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Kalfinella bulgarica gen.nov.

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Abstract

S. J. Lu, Q. Feng, J. S. Park, Vida L, Lee BS, Strausbauch M, Wettstein PJ, Honig GR, Lanza R have reported that the in vitro produced erythrocytes in embryonic stem cell cultures expel their nucleus and remain without a nucleus like the erythrocytes in human blood themselves. The reason for this phenomenon has not been discovered; however, the hypothesis of “the impossible co-existence between the erythrocyte nucleus and the unknown microorganisms living as normal flora in the red blood cell” has been supported by our experimental observations.

INTRODUCTION

In October 1992, the discovery of unknown microorganisms in non-nuclear erythrocytes of human blood was made during a series of experiments on sarcoidosis agent isolation [2] at the Referent laboratory for tuberculosis bacteria, Higher Medical Institute, Sofia. For the first time, an inoculation on human blood agar (typically not used in microbiology for ethic reasons) was performed.[3] The observations of the unknown, slowly growing microorganisms living as normal flora in human red cells, designated as erythrocyte microorganisms (EM), were prolonged for 5 months in order to examine their multiplication. Under electron microscopic examinations, formation of colonies on 10% sheep blood agar was not observed; the attempts to destroy the erythrocyte microorganisms after treatment with acetic, sulphuric, hydrochloric and nitric acid, sodium or potassium hydroxide were unsuccessful, for which it had to be admitted that the observed bodies were unknown non-nuclear microorganisms.

References to medical literature have revealed the availability of various microorganisms observed by several authors in the blood of healthy individuals, such as pleomorphic bacteria (4), bacterial ribosome DNA (5), mycoplasmata or L forms (6, 7), unknown pathogenic microorganisms (8), filterable forms (9), new bacterial structures (10), persisting bacteria (11). The fact that in most cases it referred to unstable L forms of Staphylococcus epidermidis, diphtheroids, cocci, Listeria, which are absolute saprophytes, is really absurd. In most cases, these were one-time reports. The microorganisms described were isolated from both healthy and diseased individuals and it has not been clarified if they were disease agents or some forms in the process of microorganisms’ multiplication in individual’s blood. New data for these microorganisms have been not published and the suggestion that unknown microorganisms live as normal flora in non-nuclear erythrocytes has been supported by none of the authors.

MATERIAL AND METHODS

FAST METHOD FOR EM ISOLATION IN BRAIN HEART INFUSION

100ml of BBL® Brain Heart Infusion with 0.25% sodium citrate and 0.1% vitamin K3 was distributed in sterile test tubes, 2.0 ml or 4.5 ml in each, and was sterilized in autoclave at 121°C, for 15 minutes. 0.2 ml or 0.5 ml venous human blood was inoculated in each tube containing 2 ml or 4.5 ml of the described liquid medium. Cultivation 72 hours at 43°C

ELECTRON MICROSCOPY

Fixated and unfixated, stained or non-stained preparations applied by dropping on a carbon film were used. For revelation of substructures, EM were treated with 4% and 10% sodium hydroxide, 1.5% acetate, 3% ammonium chloride, SDS, triton x 100, concentrated nitrous acid.

RESULTS

EM MULTIPLICATION IN A VITAMIN K3 CONTAINING LIQUID NUTRIENT MEDIUM. FAST METHOD FOR EM ISOLATION

1 g/L vitamin K3 is a powerful growth factor for EM. The assessment of EM culture could be performed after 48h-72h instead of 21 days. The unusual effect of vitamin K3 high concentration was empirically found and is still unexplained. On Gram’s stain, yeast-like cells situated outside the

erythrocytes were observed.

EM MULTIPLICATION IN A VITAMIN K3 CONTAINING AGAR MEDIUM

EM multiplication on an agar nutrient medium was stimulated by 1 g/L K3 vitamin. The colony size was much bigger and could be compared with those of other microorganisms. EM colonies were lucent and a coating was formed during their intensive growth. However, a 3-4 week growth period and temperature of 43ºC were still needed.

EM MORPHOLOGY

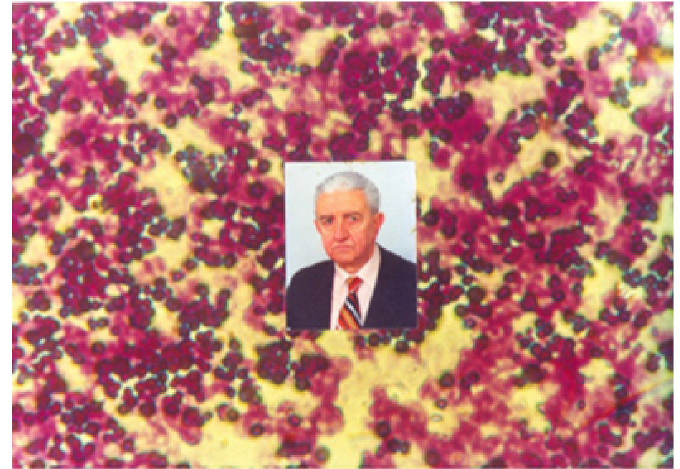
The suggestion that under unfavorable external conditions, the microorganisms would present themselves as a yeast-like form while during their multiplication in human erythrocytes they are as yeast-like forms/ fig.1 and fig 2/, or as a chlamidia-like form, was supported by our electron microscopic examinations.

Figure 1

Figure 1: Kalfinella bulgarica gen.novo in Bulgarian professors blood

Figure 2

Figure 2: Kalfinella bulgarica gen. nov. in Assoc. Prof . Kalfin´s Blood



On electron microscopic photos[12] the individual microorganism was observed as a homogenous spherical cell with neither a nucleus nor a cell wall of a Gram positive/Gram-negative microorganism. The cellular surface was overgrown with multiple tiny and fine pili more expressed in younger than in older microorganisms.

A common appearance of the yeast-like forms was revealed by light microscopy examinations. Multiplication of the yeast-like forms could be performed simple division of a daughter cell from a mother cell by squeezing of protoplasm or by clustering and binding of several divided cells.

Yeast-like forms could be presented as spore-like cells when the blood was dry in conditions of an external medium.

On electron microscopy, Chlamydia-like forms could be seen inside and outside the erythrocytes as cells with a nucleus-like formation. This nucleus-like formation could be expelled by the cell itself. In an erythrocyte, there could be both nuclear and non-nuclear cells, the latter resembling the elementary and reticular bodies in chlamidiae. On electron microscopy, the EM invasion and penetration through the erythrocyte wall, thus making it thicker, could be observed. The availability of any channels on the wall could not be précised for an insufficient magnification was used.

EM MIMICRY

The term “mimicry” is not met in microbiology textbooks and that is why the performed electron microscopy photos revealing the similarity between the new microorganisms and erythrocytes are unique. Due to their mimicry, EM could be seen dispersed among the red blood cells as “small

erythrocytes" without nuclei. Discrimination between the two kinds of cells was performed by estimating their dimensions: the new microorganisms were as big as 0.3µm to 2.6µm while the erythrocytes were several times bigger, from 3.5µm to 7.5µm. It is more than coincidence that both EM and erythrocytes expel their nuclei.

EM BIOCHEMICAL CHARACTERISTICS

On routine examination, no specific biochemical activity was demonstrated by the yeast-like EM forms as acid with no gas from glucose and maltose, but not from sucrose and lactose was produced. The urease, lysine decarboxylase and catalase tests were positive, but the ornitine decarboxylase, indole and hydrogen sulfide tests were negative.

EM were resistant to antibiotics used in routine practice but the search of specific antibiotics for curing the diseases caused by the new microorganisms was too expensive for the laboratory.

There are no sterile blood cultures/13/ because K bulgarica gen.nov. lives in blood

The EM growth was inhibited by Sodium Polyanethol Sulfonate (SPS) in blood culture media. However, after SPS removal of the medium, EM could again multiply in media assessed as sterile by routine examinations .

EM ORIGIN

Intrauterine transmission of normal flora in fetal erythrocytes was proved by inoculations of placental blood. Whether EM are available in the embryonic stem cells and in produced non-nuclear erythrocytes is still unknown.

EM PATHOGENICITY IN HUMANS

Both intensive multiplication and non-typically localized multiplication of EM might be a cause of a disease.[14151617] The study on various anemia forms in 20 patients at the Clinic of Prof. Petrov, II City Hospital, Sofia, has shown that in only 2 patients the EM had multiplied more intensively thus leading to iron deficiency anemia. The observations on 20 patients at the Department for intensive treatment of pulmonary diseases of Assoc. Prof. Osmanliev, Higher Medical Institute, Sofia, have shown that EM had multiplied more intensively in only 1 patient with chronic pneumonia, which was resistant to antibiotic treatment.

EM GENETIC ANALYSIS

BULGARIA IS THE POOREST COUNTRY IN EUROPE

Since their discovery in 1992, the unknown microorganisms in human blood were not a subject of DNA analysis at the Referent laboratory for tuberculosis bacteria, Higher Medical Institute, Sofia. The pure culture isolated in BHI was sent to the NBIMCC, where it was lyophilized in ampoules and kept at a temperature below zero for 30 years under the name of "Kalfinella bulgarica gen. nov. 3300". The NBIMCC has not yet succeeded in separating Kalfinella bulgarica gen. nov. DNA for the needs of genetic analysis in DSMZ.

The pure EM cultures isolated by the experts from the BAS and NCIPD were found impossible to be destroyed for the purposes of the DNA analysis (17). The genetic analysis of EM was delayed for the author had performed the examinations with no assistants, adhering to the classic principle that pure cultures are the alpha and omega of microbiology because they allow a comprehensive study on newly discovered microorganisms. The genetic analysis being a basis of contemporary microbiology could be carried out only after the method of pure culture isolation in a nutrient medium is known. In addition, EM genetic analysis was delayed for the author had performed the examinations with the limited funds of about a $260 month pension. However, since 2008, a team of scientists including three countries and two professors has been formed. The decoding of EM genetic structure is advancing successfully and the results will be revealed in the end of 2008.

DISCUSSION

The discovery of unknown microorganisms living as normal flora in human blood was carried out in a classic "retro style" using only the classic methods for EM isolation in a pure culture and applying no DNA analysis such as in tuberculosis bacteria, anthrax and other disease agent isolation. Due to our limited funds and the NBIMCC inability to perform the genetic analysis, the specialists from the CDC, Institut Pasteur, ECDPC and RAS, and 182 investigators from the USA (65), England (10), France (10), Germany (10) and 44 other countries were requested for supporting the genetic examinations. There was no reply from the expert research teams but the standard phrase "The blood of a healthy individual is sterile" has not been said by any author.

CONCLUSIONS

The impossible co-existence between the erythrocyte nucleus and EM has been supported by our experimental

observations and illustrated by the electron microscopic photos. Therefore, the erythrocytes expel their nuclei for the presence of unknown till now microorganisms.

The used fast method for EM isolation in brain heart infusion could answer in only 72 hours the fundamental question “Are there unknown microorganisms in the embryonic stem cells and in vitro produced erythrocytes, which live as normal flora in non-nuclear erythrocytes of human blood?”

Using the same method, the antigenic structure of the microorganisms in donor and recipient’s blood for determining compatibility in cases of transfusion, could be identified.

In 2009, a detailed DNA analysis of K. bulgarica gen.nov. will be accessible via Internet.

Modern scientists cannot or wish not to answer the question, “Is there normal flora in human blood?” Their silence would be appropriate if it turns out that human blood is sterile otherwise it is unjustifiable. Each healthy individual is born, lives and dies with normal erythrocyte flora.

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