

Riboflavin deprivation inhibits macrophage viability and activity – a study on the RAW 264.7 cell line

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Abstract

Riboflavin, or vitamin B₂, as a precursor of the coenzymes FAD and FMN, has an indirect influence on many metabolic processes and determines the proper functioning of several systems, including the immune system. In the human population, plasma riboflavin concentration varies from 3·1 nM (in a moderate deficiency, e.g. in pregnant women) to 10·4 nM (in healthy adults) and 300 nM (in cases of riboflavin supplementation). The purpose of the present study was to investigate the effects of riboflavin concentration on the activity and viability of macrophages, i.e. on one of the immunocompetent cell populations. The study was performed on the murine monocyte/macrophage RAW 264.7 cell line cultured in medium with various riboflavin concentrations (3·1, 10·4, 300 and 531 nM). The results show that riboflavin deprivation has negative effects on both the activity and viability of macrophages and reduces their ability to generate an immune response. Signs of riboflavin deficiency developed in RAW 264.7 cells within 4 d of culture in the medium with a low riboflavin concentration (3·1 nM). In particular, the low riboflavin content reduced the proliferation rate and enhanced apoptotic cell death connected with the release of lactate dehydrogenase. The riboflavin deprivation impaired cell adhesion, completely inhibited the respiratory burst and slightly impaired phagocytosis of the zymosan particles. In conclusion, macrophages are sensitive to riboflavin deficiency; thus, a low riboflavin intake in the diet may affect the immune system and may consequently decrease proper host immune defence.

Key words: Ariboflavinosis: Macrophages: Apoptosis: Proliferation: Heat shock protein 72

Riboflavin, or vitamin B₂, is a micronutrient that is necessary for maintaining human health. It is essential for the proper functioning of the nervous, endocrine, cardiovascular and immune systems⁽¹⁾. For human subjects and other animals unable to synthesise riboflavin, its main sources are food and activity of the intestinal microflora⁽²⁾. Plasma riboflavin concentration in the human population varies according to health status, being 10·4 nM in healthy adults⁽³⁾, 300 nM in people taking riboflavin supplementations⁽⁴⁾, but 3·1 nM in people with a moderate riboflavin deficiency (e.g. pregnant women)⁽⁵⁾.

In the cell, riboflavin is a precursor for both FMN and FAD synthesis, both of which participate in a range of redox reactions⁽¹⁾. A FAD-dependent enzyme, glutathione reductase, participates in the redox cycle of glutathione, which protects the organism from reactive oxygen species⁽⁶⁾. FAD is also a coenzyme for endoplasmic reticulum oxidoreductin 1 and sulphhydryl oxidases, both of which mediate the oxidative folding of secretory proteins^(7–9). Studies on human liver cells (HepG2) have shown a significant decrease

in oxidative folding and a subsequent secretion of proteins in riboflavin-deficient cells^(6,7–9). Furthermore, riboflavin deficiency causes protein and DNA damage, which are accompanied by cell cycle arrest, cell stress and increased apoptosis^(6,7–9). The expression of numerous genes is also strongly influenced by a different riboflavin status⁽⁸⁾.

Riboflavin affects the immune system, as it carries out antinociceptive and anti-inflammatory activities^(10–12) and enhances the anti-inflammatory⁽¹¹⁾ and antinociceptive⁽¹⁰⁾ effects of morphine. Moreover, an injection of vitamin B₂ reduces the mortality of mice with septic shock⁽¹³⁾ and enhances the resistance to bacterial infection⁽¹⁴⁾. On the other hand, riboflavin deficiency resulted in lowered neutrophil phagocytosis⁽¹⁵⁾, increased PG biosynthesis in rat renal medulla⁽¹⁶⁾ and impaired haematopoiesis by hindering Fe absorption⁽¹⁾. Nevertheless, the effects of riboflavin deprivation on particular types of leucocytes participating in innate and acquired immune responses have been poorly explored. The present investigation focuses on the monocytes/macrophages that participate in innate immunity and

Abbreviation: LDH, lactate dehydrogenase.

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initiate adaptive lymphocyte-mediated immunity by an efficient antigen presentation.

The aim of the present study was to determine the *in vitro* effects of riboflavin deficiency on the murine monocyte/macrophage cell line RAW 264.7, in particular on cell viability, cell cycle progression, the induction of stress proteins and the efficiency of an immune response (respiratory burst and phagocytosis).

Materials and methods

Cells

RAW 264.7 cells (a mouse monocyte/macrophage cell line) were purchased from the European Type Culture Collection (Sigma). The cells were maintained in a customised (riboflavin-depleted) RPMI-1640 medium (PAA) supplemented with riboflavin (riboflavin; Sigma) to achieve the required riboflavin concentration (riboflavin: 3.1, 10.4 and 300 nM). As a control, standard RPMI-1640 was used (riboflavin: 531 nM). The medium was supplemented with 100 µg/ml (62.5 µg/ml) streptomycin, 100 U/ml penicillin (PAA) and 10% dialysed fetal bovine serum (PAA). For the experiments, the cells were cultured for up to 4 d at 37°C in humidified air (5% CO₂) in a medium supplemented with low (3.1 nM), physiological (10.4 nM) or high (300 and 531 nM) riboflavin concentrations.

Apoptosis and necrosis assessment

Apoptotic leucocytes were identified quantitatively by the Annexin V-PE Apoptosis Detection Kit I (BD Pharmingen), according to the manufacturer's protocol. In this method, Annexin V-PE (Annexin V conjugated to phycoerythrin) binds to phosphatidylserine exposed on the outer leaflet of the plasma membrane of apoptotically dying cells (a marker of early apoptosis measured in the FL-2 channel), while 7AAD (measured in the FL-3 channel) is a vital dye that enters any dead cells (a marker of late apoptotic and necrotic cells). The measurement was performed by a FACScan cytometer (FACSCalibur™; BD Biosciences) using CellQuest software (BD Biosciences). For data analysis 10 000 events were collected.

Cell cycle analysis

Changes in cell cycle progression were recorded by flow cytometric analysis of the DNA profiles, according to the procedure described previously by Mazur *et al.*⁽¹¹⁾, by a FACScan cytometer using CellQuest software. For data analysis 100 000 events were collected.

Cytotoxicity test

The cytotoxic effect of cell culture in various riboflavin concentrations was evaluated spectrophotometrically (Expert Plus; Hitachi) by measuring the activity of a cytosolic enzyme, lactate dehydrogenase (LDH), released into the

medium from the damaged cells. The activity of LDH was examined by a commercial cytotoxicity test, according to the manufacturer's protocol (TOX7; Sigma).

Cell adhesion

Cell adhesion was estimated spectrophotometrically (Expert Plus; Hitachi) by crystal violet staining of adherent cells. In short, the cells were seeded in a ninety-six-well plate (100 000 cells/100 µl of the appropriate medium per well) for adherence to the plate surface (37°C). After 45 min incubation, the cells were fixed with formalin (final concentration 2%; 2 h), washed to remove the non-adherent cells and then dried. The adherent cells were stained with crystal violet solution (0.5% crystal violet in 20% methanol; 5 min), washed with tap water and dried again. The crystal violet was extracted by 100 µl of 100% methanol. After 10 min of shaking, the absorbance was measured at 570 nm (Expert Plus Asys/Hitech spectrophotometer; Biochrom). Each assay was carried out in triplicates.

Respiratory burst (nitro blue tetrazolium chloride test)

The respiratory burst was assessed according to Graham & Secombes⁽¹⁷⁾. In short, the cells were seeded in a ninety-six-well plate (100 000 cells/100 µl of appropriate medium per well) for adherence to the plate surface (37°C). After 2 h of incubation, a solution of nitro blue tetrazolium chloride (10 mg/ml; Sigma Chemicals) or nitro blue tetrazolium chloride with phorbol myristate acetate (1 µg/ml; Sigma Chemicals) was added to the wells. After 1 h of incubation, the cells were washed, fixed with 70% methanol and dried. Intracellular formazan was extracted by 120 µl of 2 M-KOH and 140 µl of 99.5% dimethyl sulphoxide per well. After 10 min of shaking, the absorbance was measured at 630 nm (Expert Plus Asys/Hitech spectrophotometer). Each assay was carried out in triplicates.

Phagocytosis

The cells were incubated with zymosan particles (30 min; 37°C) in a proportion of 1:50 in a twenty-four-well plate. After incubation, the cells were washed in PBS and fixed in formalin (the final concentration was 2%). Both the percentage of cells that take up the particles and the number of particles per cell were recorded.

Cytometric analysis of heat shock protein 72 expression

Heat shock protein 72 was detected in cells by flow cytometry (FACSCalibur™; BD Biosciences using CellQuest software) after labelling with a fluorescent antibody. Briefly, after incubation, the cells were detached from the substratum (Accutase; 10 min; 37°C; PAA), blocked with Fc-block (1:200; 20 min; 4°C; BD Biosciences), fixed and permeabilised according to the manufacturer's instruction (Cytotfix/Cytoperm; BD Biosciences). Then, the cells were incubated with the biotin anti-heat shock protein 72 antibody (1:200; 20 min;



4°C; StressGen) and PerCP-Cy5.5 Streptavidin (1:200; 20 min; 4°C; BD). For data analysis, 10 000 events were collected.

Statistical analysis

Data were analysed by a one-way ANOVA. The level of statistical significance was set at 0.05. All data were expressed as means with their standard errors.

Results

Apoptotic cells

The culture of RAW 264.7 cells in a riboflavin-deficient medium (3.1 nM) resulted in a significant increase in the number of apoptotic cells, both at the early and late stages of the apoptotic processes, compared with cells cultured in a medium with riboflavin at higher concentrations, i.e. 10.4 nM to 531 nM (Fig. 1). These results indicate that riboflavin is important for the survival of macrophage RAW 264.7 cells.

Cell cycle

As presented in Fig. 2, proper riboflavin content is essential for proliferation of the monocyte/macrophage cell line. After 4 d of incubation in a medium with a low riboflavin concentration (3.1 nM), the percentage of proliferating cells, i.e. those at the S and the G2M phases of the cell cycle, was statistically significantly lower than the percentage of cells cultured in a

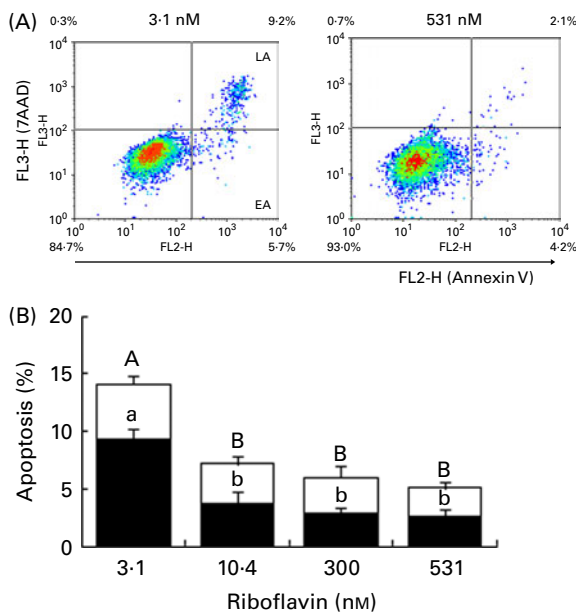


Fig. 1. The effect of 4 d incubation in a medium with various riboflavin concentrations on apoptosis of RAW 264.7 cells. (A) Representative dot-plots for 3.1 and 531 nM. LA, late apoptotic; EA, early apoptotic. (B) Percentage of apoptotic cells in early (□) and late phase of apoptosis (■). Values are means with their standard errors (n 4–6). ^{A,B} Mean values with unlike letters were significantly different according to *t* test (*P*<0.05). ^{a,b} Mean values with unlike letters were significantly different according to *t* test (*P*<0.05). (A colour version of this figure can be found online at <http://www.journals.cambridge.org/bjn>).

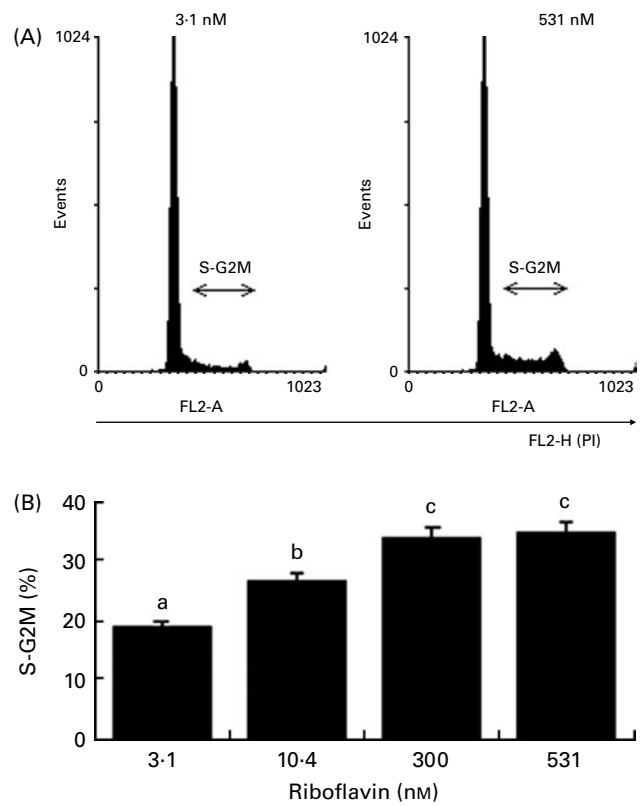


Fig. 2. Cell cycle analysis in RAW 264.7 cells after 4 d of culture in a medium with various riboflavin concentrations. (A) Representative histograms for 3.1 and 531 nM. (B) Percentage of proliferating cells (in phase S-G2M). Values are means with their standard errors (n 4–6). ^{a,b,c} Mean values with unlike letters were significantly different according to *t* test (*P*<0.05).

physiological riboflavin concentration (10.4 nM). Cell proliferation increased further at higher riboflavin concentrations, i.e. at 300 and 531 nM. In conclusion, the reduction of riboflavin concentration resulted in a decreased rate of cell proliferation.

Cytotoxicity test

On the 4th day of incubation, the level of LDH released from the RAW 264.7 cells was statistically significantly higher in the medium with the lowest riboflavin concentration (3.1 nM) than in wells with a physiological and higher riboflavin content (10.4–531 nM) (Fig. 3(A)). Such an increase of LDH putatively released from damaged or dead cells was recorded already on day 3 (data not shown).

Cell adhesion

After 4 d of culture in a riboflavin-deficient medium (riboflavin: 3.1 nM), the amount of crystal violet extracted from the adherent cells was statistically significantly lower in wells with a 3.1 nM riboflavin concentration than in wells with a physiological or higher concentration of riboflavin (Fig. 3(B)). Such a loss of ability to adhere to the substratum

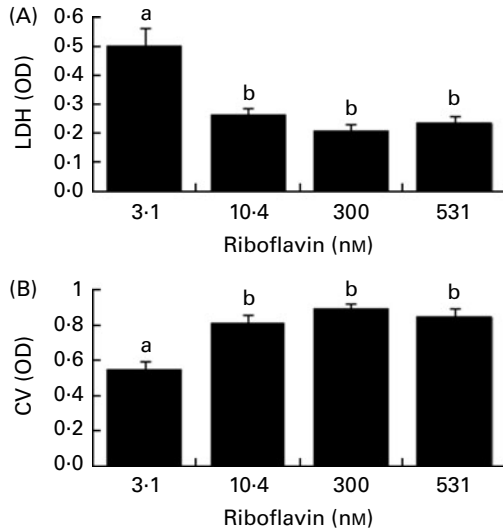


Fig. 3. Effects of 4 d incubation in a medium with various riboflavin concentrations on (A) lactate dehydrogenase (LDH) released in cytotoxicity test and (B) RAW 264.7 cell adhesion in crystal violet (CV) test. OD, optical density. Values are means with their standard errors (n 4–6). ^{a,b} Mean values with unlike letters were significantly different according to ANOVA ($P < 0.05$).

was probably caused by decreased cell viability, which corresponded to increased apoptotic death and a decreased proliferation rate in the riboflavin-deficient medium.

Respiratory burst

Various concentrations of riboflavin in the culture medium had no effect on the spontaneous respiratory burst measured on the 4th day of incubation (open bars in Fig. 4). In sharp contrast, a phorbol myristate acetate-induced respiratory burst was clearly dependent on the riboflavin concentration (the solid bars in Fig. 4). At a 3.1 nM riboflavin concentration, both the spontaneous and phorbol myristate acetate-induced nitro blue tetrazolium chloride reduction to formazan were similar; this indicates that a respiratory burst is absent in suboptimal riboflavin concentrations.

Phagocytosis

On the 4th day of culture in the riboflavin-deficient medium (3.1 nM), the RAW 264.7 cells exhibited a statistically insignificant decrease in phagocytic capacity *v.* that at 10.4 nM, while both the percentage of phagocytic cells (Fig. 5(A)) and the number of zymosan particles within one phagocytic cell (Fig. 5(B)) were significantly lower than those at the 300 and 531 nM riboflavin concentrations (Fig. 5).

Heat shock protein 72 induction

A riboflavin deficiency (3.1 nM in the culture medium) is a stressing factor for RAW 264.7 cells, as it induces heat shock protein 72 expression above the level that is characteristic for resting cells cultured at higher riboflavin concentrations, *i.e.* from 10.4 to 531 nM (Fig. 6).

Discussion

Macrophages are leucocytes within tissues that are produced by the differentiation of monocytes. Under *in vivo* conditions, monocyte-derived macrophages play many immunologically important roles. As phagocytes, they rid the body of damaged cells and cell artifacts. Besides phagocytosis, macrophages fulfill their role in the innate immune response as secretory cells, *i.e.* they produce a wide array of cytokines, enzymes and reactive oxygen species. Furthermore, macrophages are crucial for initiating the adaptive immune response by presenting antigens to the T helper cells⁽¹⁸⁾.

In the RAW 264.7 cell model, deprivation of riboflavin from the culture medium resulted in a lowered cell viability and a rapid rate of cell death, with a strikingly high fraction of late apoptotic cells with compromised membranes. As apoptosis is an active energy-demanding mechanism, energy-depleted dying cells convert from apoptosis to necrosis in a process known as a secondary necrosis⁽²²⁾. Riboflavin deficiency interferes with electron transport along the respiratory chain⁽⁶⁾; thus, riboflavin-deficient cells deal with decreased energy production and are not able to maintain the intact cell membrane of early apoptotic cells. As a result, riboflavin-deficient cells go through the stage of early apoptosis very quickly to yield a higher level of secondary necrosis. This has been confirmed by the results of the cytotoxicity test, as a significantly increased amount of LDH was released from the damaged or dead cells in the riboflavin-deprived group (3.1 nM).

The present data are consistent with studies on HepG2 liver cells, in which riboflavin deficiency caused cell cycle arrest and inhibited the proliferation rate within 4 d⁽¹⁹⁾. This was accompanied by an increased expression of GADD153⁽¹⁹⁾, a molecule that plays a role in cell growth arrest and apoptosis⁽²⁰⁾. Studies on hybridoma cells cultured in media without riboflavin have shown lowered cell viability accompanied by a high percentage of late apoptotic cells already after 3 d of maintenance⁽²²⁾.

Currently, it is unknown whether impaired cell viability and proliferation are both mediated by a riboflavin deficiency *per se* or by an alteration in the metabolite profiles that are secondary to the riboflavin deficiency. Zempleni & Mock⁽²³⁾ reported that riboflavin influx significantly increases during

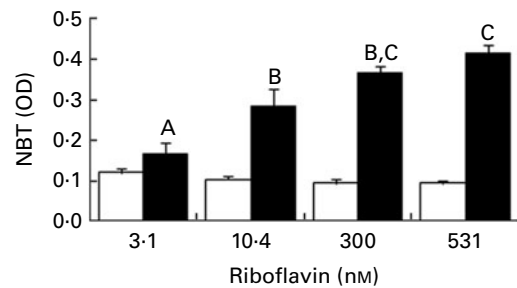


Fig. 4. Effects of 4 d culture in a medium with various riboflavin concentrations on intensity of respiratory burst in non-stimulated (phorbol myristate acetate (-PMA, □) and stimulated RAW 264.7 cells (+PMA, ■). NBT, nitro blue tetrazolium chloride; OD, optical density. Values are means with their standard errors (n 4–6). ^{A,B,C} Mean values with unlike letters were significantly different according to ANOVA ($P < 0.05$).

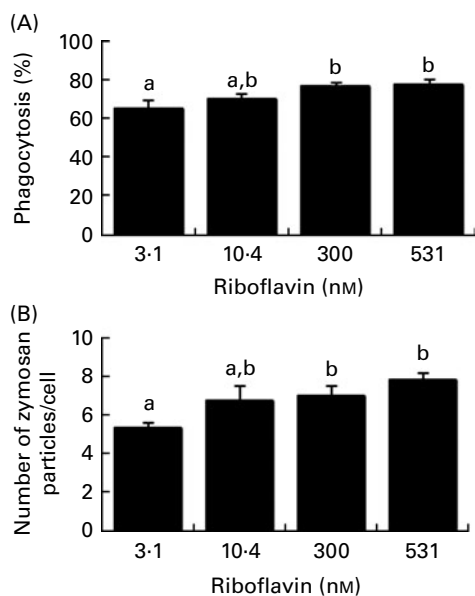


Fig. 5. Phagocytosis of zymosan particles by RAW 264.7 cells after 4 d of culture in a medium with various riboflavin concentrations. (A) Percentages of phagocytosing cells. (B) Number of zymosan particles phagocytosed per cell. Values are means with their standard errors (n 4–6). ^{a,b}Mean values with unlike letters were significantly different according to ANOVA (P <0.05).

mononuclear blood cell proliferation. On the other hand, riboflavin deficiency is associated with an accumulation of unfolded proteins and causes protein carbonylation and DNA damage⁽¹⁹⁾. It is not unreasonable to propose that this damage also takes place in RAW 264.7 cells and triggers apoptosis and cell cycle arrest.

The elevated level of the heat shock protein 72 protein in resting RAW 264.7 cells cultured in a low riboflavin medium (riboflavin; 3.1 nm) suggested that riboflavin deprivation is a stressing factor for these cells. Moreover, since the macrophages' ability to function appropriately is dependent on their adherence, a riboflavin deficiency may inhibit the immune response by interfering with the adherence capability of these leucocytes, as was demonstrated herein. However, the mechanism by which riboflavin supply influences cell adherence remains unexplained. On the one hand, this cell detachment may be connected with an undergoing apoptotic

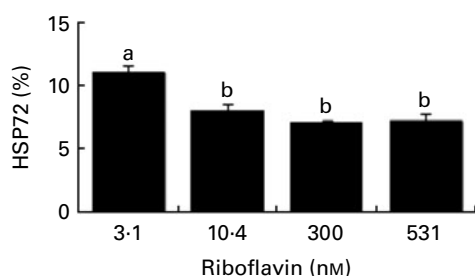


Fig. 6. Heat shock protein 72 (HSP72) activation in RAW 264.7 cells after 4 d of culture in a medium with various riboflavin concentrations. Values are means with their standard errors (n 4). ^{a,b}Mean values with unlike letters were significantly different according to ANOVA (P <0.05).

process, e.g. apoptotic neutrophils have been proved to develop a reduced capacity for adhesion due to modified integrin expression and activation⁽²⁴⁾.

The loss of adherence might be a result of inhibited flavoprotein formation in a riboflavin deficiency. It has been shown that the expression of certain cell adhesion molecules (vascular cell adhesion molecule 1 and intercellular adhesion molecule 1) depends on flavoproteins, such as NADH/NADPH oxidase⁽²⁵⁾. The proper level of these flavoproteins may be unsettled in a stage of riboflavin deprivation. Last but not least, the oxidative folding of integrins might be impaired in riboflavin-deficient macrophages. A common factor of all integrins is the high content of cysteine (e.g. one integrin potentially forms twenty-one disulphide bonds)⁽²⁶⁾ and therefore they undergo oxidative folding (the formation of sulphide bonds) in the endoplasmic reticulum. FAD is a coenzyme for endoplasmic reticulum oxidoreductin 1 and sulfhydryl oxidase, both of which mediate oxidative folding. When riboflavin deficiency depletes cellular FAD, the activity of both endoplasmic reticulum oxidoreductin 1 and sulfhydryl oxidase decreases and impairs proper protein folding. This is what happened to the HepG2 cells cultured in riboflavin-depleted medium, and it even triggered an unfolded protein response^(8,9).

Macrophages as professional phagocytes play a crucial role in pathogen elimination. The present results indicate that this ability may be significantly limited in macrophages with riboflavin deficiency. These observations confirmed earlier findings gathered on neutrophil populations⁽¹⁵⁾. Furthermore, it is well known that macrophages ingesting certain particles, such as bacteria, exhibit respiratory burst and produce reactive oxygen species, which give them the ability to destroy the ingested cells. The crucial enzyme for reactive oxygen species production, i.e. the NADPH oxidase enzyme complex, depends on FAD, which is one of the NADPH oxidase enzyme components⁽²⁷⁾. The lack of a respiratory burst recorded during the present study in riboflavin-deficient macrophages (3.1 nm) could be caused by the reduction of NADPH oxidase enzyme activity as a result of a low FAD content in these cells. The findings of the present study suggest that riboflavin-deficient macrophages may exhibit an impaired ability of phagocytosis and killing of ingested particles. The present results suggest that riboflavin deprivation can reduce the viability of macrophages and their capability to properly function during the immune response.

Contrary to Jurkat cells (T lymphocyte cells), which are rather resistant to moderate riboflavin depletion⁽⁸⁾, monocyte/macrophage RAW 264.7 cells are sensitive to riboflavin deficiency.

In conclusion, the results of the present study provide evidence that RAW 264.7 macrophages are sensitive to riboflavin concentration in culture medium and show distinct signs of riboflavin deficiency already within 4 d. The culture of RAW 264.7 cells in a medium with a low riboflavin content (3.1 nm) leads to suppressed cell proliferation and enhanced apoptotic death connected with the release of LDH by the dying cells, impairs cell adherence to the substratum but hardly affects their phagocytic activity and inhibits a phorbol

myristate acetate-induced respiratory burst, which is enhanced in high riboflavin concentrations (300 and 531 nM). Collectively, the findings of the present study provide insight into how riboflavin depletion may affect the immune system and may consequently decrease proper host immune defence.

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