Plasmodium falciparum: Effective use of the CO[2]-NaHCO[3] Buffer System for Evaluating Chloroquine Resistance

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(Degree)
博士（医学）

(Date of Degree)
2000-03-31

(Resource Type)
doctoral thesis

(Report Number)
甲2111

(URL)
https://hdl.handle.net/20.500.14094/D1002111

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博士の専攻分野の名称	博　士(医　学)
学位記番号	博い第1254号
学位授与の要件	学位規則第4条第1項該当
学位授与の日付	平成12年3月31日
学位論文題目	*Plasmodium falciparum* : Effective use of the CO_2—$NaHCO_3$.Buffer System for Evaluating Chloroquine Resistance (熱帯熱マラリア原虫 *in vitro* クロロキン耐性試験における適正な CO_2—$NaHCO_3$. Buffer System の使用法)
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論文内容の要旨

INTRODUCTION

Since the establishment of the *in vitro* culture system of *P. falciparum* by Trager and Jensen in 1976, their methods have been extensively used for a variety of studies on this parasite. *P. falciparum* has conventionally been cultured in RPMI 1640 medium with constant buffer (24 mM $NaHCO_3$ and 25 mM HEPES) supplemented with 10% human serum at various gas phases (1－7% CO_2 and 1－18% O_2), including the gas phases in candle jars. In malaria field studies, *in vitro* drug sensitivity assays for *P. falciparum* have often been performed using candle jars with the conventional medium mentioned above. However, we have recently noticed that the effect of chloroquine (CQ) on the growth of *P. falciparum* in candle jars is considerably different from that in incubators at 5% CO_2. Media with 24mM $NaHCO_3$ were originally designed for maintaining the pH of culture media around the physiological range at 5% CO_2. Theoretically, lower CO_2 concentrations increase pH and vice versa, for media with constant $NaHCO_3$ concentrations. On the other hand, there is accumulating evidence that the antimalarial effects of CQ are dependent on the pH of the culture media. Thus, the effect of CQ may vary at different pHs caused by various CO_2 concentrations in the presence of a constant $NaHCO_3$ concentration. In this study, we investigated the inhibitory effects of CQ on parasite growth in incubators at different CO_2 concentrations and in candle jars using conventional medium for *P. falciparum*.

MATERIALS AND METHODS

Two strains of *P. falciparum*, the CQ resistant K1 strain and the CQ sensitive MAD20 strain were used. Parasites were maintained in asynchronous culture as described by Trager and Jensen with slight modiffication. Effects of antimalarial drugs on parasite growth were assayed mainly by monitoring uptake of [^{3}H] hypoxanthine at various CO_2 concentrations or in candle jars at 37℃.

RESULTS

First we compared the effects of CQ on the growth of the K1 strain in incubators at different CO_2

concentrations（7％, 5％, and 2.7%）and in candle jars. The inhibition of parasite growth was reduced progressively with incresing CO_2 concentrations. The inhibition in candle jars (2.7%, CO_2) was nearly the same as that in incubators at 2.7%CO_2. The inhibitory effect of CQ on the CQ sensitive MAD20 strain in incubators at 7 %CO_2 was not as reduced as on the K 1 strain. The IC50 for the K 1 strain in incubators at 7 %CO_2 was approximately ninefold greater than in incubators at 2.7% CO_2 and in candle jars ($P<0.01$). The IC50 values in incubators at 2.7% CO_2 and in candle jars were quite close to each other. The pH of Pf-medium [+] at different CO_2 concentrations were examined. The higher the CO_2 concentrations were, the lower the pH values of media were. Although the differences in pH seemed small (at most 0.26), they were significant ($P<0.01$). The pH values of Pf-medium [+] in incubators at 2.7% CO_2 and in candle jars were very close. The pH of Pf-medium [+] containing a reduced concentration of $NaHCO_3$ (16mM) in candle jars was almost the same (pH7.50) as that of Pf-medium [+] containing 24mM $NaHCO_3$ at 5 % CO_2, and the effect of CQ on parasite growth turned to be nearly identical under the two culture conditions.

DISCUSSION

The *in vitro P. falciparum* continuous culture method were designed to maintain asexual multiplication of the parasite. One of the important requirements for the continuous culture was gas phases with relatively high CO_2 concentrations and low O_2 concentrations. However, in this study, we found that CO_2 concentrations dramatically alter the inhibitory effect of CQ on the growth of the chloroquine resistant K 1 strain with stronger inhibition by CQ at lower CO_2 concentrations, due to changes in pHs of media resultant from inappropriate use of CO_2－$NaHCO_3$ buffer system.

The CO_2－$NaHCO_3$ buffer system has not yet been used appropriately in many studies, even when pH dependency of the antimalarial effect of CQ has been examined. It is noteworthy that slight but significant changes in pHs caused marked difference in IC50 of CQ, indicating that the antimalarial effects of CQ are highly sensitive to marginal fluctuations in the pH of the media. Therefore, it is of prime inportance to keep the pH of the media around 7.4 when evaluating sensitivities of *P. falciparum* strains to CQ or when investigating mechanisms of action of CQ or resistance to CQ. We strongly recommend that the conventional medium for *P. falciparum* culture with constant $NaHCO_3$ (24mM) be modified according to the CO_2 concentrations used in order to avoid confusion of interpreting the obtained data. The WHO kit for *in vitro* chloroquine sensitivity test should be modified accordingly. Our recommendation should also be valid for other antimalarials and even for other drugs unrelated to antimalarials, whose effects are pH-dependent. In this study, we also worked out the appropriate $NaHCO_3$ concentrations for achieving media with a pH of 7.4 at various CO_2 concentrations.

論 文 審 査 の 結 果 の 要 旨

熱帯熱マラリア原虫の *in vitro* 培養法は1976年に確立され，マラリアに関する種々の基礎的研究を飛躍的に発展させた。なかでも，*in vitro* 培養マラリア原虫を用いた薬剤感受性の評価法は汎用されており，マラリア原虫の薬剤耐性に関する多くの知見の集積に貢献してきた。熱帯熱マラリア原虫の培養には低酸素ガス条件と培養液中の高濃度の human serum が重要であることがわかっている。しかし，ある程度の異なったガス条件（1－7％CO_2, 1－18%O_2）で，同じ組成の培養液（RPMI1640 with 24 mM $NaHCO_3$ and 25 mM HEPES）を用いてもマラリア原虫の増殖には大きな差は認められないことから，研究者によって異なるガス条件が選択されてきている。また，低酸素条件を形成するため

に candle jar （点灯したろうそくを自然消灯させた密閉容器）が利用され，現在でも，発展途上の流行地における薬剤感受性評価には candle jar を用いた培養が行われている（１％CO_2，17％O_2）。ところが，予備的実験により，*in vitro* 培養のガス条件の差により，マラリア原虫のクロロキン感受性が，大きく異なることが示された。本研究は，*in vitro* 培養のガス条件がクロロキン感受性を変化させた機序を明らかにすることを目的に行われた。

方法

クロロキン耐性熱帯熱マラリア原虫Ｋ１株，およびクロロキン感受性熱帯熱マラリア原虫 MAD20 株を *in vitro* 培養し，異なるガス条件下でのクロロキン感受性を parasiemia および［^{3}H］hypoxanthine 取込みを指標にした assay で検討した。

結果と考察

クロロキン耐性Ｋ１株では，クロロキン感受性はCO_2濃度が2.7％から７％まで上がるにしたがって，低下することを明らかにした。一方，クロロキン感受性 MAD20株では，クロロキン耐性Ｋ１株ほどクロロキン感受性の低下は著しくなかった。また，クロロキン感受性はO_2濃度には影響を受けないことも明らかになった。マラリア原虫のクロロキンの感受性が培養液の pH が低ければ，低下することが報告されているため，種々のCO_2濃度条件下で培養液の pH を測定したところ，CO_2濃度が2.7％から７％まで上がるにしたがって，pH が7.5から7.25に低下することが分かった。異なるCO_2ガス条件下で，同じ濃度の$NaHCO_3$を用いると pH が変化することが予想されるため，$NaHCO_3$の濃度を変化させることにより pH を7.4に固定したところ，確かに同じクロロキン感受性を得ることが証明された。

以上の結果から，ガス条件による熱帯熱マラリア原虫のクロロキン感受性の変化は，異なるCO_2濃度下で一定濃度の$NaHCO_3$を用いていることによる微妙な pH の差によることが明かとなった。

本研究は熱帯熱マラリア原虫の異なる培養ガス条件によるクロロキン感受性の変化の機構を明らかにしたものであり，従来，熱帯熱マラリア原虫のクロロキン感受性の判定に不適切に用いられてきた培養液組成を訂正し，今後のマラリア原虫のクロロキン耐性機構に関する研究に重要な情報を提供する価値ある業績である。よって，本研究者は，博士（医学）の学位を得る資格があると認める。