Journal of Cellular Biochemistry 83:200–203 (2001)

Novel Role of Extracellular Carbon Dioxide in Lymphocyte Proliferation in Culture

Ranjana Chakrabarti[1] and Rabindranath Chakrabarti[2]*

[1]Molecular Biology Unit, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, India
[2]Department of Biology, Acadia University, Wolfville, Nova Scotia, B0P1X0, Canada

Abstract CO_2/HCO_3^- buffering system is indispensable to maintain the pH of culture media for long-term cell culture. Now-a-days, the zwiterionic hydrogen buffer HEPES is widely used as an additional buffer in the commonly used culture media. There are reports on the successful use of HEPES-buffered media, under CO_2/HCO_3^- free conditions, for long-term cell cultures. However, still CO_2/HCO_3^- buffering system is widely used. We aimed at investigating the reason for this. We found that lymphocytes proliferate in response to concanavalin A only in HCO_3^--buffered medium in the presence of 5% CO_2, but not in the HEPES-buffered medium in the absence of CO_2. However, lymphocyte proliferation was observed in HEPES-buffered medium in the presence of 5% CO_2 and in the absence of HCO_3^-. On the other hand, a low level proliferation was observed in HEPES-buffered medium supplemented with HCO_3^- in the absence of CO_2. Supplementation of the culture medium with TCA cycle intermediates and the precursors for the salvage pathway of nucleotide synthesis did not support the lymphocyte proliferation at all. Based on these findings and other reports, we suggest that extracellular CO_2 plays a novel role in cell proliferation. J. Cell. Biochem. 83: 200–203, 2001. © 2001 Wiley-Liss, Inc.

Key words: HEPES; CO_2; cell culture; cell growth

Use of continuous supply of gaseous CO_2 in the incubation chamber is indispensable for the growth and long-term maintenance of animal cells in culture. Exogenous supply of CO_2 is required, in conjunction with a HCO_3^- ion (in the form of $NaHCO_3$), mainly to maintain the pH of the culture medium. However, CO_2/ HCO_3^- system has several disadvantages, such as need to continuously maintain the CO_2 enriched atmosphere, need for an expensive CO_2 incubator, and sub-optimal buffering in the physiological pH range due to the 6.1 pKa of $NaHCO_3$. To circumvent this, several attempts were made to replace CO_2/HCO_3^- buffering system with some other zwiterionic hydrogen buffers, notably HEPES. Initially, HEPES was introduced as a buffer for studying the mitochondrial metabolism [Good et al., 1966]. This was followed by the use of HEPES to replace the CO_2/HCO_3^- buffering system for the long-term culture of mammalian organ and cells, including human peripheral blood lymphocytes [Shipman, 1969; Fisk and Pathak, 1969; Darzynkiewicz and Jacobson, 1971; Medzon and Gedies, 1971; Itagaki and Kimura, 1974]. These reports also showed the better buffering capacity of HEPES, as compared to CO_2/HCO_3^- buffer [Shipman, 1968; Darzynkiewicz and Jacobson, 1971]. However, despite these reports on the successful use of zwiterionic buffers, CO_2/ HCO_3^- buffering system is still widely used for cell culture. Any undesirable effect of HEPES as the possible reason is ruled out from the fact that this compound has been shown to be non-toxic [Shipman, 1968; Darzynkiewicz and Jacobson, 1971; Itagaki and Kimura, 1974] and is widely used now-a-days as an additional buffer in culture media commonly used for animal cell culture.

In the present work, we aimed to investigate why CO_2/HCO_3^- buffering system could not be replaced by HEPES buffering system widely. We found that lymphocytes proliferate in response to concanavalin A (ConA) only in the presence of CO_2 in HCO_3^--buffered medium, but not in the absence of CO_2 in HEPES-

*Correspondence to: Rabindranath Chakrabarti, Department of Biology, Acadia University, Wolfville, Nova Scotia B0P 1X0, Canada. E-mail: robinchak@yahoo.com

Received 19 March 2001; Accepted 7 May 2001

© 2001 Wiley-Liss, Inc.
DOI 10.1002/jcb.1218

buffered medium. However, supply of CO_2 can restore the lymphocyte proliferation in response to ConA in HEPES-buffered medium, in the absence of HCO_3^-. On the other hand, presence of HCO_3^- in HEPES-buffered medium, in the absence of CO_2, supported very little lymphocyte proliferation in response to ConA. These results showed that extracellular CO_2 plays a novel role, other than pH maintenace, in cell growth in culture.

MATERIALS AND METHODS

Reagents

4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) and 3-(4,5dimethyl-thiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO). ConA was obtained from Pharmacia AB (Upsula, Sweeden). [^{3}H]thymidine (specific activity 18,000 mCi/mmol) was purchased from Bhaba Atomic Research Center (Mumbai, India). Rosewell Park Memorial Institute medium (RPMI-1640) was from HiMedia Laboratories Limited (Mumbai, India).

Measurement of Lymphocyte Proliferation

Lymphocytes were isolated from the spleen of Balb/C mice in RPMI-1640 medium supplemented with 2 mM L-glutamine, gentamycin (0.2 mg/ml), streptomycin (0.1 mg/ml), and heat-inactivated fetal bovine serum (complete medium) as described before [Chakrabarti et al., 1995]. The culture medium was buffered with either $NaHCO_3$ (0.2% (w/v)) or 25 mM HEPES, depending on the experiment. However, the pH of all the culture media was always about 7.4.

Cell proliferation was measured by the incorporation of [^{3}H]thymidine into the replicating DNA. Thus, lymphocytes (10^5 cells/well of 96-well culture plate) were stimulated for 60 hr with 2.5 μg/ml ConA in complete medium buffered with either $NaHCO_3$ or 25 mM HEPES, in the presence or absence of 5% CO_2 in a humidified incubator at 37°C. The cells were pulsed at 24 hr, harvested at 60 hr of culture, and [^{3}H]thymidine incorporation was measured by a liquid scintillation counter.

Determination of Cell Viability

The survivability of lymphocytes in culture was measured by MTT assay [Mossmamm, 1983]. Cells were incubated with 2.5 mg/ml MTT for 4 hr, washed with normal saline, and formazan crystals were solubilized in acidic isopropanol (with 0.04 N HCl). The absorbance of the solution was measured at 492 nm λ in an E_{max} microplate reader (Molecular Devices).

RESULTS AND DISCUSSION

Initially, we determined the proliferation of lymphocytes in response to ConA under different conditions. The lymphocytes proliferated in response to different doses of ConA in HCO_3^--buffered medium in the continuous presence of 5% CO_2 in the incubation chamber (Table I). However, no proliferation was observed in HEPES-buffered medium in the absence of CO_2 supply (Table I), although the pH of the culture medium and cell viability remained unaltered (data not shown). These results clearly showed that HEPES couldn't replace the CO_2/HCO_3^- buffering system to support the growth of the lymphocytes in culture. This suggests that CO_2/HCO_3^- has a role in cell growth, other than maintaining the pH of the extracellular medium.

TABLE I. ConA-Stimulated [^{3}H]thymidine Incorporation in Lymphocytes Cultured in HCO_3^--Buffered RPMI in the Presence of 5% CO_2 in Air ($CO_2 + HCO_3^-$) or in HEPES-Buffered RPMI in Air (HEPES), Either in a Regular Open System or a Close System*

	[^{3}H]thymidine incorporation (cpm/10^5 cells)		
ConA (μg/ml)	HEPES (open)	HEPES (close)	$CO_2 + HCO_3^-$
0	119±12	103±11	125±55
0.25	114±8	107±3	1710±145
0.5	201±30	139±14	3493±268
1.0	151±26	129±32	9483±624
2.5	195±112	175±96	16862±1112
5.0	145±55	148±48	12296±2751

*(n=3).

TABLE II. ConA-Stimulated [^{3}H]thymidine Incorporation in Lymphocytes Cultured in HEPES-Buffered RPMI in the Presence of 5% CO_2 (HEPES + CO_2) or HCO_3^- (HEPES + HCO_3^-)*

	[^{3}H]thymidine incorporation (cpm/10^5 cells)	
ConA (μg/ml)	HEPES + HCO_3^-	HEPES + CO_2
0	239±45	121±36
2.5	1588±204	18014±1377

*(n=3).

In the next step, we examined the effect of HCO_3^- and exogenous CO_2 separately on the ConA-stimulated proliferation of lymphocytes in HEPES-buffered medium. As shown in Table II, while very little proliferation was observed in the presence of HCO_3^-, a huge amount of proliferation was observed in the presence of CO_2. This result clearly showed that extracellular CO_2 plays an essential role in cell growth, other than maintaining pH of the medium. That is why HCO_3^- supported only a low level proliferation in HEPES-buffered medium in the absence of CO_2 (Table II), because most of the CO_2 generated from HCO_3^- would be lost in the air. The other role of CO_2 may appear nutritional, as it is involved in the regeneration of TCA cycle intermediates and the synthesis of nucleotides. However, this is ruled out because of several reasons. First and the most important, the required amount of CO_2 for these process is directly supplied from mitochondrial respiration. Even if we consider that the presence of exogenous CO_2 reduces the loss of endogenous CO_2, still significant proliferation should have occurred in the absence of CO_2 supply in HEPES-buffered medium, because it is unlikely that in the absence of exogenous CO_2 all of the endogenous CO_2 is lost immediately after its generation without participating in cellular metabolism. Second, exogenous supply of TCA cycle intermediates (succinic acid, Na-succinate), pyruvic acid which generates TCA cycle intermediate (oxloacetate), hypoxanthine and uridine (for nucleotide synthesis by salvage pathways), alone or in combination, did not support the proliferation of lymphocytes in HEPES-buffered medium in the absence of CO_2 supply (data not shown). These compounds should have recovered the lymphocyte growth at least to some extent, if exogenous CO_2 was essential only to sustain the synthesis of nucleotides and TCA cycle intermediates. Third, culturing in a close system, to prevent CO_2 loss, did not support lymphocyte proliferation in HEPES-buffered medium (Table I). Fourth, not all the works, showing cell growth in the absence of CO_2, did use culture media supplemented with TCA cycle intermediates, nucleotides, or nucleotide precursors. No cell growth should have occurred in those cases, if CO_2 supply was required to sustain the synthesis of nucleotides and TCA cycle intermediates.

The above results showed that extracellular CO_2 has a role, other than pH maintenance and the synthesis TCA cycle intermediates and nucleotides, in cell growth. It is not clear at this moment, why in few isolated cases cells grew in the absence of exogenous CO_2 supply. This perhaps reflects the species and cell type differences in the amount of extracellular CO_2 required to support the cell growth. For those cells, which did not require CO_2 supply for growth, perhaps CO_2 released from the cell was sufficient to fulfil the requirement of extracellular CO_2. That might explain why in those works a better cell growth was obtained at relatively higher cell densities or when cultured in a close system [Shipman, 1969; Darzynkiewicz and Jacobson, 1971; Itagaki and Kimura, 1974]. Thus, considering our results and the other findings, we postulate that extracellular CO_2 plays a novel role in cell growth.

ACKNOWLEDGMENTS

We acknowledge the financial support from UGC and DBT, Government of India, and the technical assistance from Sanjeev Kumar.

REFERENCES

Chakrabarti R, Chang JY, Erickson KL. 1995. T cell receptor mediated Ca^{2+} signalling: release and influx are independent events linked to different Ca^{2+} influx pathways in the plasma membrane. J Cell Biochem 58:344–359.

Darzynkiewicz Z, Jacobson B. 1971. HEPES-buffered media in lymphocyte cultures. Proc Soc Exp Biol Med 136:387–393.

Fisk A, Pathak S. 1969. HEPES-buffered medium for organ culture. Nature 224:1030–1031.

Good NE, Winget GD, Winter W, Conolly TN, Izawa S, Sing RMM. 1966. Hydrogen ion buffers for biological research. Biochemistry 5:467–477.

Itagaki A, Kimura G. 1974. TES and HEPES buffers in mammalian cell cultures and viral studies: problem of carbon dioxide requirements. Exp Cell Res 83:351–361.

Medzon EL, Gedies A. 1971. Substitution of 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) for bicarbonate in protein-free animal cell culture medium: application to vaccinia virus quantitation and fluorogenic acetylesterase assay in living M cells. Can J Microbiol 17:651–653.

Mossmamm T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. J Immunol Methods 65:55–63.

Shipman C Jr. 1969. Evaluation of 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) as a tissue culture buffer. Proc Soc Exp Biol Med 130:305–310.