartis), 10 ng/mL G-CSF (Amgen), and 10 ng/mL GM-CSF (Immunex, Seattle, WA). The cell suspension was then inoculated into T25 flasks (10 mL/flask) or T75 flasks (30 mL/flask) (Falcon, Lincoln Park, NJ) and incubated at 37° C in a fully humidified atmosphere of 5% CO_2 in air. Starting at day 5, the flasks were sampled daily and analyzed for total nucleated cells using a Coulter Multisizer IIe (Coulter, Hialeah, FL) after treatment with cetrimide (Sigma, St.Louis, MO) to release the nuclei. Cells were also prepared for colony-forming cell assays and flow cytometry as described below. Glucose and lactate concentrations were measured daily using a YSI model 2700 glucose/lactate analyzer (Yellow Springs Instruments, Yellow Springs, OH). Medium osmolality was measured using an Advanced Instruments model 3D3 osmometer (Advanced Instruments Inc., Norwood, MA), and pH was measured using a model 1306 blood gas analyzer (Instrumentation Laboratories, Lexington, MA).

Feeding Protocols. Flasks were fed in one of four different ways: (1) Fed + Lactate + HCl: flasks were fed daily by 1/2 medium exchange. The pH of the fresh medium was adjusted using 1 M HCl (Sigma) to match the pH of the medium before feeding. Also, 1 M sodium lactate (Sigma) was added to the fresh medium to obtain a concentration equivalent to that in the culture prior to feeding (prefeeding concentration). (2) Fed + Lactate: flasks were fed daily by 1/2 medium exchange. Sodium lactate was added to the fresh medium at a concentration equivalent to the prefeeding concentration. (3) Fed + NaCl: flasks were fed daily by 1/2 medium exchange; 1 M NaCl was added to the fresh medium to obtain a concentration equivalent to the concentration of sodium lactate added in the {Fed + Lactate} flasks. (4) Fed: flasks were fed daily by 1/2 medium exchange. For all flasks that were fed, medium exchange was performed by removing 1/2 of the spent medium and spinning down the cells at 300*g* for 10 min using an IEC CL3R centrifuge (IEC, Needham Heights, MA). The supernatant was carefully removed, so as not to disturb the cell pellet. Fresh medium (adjusted as described above and pre-equilibrated in a 5% CO_2 atmosphere) was then added to resuspend the cell pellet, and the cell suspension was added back to the original flask. Two unfed cultures were also established as follows: (1) Not Fed: flasks were unfed for the culture duration. (2) Not Fed + NaOH: flasks were unfed for the culture duration. However, 1 M NaOH was added daily to adjust the pH to match that of the Fed cultures after feeding. In {Not Fed} and {Not Fed + NaOH} cultures, 10 ng/mL G-CSF was added every other day to prevent depletion of G-CSF due to instability (*25*).

Colony-Forming Cell Assays. Myeloid and erythroid progenitor cells were enumerated using methylcellulose colony assays (*3*). Briefly, 4,000−10,000 cells/mL were inoculated into a solution of 1.1% methylcellulose supplemented with 50 ng/mL SCF, 5 ng/mL IL-3, 10 ng/mL IL-6, 1.5 ng/mL G-CSF, 4 ng/mL GM-CSF, and 3 U/mL erythropoietin (Epo, Amgen). One milliliter aliquots were then plated in gridded 35-mm dishes (Nunc, Naperville, IL), and cultured in a fully humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2 for 14 days. Colonies of ≥50 cells were classified as CFU-GM or BFU-E through inspection on a dark-field stereomicroscope (Zeiss, Batavia, IL).

Flow Cytometry. Lineage distribution of the cell product was analyzed by flow cytometry as previously described (*3*). Briefly, 250,000−500,000 cells were washed twice in a solution (PAB) of bovine serum albumin (0.5%, Intergen, Purchase, NY) and sodium azide (0.1%, Sigma) in phosphate buffered saline (PBS, Sigma). After washing, 10 μL of fluorescence-conjugated antibody was added to the cell pellet and allowed to incubate for 20 min at 4 °C. The following antibodies were used for staining the cell samples: FITC-CD15, PE-CD11b (Becton Dickinson (BD), San Jose, CA), and PE-Cy5 CD14 (Immunotech, Miami, FL). After incubation, contaminating red cells were lysed using a solution of ammonium chloride (0.83%, Sigma) and potassium bicarbonate (0.1%, Sigma). After red cell lysis, cells were washed one final time with PAB. Samples were run on a FACScan (BD) flow cytometer equipped with a 488 nm argon laser. Ten thousand events were acquired from each sample in list-mode using FACScan Research Software (BD), and analyzed using CellQuest (BD). The cell population was discriminated from debris on the basis of forward scatter vs side scatter plots.

Calculations. Specific glucose consumption rates at day *n* were calculated using the following time-weighted average:

$$q_{\mathrm{glu},n} = \frac{\Delta t_{\mathrm{b}}\left(\dfrac{-\Delta G_{\mathrm{a}}}{(CD_{\mathrm{LM,a}})(\Delta t_{\mathrm{a}})}\right) + \Delta t_{\mathrm{a}}\left(\dfrac{-\Delta G_{\mathrm{b}}}{CD_{\mathrm{LM,b}}(\Delta t_{\mathrm{b}})}\right)}{(\Delta t_{\mathrm{a}} + \Delta t_{\mathrm{b}})}$$

where Δt_a and Δt_b are the intervals in hours immediately after day n ($t_{n+1} - t_n$) and before day n ($t_n - t_{n-1}$), respectively. ΔG is the change in glucose concentration for the indicated period, and CD_{LM} is the log mean cell density for the indicated period.

Specific lactate production rates at day n were calculated using a similar average:

$$q_{\mathrm{lac},n} = \frac{\Delta t_{\mathrm{b}}\left(\dfrac{\Delta L_{\mathrm{a}}}{(CD_{\mathrm{LM,a}})(\Delta t_{\mathrm{a}})}\right) + \Delta t_{\mathrm{a}}\left(\dfrac{\Delta L_{\mathrm{b}}}{CD_{\mathrm{LM,b}}(\Delta t_{\mathrm{b}})}\right)}{(\Delta t_{\mathrm{a}} + \Delta t_{\mathrm{b}})}$$

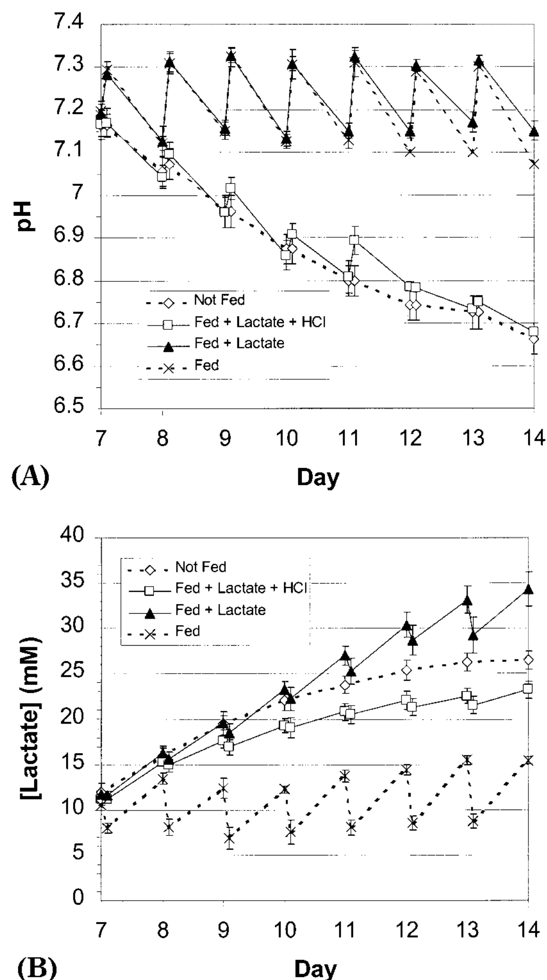


Figure 1. (a) pH and (b) [lactate] from days 7–14 for the indicated cultures. The various feeding protocols are defined in Materials and Methods. For cultures that were fed, values are shown before and after feeding. The mean ± SEM of nine experiments is shown. No data are shown for days 0–6 in these or subsequent figures because there were only small differences between the values for the different culture conditions.

where ΔL is the change in lactate concentration for the indicated period.

Statistics/Data Analysis. All results are presented as the mean ± standard error of the mean (SEM) of replicate PB MNC samples. Statistically significant differences are defined as $p < 0.05$ based on a two-tailed paired Student's *t*-test.

Results

pH and Lactate Concentration. Our main goal was to understand whether growth inhibition in unfed batch cultures could be explained by the accumulation of lactate ion, the accompanying decrease in medium pH, or by other factors, such as nutrient depletion. Toward this end, four cultures were set up to isolate the variables in question. The pH and lactate profiles for these cultures are shown in Figure 1. In the {Not Fed} cultures, which have the additional possibility of nutrient depletion, the pH decreased to 6.66 (Figure 1a) and [lactate] increased to 26.5 mM (Figure 1b) by day 14. In contrast, the Fed cultures were maintained at a relatively constant pH (7.1–7.3) and low [lactate] (<15 mM) for the culture duration. The {Fed + Lactate} cultures were also maintained at a relatively constant pH, but lactate accumulated to 34.3 mM by day 14. In the {Fed + Lactate + HCl} cultures, neither pH, which dropped to 6.68, nor [lactate], which increased to 23.2 mM, were maintained within a constant range. In all cultures, the changes in pH and/or [lactate] occurred gradually, as is typically observed in batch {Not Fed} cultures.

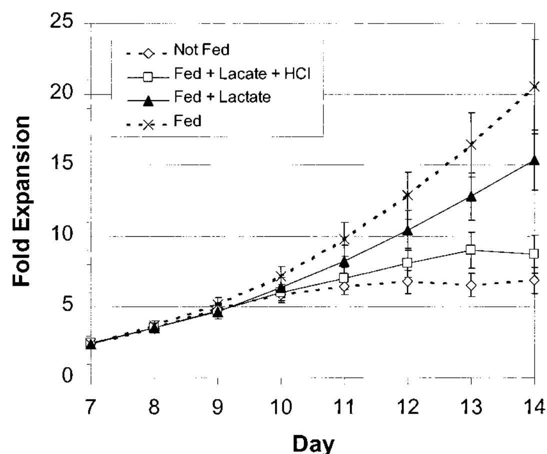


Figure 2. Total cell expansion from days 7–14 for the indicated cultures. The mean ± SEM of nine experiments is shown. Statistically significant differences ($p < 0.05$) were observed when {Not Fed} cultures were compared to Fed cultures on days 9–14, {Fed + Lactate} cultures on days 12–14, and {Fed + Lactate + HCl} cultures on days 12–14. Statistically significant differences were observed when {Fed + Lactate + HCl} cultures were compared to Fed cultures on days 10–14 and {Fed + Lactate} cultures on days 13–14. Statistically significant differences were observed when {Fed + Lactate} cultures were compared to Fed cultures on days 8–14.

Cell Proliferation. Total cell expansion for each of the four cultures is shown in Figure 2. As expected, the {Not Fed} cultures exhibited the smallest expansion at day 14 (6.9-fold), representing an inhibition of approximately 66% relative to the Fed cultures (20.5-fold). The presence of lactate ion in the {Fed + Lactate} cultures inhibited expansion only slightly (15.4-fold; approximately 25% inhibition), even though the final [lactate] was 30% higher than in the {Not Fed} cultures (Figure 1b). In contrast, the presence of both low pH and the lactate ion in the {Fed + Lactate + HCl} cultures significantly inhibited cell expansion (8.9-fold expansion; approximately 60% inhibition), even though [lactate] was slightly lower than in the {Not Fed} cultures.

Because the feeding process in the {Fed + Lactate} cultures involved adding sodium lactate to the fresh medium, an increase in medium osmolality was to be expected. Indeed, when day 14 supernatants were analyzed, the {Fed + Lactate} cultures had a significantly higher medium osmolality than the Fed cultures (343 vs 306 mOsm/kg, respectively; $p < 0.05$). Parallel {Fed + NaCl} cultures were therefore employed to determine if the inhibition observed in the {Fed + Lactate} cultures (and part of the inhibition in the {Fed + Lactate + HCl} cultures; 340 mOsm/kg) could be explained by the increase in medium osmolality. The culture conditions at day 14 for this experiment are shown in Table 1. Total cell expansion for the {Fed + Lactate} cultures and the {Fed + NaCl} cultures were similar for the duration of the experiment (Figure 3). By day 14, total cell expansion was inhibited by approximately 20% in both the {Fed + Lactate} cultures (11.7-fold expansion) and the {Fed + NaCl} cultures (11.8-fold expansion), relative to the Fed cultures (14.8-fold expansion).

Daily pH control was also implemented in {Not Fed} cultures to determine the extent to which the pH decrease due to lactate accumulation contributes to growth inhibi-

Table 1. Culture Conditions at Day 14 for the Indicated Cultures (mean ± SEM of five experiments)

	Fed	Fed + Lactate	Fed + NaCl	Not Fed	Not Fed + NaOH
pH	7.10 ± 0.12	7.14 ± 0.07	7.05 ± 0.16	6.67 ± 0.14	7.14 ± 0.03
[lactate] (mM)	14.7 ± 5.0	34.2 ± 10.7	14.7 ± 3.8	25.0 ± 3.0	34.2 ± 5.1
osmolality (mOsm/kg)	303 ± 4	347 ± 6	348 ± 6	318 ± 11	329 ± 2

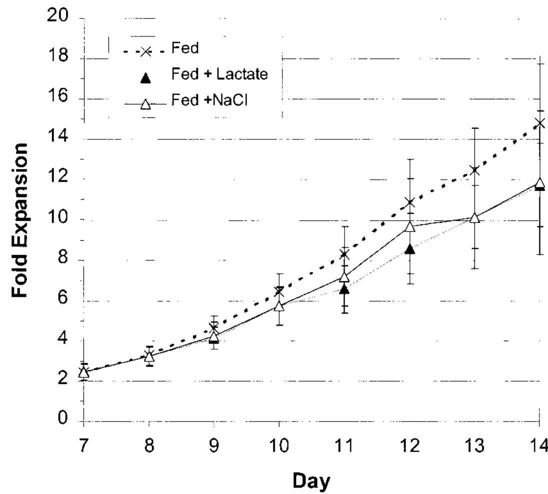


Figure 3. Total cell expansion from days 7−14 for the indicated cultures. The mean ± SEM of five experiments is shown. Statistically significant differences ($p < 0.05$) were observed when Fed cultures were compared to {Fed + Lactate} cultures on days 9−10,12 and {Fed + NaCl} cultures on days 13−14. There were no statistically significant differences between {Fed + Lactate} and {Fed + NaCl} cultures.

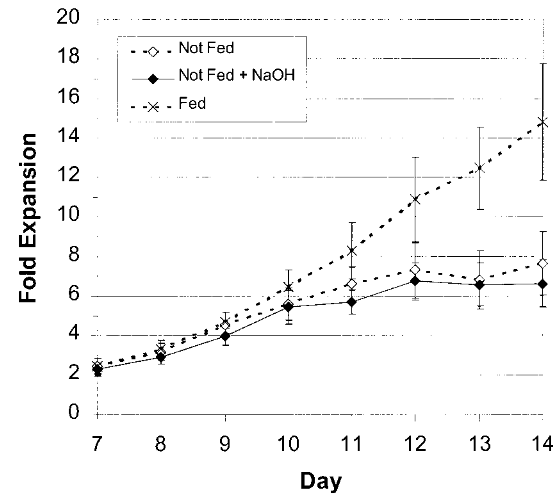


Figure 4. Total cell expansion from days 7−14 for the indicated cultures. The mean ± SEM of five experiments is shown. Statistically significant differences ($p < 0.05$) were observed when Fed cultures were compared to {Not Fed} cultures on days 10,12−14 and {Not Fed + NaOH} cultures on days 12−14. There were no statistically significant differences between {Not Fed} and {Not Fed + NaOH} cultures.

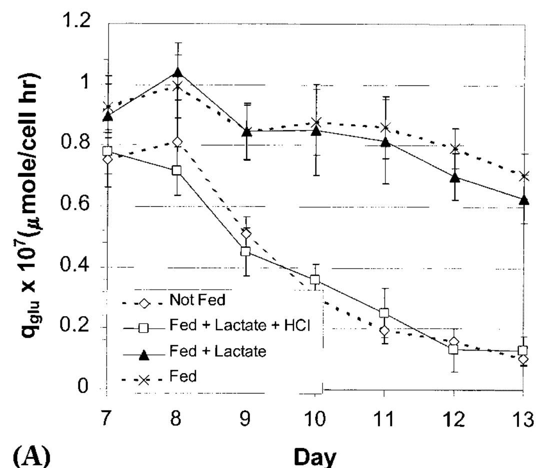
tion in the {Not Fed} cultures. As shown in Figure 4, there is no improvement in total cell expansion for the culture duration despite pH control (Table 1). By day 14, both {Not Fed} and {Not Fed + NaOH} cultures exhibited only 6- to 8-fold expansion of total cells, compared to 14.8-fold expansion in Fed cultures.

Cell Metabolism. We examined profiles of the specific glucose uptake rates (q_{glu}) and specific lactate production rates (q_{lac}) to determine if lactate accumulation affects glucose metabolism. As shown in Figure 5, both q_{glu} and q_{lac} decreased only slightly with time in the Fed cultures. In contrast, in the {Not Fed} cultures, q_{glu} declined from 0.75 to 0.10 $\times$ 10^{-7} μmol/cell h, and q_{lac} declined from 1.43 to 0.10 $\times$ 10^{-7} μmol/cell h. This represents a 87% and 93% decrease in q_{glu} and q_{lac}, respectively, when compared to Fed cultures at day 13. Similar to cell proliferation, the lactate ion in the {Fed + Lactate} cultures had little effect on either q_{glu} or q_{lac} (11% and 12% decrease, respectively, when compared to Fed cultures at day 13), while the presence of both low pH and high [lactate] in the {Fed + Lactate + HCl} cultures resulted in large decreases (82% and 66%, respectively).

We next examined to what extent the decreases in q_{glu} and q_{lac} in the {Not Fed} cultures could be explained by medium acidification from lactate accumulation. As shown in Figure 6, both q_{glu} and q_{lac} were greater in the {Not Fed + NaOH} cultures than in the {Not Fed} cultures between days 9 and 12 (although they were still lower than in the Fed cultures). By day 13, however, these differences with the {Not Fed} cultures were greatly reduced, possibly as a result of the fact that [glucose] in the {Not Fed + NaOH} cultures approached 0 mM at day 13 in two of the five experiments (7.6 ± 0.4 vs 1.4 ± 0.6 mM in {Not Fed} and {Not Fed + NaOH} cultures, respectively; $n = 5$).

Cell Differentiation. Unlike the case for cell expansion and metabolism, lactate accumulation and pH decreases had little effect on cell differentiation. Figure 7a,b shows the percentages of CFU-GM and BFU-E, respectively, for the various cultures. Although there are scattered instances of statistically significant differences between some of the cultures, the differences are not very large. Furthermore, accurately determining these differences is very difficult given the small fraction of progenitor cells (<1%) at late culture times.

We also quantified the percentages of post-progenitors, which comprise the bulk of the total cell product. Since the cytokine combination used in these studies favors the production of granulocytes and monocytes, we chose appropriate cellular markers to identify both mature monocytes ($CD14^+$ cells), immature granulocytes ($CD15^+CD11b^-$ cells), and mature granulocytes and mature monocytes ($CD15^+CD11b^+$ cells). While there were few differences in the fraction of $CD14^+$ monocytes between the four cultures (Figure 8a), there was a marked difference in the distribution of granulocytes between the {Not Fed} and Fed cultures (Figure 8b,c). In the Fed cultures, the fraction of immature granulocytes ($CD15^+CD11b^-$ cells) steadily decreased from days 7 to 14 (from approximately 25% to 5%), concomitant with a rise in the fraction of mature granulocytes/mature monocytes ($CD15^+CD11b^+$ cells; from approximately 40% to 60%). In contrast, the {Not Fed} cultures maintained the percentage of $CD15^+CD11b^-$ cells at nearly constant levels past day 10. By days 12−14, the {Not Fed} cultures had nearly 3 times as great a percentage of $CD15^+CD11b^-$ cells as the Fed cultures. When pH was controlled in the {Not Fed + NaOH} cultures, this trend was maintained (data not shown). Furthermore, the fraction of $CD14^+$ monocytes at day 14 increased from 30% in the {Not Fed} cultures to 50% in the {Not Fed + NaOH} cultures ($p <$ 0.05; data not shown). The percentages of various cell types in both {Fed + Lactate} and {Fed + Lactate + HCl} cultures were similar to those in the Fed cultures at all times, although the $CD15^+CD11b^+$ cell content was



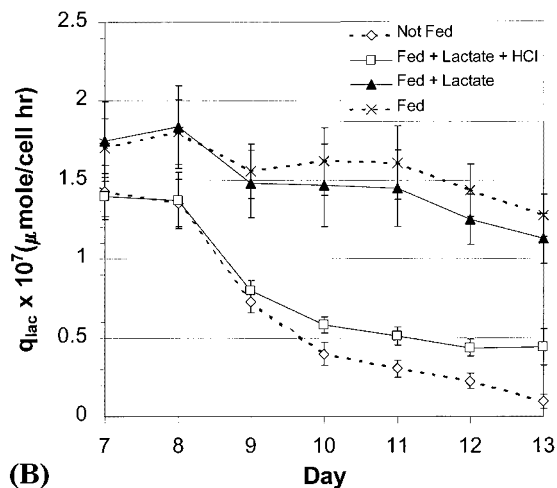
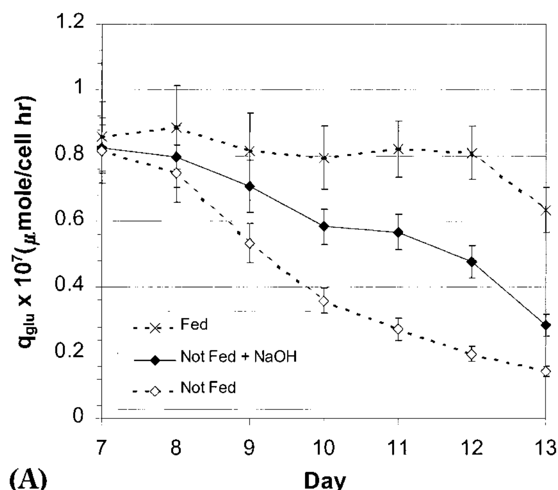


Figure 5. Specific glucose consumption rate (a) and specific lactate production rate (b) from days 7–14 for the indicated cultures. The mean ± SEM of nine experiments is shown. For specific glucose consumption rates, statistically significant differences ($p < 0.05$) were observed when {Not Fed} cultures were compared to Fed cultures on days 8–13 and {Fed + Lactate} cultures on days 8–13. Statistically significant differences were observed when {Fed + Lactate + HCl} cultures were compared to Fed cultures on days 8–13 and {Fed + Lactate} cultures on days 8–13. Statistically significant differences were observed when {Fed + Lactate} cultures were compared to Fed cultures on days 12 and 13. For specific lactate production rates, statistically significant differences were observed when {Not Fed} cultures were compared to Fed cultures on days 8–13, {Fed + Lactate} cultures on days 8–13, and {Fed + Lactate + HCl} cultures on days 10–13. Statistically significant differences were observed when {Fed + Lactate + HCl} cultures were compared to Fed cultures on days 8–13 and {Fed + Lactate} cultures on days 8–13. Statistically significant differences ($p < 0.05$) were observed when {Fed + Lactate} cultures were compared to Fed cultures on days 12 and 13.



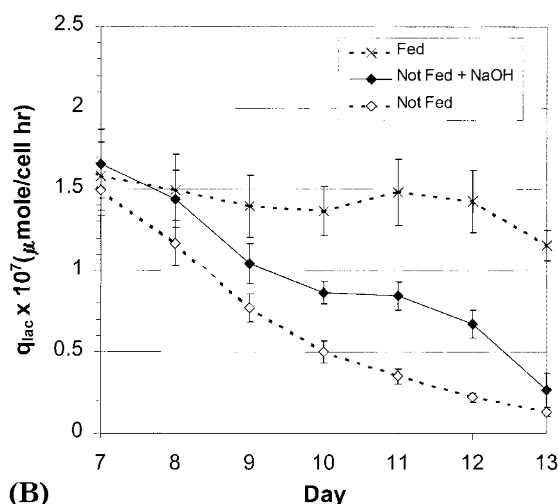


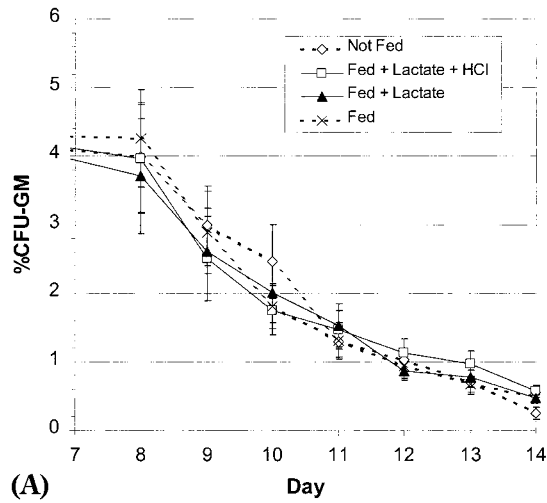
Figure 6. Specific glucose consumption rate (a) and specific lactate production rate (b) from days 7–14 for the indicated cultures. The mean ± SEM of five experiments is shown. For specific glucose consumption rates, statistically significant differences ($p < 0.05$) were observed when {Not Fed} cultures were compared to Fed cultures on days 10–13 and {Not Fed + NaOH} cultures on days 10–13. Statistically significant differences were observed when {Not Fed + NaOH} cultures were compared to Fed cultures on days 11–13. For specific lactate production rates, statistically significant differences were observed when {Not Fed} cultures were compared to Fed cultures on days 9–13 and {Not Fed + NaOH} cultures on days 8 and 10–12. Statistically significant differences were observed when {Not Fed + NaOH} cultures were compared to Fed cultures on days 12–13.

somewhat lower at day 14 in the {Fed + Lactate + HCl} cultures.

Discussion

Previous efforts to study lactate inhibition of cell proliferation and metabolism relied on a strategy whereby different concentrations of a lactate salt were initially added to batch cultures (with or without pH adjustment); conclusions were then drawn from the corresponding growth and metabolism kinetics. Although easy to implement, this approach is limited in two ways. First, it does not allow for the possibility of cell adaptation to adverse culture conditions. For example, it has been well documented that hybridomas are capable of adapting to step changes in ammonia concentration, given enough time. Miller et al. *(15)* and Matsumura et al. *(11)* examined step changes of varying levels in continuous culture (Δ[ammonium] ranging from 2.5 to 12.6 mM) and noted that, after a transient decline (lasting 3–10 days), the viable cell concentration recovered to its initial value. Newland et al. *(16)* further noted in fed-batch cultures that if an increase in [ammonium] is performed gradually over several days, the cells adjust even better, with no observed effect on their ability to proliferate. In this regard, it is possible that the concentrations of lactate and/or pH that were found to be inhibitory in previous studies (where lactate was initially added to batch cultures) might be inaccurate.

A second potential limitation in the previous studies is the fact that lactate was added exogenously to the cultures. It has been postulated that exogenously added ammonia does not inhibit cultures in the same manner



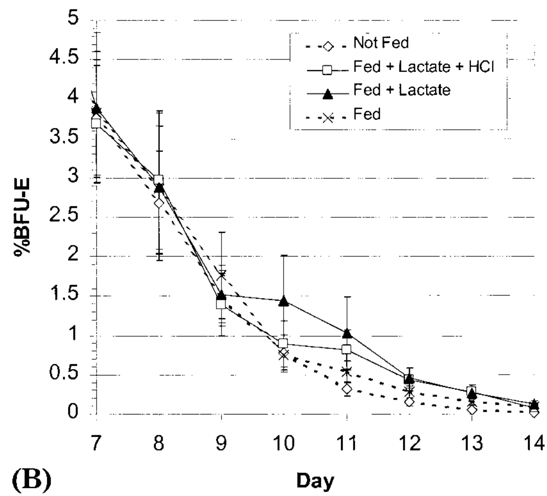
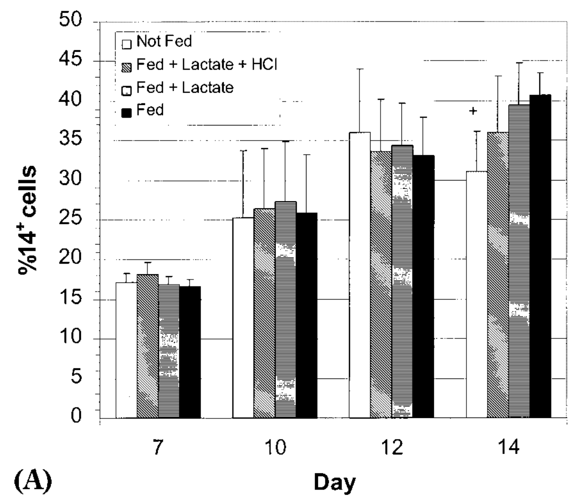
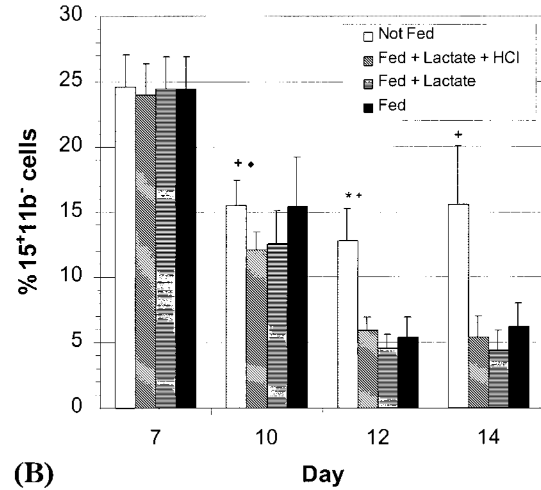


Figure 7. Progenitor cell content, (a) % CFU-GM and (b) % BFU-E, from days 7−14 for the indicated cultures. The mean ± SEM of nine experiments is shown. For % CFU-GM, statistically significant differences ($p < 0.05$) were observed when {Not Fed} cultures were compared to Fed cultures on days 10 and 14 and {Fed + Lactate + HCl} cultures on days 9−10,14. Statistically significant differences were observed when {Fed + Lactate + HCl} cultures were compared to Fed cultures on day 12 and {Fed + Lactate} cultures on day 12. Statistically significant differences were observed when {Fed + Lactate} cultures were compared to Fed cultures on day 8. For % BFU-E, statistically significant differences were observed when {Not Fed} cultures were compared to Fed cultures on days 11,13,14; {Fed + Lactate} cultures on days 12−14, and {Fed + Lactate + HCl} cultures on days 11−14. Statistically significant differences were observed when {Fed + Lactate + HCl} cultures were compared to Fed cultures on days 12 and 14. Statistically significant differences were observed when {Fed + Lactate} cultures were compared to Fed cultures on days 12 and 14.





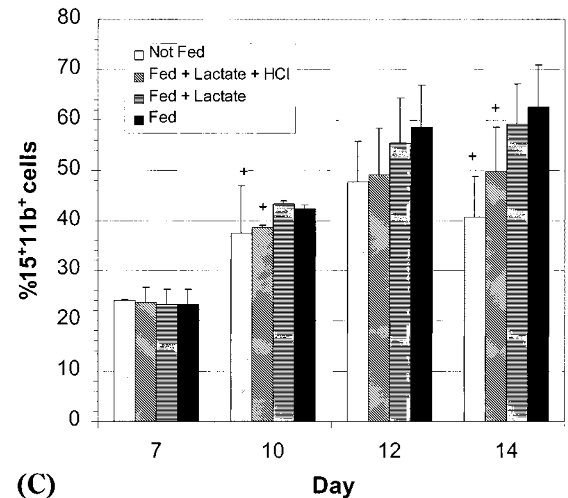


Figure 8. Post-progenitor cell content, (a) % $CD14^+$, (b) % $CD15^+CD11b^-$, and (c) % $CD15^+CD11b^+$ cells, from days 7−14 for the indicated cultures. The mean ± SEM of five experiments is shown. Statistically significant differences ($p < 0.05$) are denoted by * when compared to Fed cultures, + when compared to {Fed + Lactate} cultures, and ♦ when compared to {Fed + Lactate + HCl} cultures.

as if ammonia were produced by the cell and released into the medium (*10*). It is not known if the same phenomenon applies to lactate. Therefore, instead of adding lactate to our cultures, we merely replaced the lactate that had already been produced by the cells.

Within this experimental framework, we have shown that the decrease in pH accompanying lactate accumulation significantly inhibits cell proliferation. This inhibition (~60%) became apparent by day 10 (Figure 2) when pH decreased to approximately 6.9 and [lactate] increased to ~20 mM in the {Fed + Lactate + HCl} cultures (Figure 1). In contrast, only slight inhibition (20−25%) was observed in the {Fed + Lactate} cultures, where pH was maintained relatively constant (7.1−7.3) and lactate was allowed to accumulate (>30 mM). As shown by the {Fed + NaCl} cultures (Figure 3), this residual growth inhibition that we observed from the lactate ion can be accounted for by the corresponding increase in medium osmolality. It is important to note, however, that this increase in osmolality is an artifact

of the feeding process used. In typical unfed hematopoietic cultures, the effect of lactate production on medium osmolality is counterbalanced by the consumption of nutrients, resulting in a fairly constant medium osmolality over time (314 vs 306 mOsm/kg in {Not Fed} and Fed cultures, respectively, at day 14). Thus, our results show that as long as pH is reasonably well controlled, cellular production of lactate should not prove detrimental to culture performance. Obviously, the concentration of lactate at which pH becomes inhibitory is dependent on the buffering capacity of the medium. Therefore, careful consideration should be given to choosing a medium that can handle the expected concentrations of lactate. Alternatively, appropriate pH control strategies (base addition, gas headspace manipulation, etc.) can be implemented to ensure that constant pH is maintained for the culture duration.

Our results are consistent with those of previous studies that have shown that the proliferation of various cell lines (e.g., hybridoma, BHK, Vero, and CHO) is unaffected by [lactate] as high as 40−50 mM, provided pH is maintained at an optimal value (*4*, *7*−*9*, *15*, *18*, *20*, *23*, *26*). Since these earlier studies relied on the addition of exogenous lactate, our results suggest that endogenously produced lactate inhibits proliferation similarly, at least qualitatively. Only a direct comparison using the two experimental approaches can confirm if the inhibition is quantitatively similar as well.

We have also shown that the associated decrease in pH accompanying lactate accumulation greatly inhibits glucose metabolism. Similar to cell proliferation, we observed significant reductions of q_{glu} and q_{lac} in {Fed + Lactate + HCl} cultures and only slight reductions in {Fed + Lactate} cultures (Figure 5). Medium acidification has been shown to decrease q_{glu} and q_{lac} in a variety of cell lines in both batch and continuous cultures (*7*, *14*, *19*). A likely explanation for the reduced glycolytic flux involves the alteration of intracellular pH (pH_i). McQueen and Bailey *(13)* and Ozturk et al. *(20)* have shown that decreasing extracellular pH can result in cytoplasmic acidification in hybridomas. Decreases in pH_i are known to decrease the activity of key glucose metabolizing enzymes (i.e., hexokinase, phosphofructokinase), resulting in decreased consumption of glucose and production of lactate (*5*). In our studies, we see significant deviations between the Fed and {Fed + Lactate + HCl} cultures for both q_{glu} and q_{lac} by day 9, by which time extracellular pH was approximately 7.15 and 6.95, respectively (Figure 1a). pH_i will have to be measured in these cultures to confirm this hypothesis.

Finally, we observed very few differences in cell composition between the Fed, {Fed + Lactate} and {Fed + Lactate + HCl} cultures, showing that lactate accumulation and pH decreases have little effect on the fractions of progenitor (Figure 7) and post-progenitor (Figure 8) cells in daily fed cultures. McAdams et al. also examined the effects of pH on the fraction of BFU-E and CFU-GM in PB MNC and cord blood MNC cultures. They observed no effect on the percentage of CFU-GM as pH decreased from 7.35 to 7.15 and only a slight increase in the percentage of BFU-E (*12*). Interestingly, we observed a higher fraction of immature granulocytes ($CD15^+CD11b^-$ cells) in both {Not Fed} and {Not Fed + NaOH} cultures. This would suggest that factors other than medium acidification and lactate accumulation are responsible for this phenomenon. Since the production of immature granulocytes is likely to be important for mediating short-term neutrophil engraftment, isolating these factors will undoubtedly be important.

Although medium acidification can account for much of the difference observed between the {Not Fed} and Fed cultures, it is not the only factor involved. We have shown that pH control in the {Not Fed} cultures does not alleviate the inhibition of growth (Figure 4) and only partially alleviates the inhibition of glucose metabolism (Figure 6). Also, as mentioned above, lactate and pH decreases cannot explain the blocking of granulocyte differentiation in the {Not Fed} cultures. Clearly, there are other inhibitors that must be identified. One possible candidate is ammonia, whose concentration in the {Not Fed} and {Not Fed + NaOH} cultures at day 14 was approximately 3 mM (vs 1.5 mM in Fed cultures). Several studies have shown that comparable concentrations of ammonia (1−5 mM) can inhibit growth by 50% in a variety of cell lines (*4*, *15*, *20*, *26*). Interestingly, in contrast to our results, the inhibitory concentrations of ammonia were found to increase both q_{glu} and q_{lac} in some of these studies (*15*, *20*). It is also possible that key nutrients (i.e., amino acids) have been depleted in the {Not Fed} cultures, inhibiting both growth and metabolism. In this regard, because nutrients are depleted more rapidly in {Not Fed + NaOH} cultures, the similar cell growth in {Not Fed} and {Not Fed + NaOH} cultures does not indicate that medium acidification is not the primary limitation in {Not Fed} cultures. It simply indicates that pH control alone is not sufficient to significantly improve performance in unfed cultures. Only a systematic approach of analyzing nutrient concentrations and studying potential inhibitors will shed further light on this issue.

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