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ORIGINAL STUDY

Are Lactobacillus Bulgaricus and Bacillus Calmette-Guérin vaccine suitable for patient protection against SARS-CoV-2 infection?

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ABSTRACT

Before COVID-19 infection caused the global pandemic in 2020, coronavirus diseases were mainly of veterinary interest. This pandemic necessitated the development of protective and therapeutic measures against the spread of SARS-CoV-2. Foods containing representatives of the genus Lactobacillus are an integral part of the daily menu of the Bulgarian people. Our hypothesis is based on studies examining its potential for competitive inhibition of viruses and bacteria by attachment to the surface of enterocytes.

Bacillus Calmette-Guérin (BCG) is an integral part of the vaccination calendar in the Republic of Bulgaria. In the literature, many clinical studies show that the administration of BCG vaccine limits the SARS-CoV-2 antigens and, consequently, is able to induce protection for COVID-19, by activating the specific, innate immune system.

The lack of definitively approved treatment necessitates finding ways to limit the spread of COVID-19 until final drug approval. We believe that the use of dietary components in the context of competitive inhibition and the vaccination schedule for protection in coronavirus-related diseases is applicable. We hypothesize that Lactobacillus and BCG may play a protective effect against SARS-CoV-2 infection alone or in combination in healthy individuals.

KEYWORDS: Lactobacillus, BCG vaccine, emerging coronavirus, SARS-CoV-2, viral entry.

INTRODUCTION

Coronavirus is a single stranded RNA virus, which was first identified in 1937. Coronaviruses (CoVs) are members of the Coronaviridae family (subfamily Coronavirinae, in Nidovirales order) described in the mid-1960s. They are divided into four different subfamilies: Alpha-coronavirus, Beta-coronavirus (lineages A–D), Gamma-coronavirus and Delta-coronavirus[1-3].

We will first review the mechanisms of capture and penetration into the target cell of coronaviruses. The Coronaviridae family includes a group of large enveloped non-segmented viruses with a positive sense RNA genome of up to 32 kilobase (kb) and share common morphological characteristics[4-6]. The virion envelope contains in its sequence the following viral proteins: the spike protein (S), envelop protein (E), membrane or matrix protein (M), accessory proteins (orf 3, 6, 7a, 7b, 8 and 9b), nucleoprotein (N), and 3' untranslated region (3' UTR)[7]. On the surface of the virion, spike proteins group into trimers and form the distinctive "corona". These S proteins present a receptor-binding domain (RBD) which contains receptor binding motifs (RBM), responsible for the specific binding to host angiotensin-converting enzyme 2 (ACE2) receptors[8]. The comparison of the genome of SARS-CoV-2 (severe acute respiratory syndrome coronavirus) with that of severe acute respiratory syndrome (SARS) reveals that the sequence encoding the spike protein has 27 amino acid substitutions - six located within the region of the receptor-binding domain (RBD) and another

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six substitutions in the underpinning subdomain (SD)[1]. Phylogenetic analyses have shown 88% similarity between SARS-CoV-2 and two SARS-like Coronaviruses, which originate from bat (bat-SL-CoVZC45 and bat-SL-CoVZXC21). Additionally, SARS-CoV-2 has 79% genetical similarity with SARS-CoV and nearly 50% with MERS-CoV (Middle East respiratory syndrome coronavirus)[2,4,9].

Angiotensin-converting enzyme 2 (ACE2) is designated as the receptor providing SARS-CoV-2 viral entry and its expression on type II pneumocytes and enterocytes (considered major viral target cells) has been established. An alternative receptor for SARS-CoV has also been reported. This is the lectin-specific intercellular adhesion molecule-3 capturing non-integrin (L-SIGN), which is expressed on the endothelial cells of the liver and lung. Some coronaviruses contain a hemagglutinin esterase (HE) and use sialic acid binding activity. Beta-coronaviruses have the ability to bind carbohydrates. In lineage A beta-coronaviruses (A-βCoVs), a group of human clinical and veterinary relevance, binding to O-acetylated Sias (O-Ac-Sias), is mediated by the hemagglutinin esterase (HE), a homodimeric type I envelope glycoprotein[10-13].

Until 2020, six types of coronaviruses were known to infect humans, including HCoV-229E, HCoV-NL63, HCoV-OC43, HCoV-HKU1, SARS-CoV and MERS-CoV, all of them with zoonotic origin. Their course varies from the common cold to more severe and often fatal respiratory disease in humans[1]. SARS-CoV-2 is the seventh human coronavirus identified. This virus, responsible for coronavirus disease COVID-19, emerged from the city of Wuhan, Hubei province, China, starting from December 2019. It had a significant impact on international social and economic activities, its case fatality rate (CFR) being higher than seasonal influenza.

The first four (HCoV-OC43, HCoV-HKU1, HCoV-229E and HCoV-NL63) present different degree of amino acid homology with SARS-CoV-2 and recent studies identified the presence of SARS-CoV-2 cross-reactive CD4 T cells, with high specificity for spike[14]. Whilst SARS-CoV, MERS-CoV and SARS-CoV-2 are associated with increased mortality, other coronaviruses are found to determine only mild forms of upper respiratory tract diseases[15]. Recent studies show that the risk of spreading zoonotic viruses has always remained high. They have high mutagenic levels and, in addition to creating new strains, this allows them to adapt to a wide range of hosts[16].

A North American study proposes a hypothetical conceptual model demonstrating the possible spread of SARS-CoV-2 from humans to animals through the gastrointestinal tract, where the faeces of COVID-19 infected patients contaminate water pools and reach wildlife hosts[17]. This study led us to accept the possible hypothesis for infecting humans through the gastrointestinal tract by animal species.

The known symptoms of COVID-19 infection are weakness, fatigue, fever, headache, gastrointestinal complains with diarrhea, neurological diseases, and respiratory symptoms such as coughing, dyspnea, breathlessness, which may or may not progress to acute respiratory distress syndrome (ARDS), pneumonia and/or pulmonary vasculitis, multiorgan failure or even death in severe cases[18,19]. In patients with coronavirus infection, a predominance of metabolic illnesses is observed, such as obesity, cardiovascular disease, hypertension and diabetes, as well as chronic respiratory disease and cancer. The virus enters its host cell through the ACE2 receptor. SARS-CoV-2 nucleic acids can be detected from samples[20] such as bronchoalveolar lavage fluid, sputum, nasal or pharyngeal swabs, fiber biopsy specimen with a fiber bronchoscope, faeces, blood and urine, each of them with different levels of diagnostic efficiency. The pathogen has been found to be transmitted from person to person through airway secretions, air droplets or direct contact with the host.

Coronavirus infection has a zoonotic origin. In order to facilitate the understanding of the mechanism of infection in humans, we consider it appropriate to first focus in detail on that in animals. Research on wild and domestic animals is much more numerous, they are at a much more advanced stage and the experience gained in veterinary medicine can be invaluable for us to understand the mechanism of action of the virus in humans and help us in treatment development. To substantiate our hypothesis of possible transmission of the infection through the gastrointestinal tract, we will first consider in detail the mechanism of coronavirus infection in animals and humans.

MECHANISM OF INFECTION IN ANIMALS

Five porcine coronaviruses have been identified, belonging to three of the four genera. Porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV) and the natural TGEV deletion mutant porcine respiratory virus (PRCoV) belong to the Alpha-coronavirus genus. PEDV and TGEV replicate importantly, almost exclusively within the epithelial cells of the small intestine, producing villus atrophy, with subsequent malab-

sorption and severe diarrhea. PRCoV infects especially the epithelial cells of the respiratory tract and alveolar macrophages, resulting a mild or often subclinical respiratory disease[21].

The porcine hemagglutinating encephalomyelitis virus (PHEV) is part of the Beta-coronavirus genus, has tropism for respiratory and neuronal tissues, being able to induce vomiting, wasting disease and neurological disorders in seronegative piglets.

The recently identified porcine deltacoronavirus (PDCoV) of the Delta-coronavirus genus targets the intestine, causing mild to moderate disease in young piglets[22].

Feline coronaviruses (FcoV) belong to the Alpha-coronavirus genus. Feline enteric coronavirus (FECV) gives asymptomatic to mild enteric tract infection and may induce viral persistence in the host (chronic infection). Feline infectious peritonitis virus (FIPV) is yet another example of a coronavirus permissive to target immune cells (monocytes and macrophages), in order to achieve systemic dissemination. A striking characteristic of FIPV is its ability to replicate within the macrophages and monocytes[23]. It is supposed that this switch in cells tropism, from gut epithelium to motile cells (monocytes/macrophages) is a tipping point in the development of the pathogenesis of FIP. FIPV causes feline infectious peritonitis (FIP), a fatal immune-mediated disease[24,25]. In the persistently infected host, mutations may cause the virulent FIPV. All these sustain the hypothesis that mutations in the spike gene have a key role in the transition of tropism from gut epithelial cells to macrophages[26,27].

Another representative of Coronaviridae family, mouse hepatitis virus (MHV) infects mainly the brain and liver. Different patterns of disease are associated with various strains of MHV[28].

Infectious bronchitis virus (IBV) is a frequent fowl pathogen that replicates in the respiratory tract and in the epithelial kidney cells, the oviduct and the gut.

MECHANISM OF HUMAN INFECTION

The ability of a single virus to cause pathologic different expressions in humans is commonly observed. This is due to multiple contributory factors, including the size of the viral inoculum, genetic system of patients and the presence of comorbidities. An adaptive immunity towards the closely related viruses or other microbes can reduce susceptibility or enhance disease severity. The viruses trigger antibodies and T-cell responses in infected patients[14]. The antibody levels decrease faster than T cells. It was observed that SARS-CoV specific antibodies fall below the detection limit within 2-3 years, whereas SARS-CoV specific memory T cells can be detected even 11 years after SARS infection[14,29]. These findings allow us to suggest that a similar relationship may be observed with SARS-CoV-2 due to the great similarity of SARS-CoV and SARS-CoV-2 (79% similarity). The data in the literature is still insufficient about the pre-existence of T cells in humans, that are able to recognize SARS-CoV-2 virus. Le Bert et al. studied T-cell reactions to structural (nucleocapsid protein, NP) and non-structural (NSP-7 and NSP-13 of ORF1) regions of SARS-CoV-2 in humans cured from COVID-19. The authors oversee in the observed group the presence of CD4 and CD8 T cells recognizing multiple regions of the nucleocapsid protein and detected SARS-CoV-2 specific T cells in individuals without history of SARS or with direct contact with SARS. Le Bert et al. come to the conclusion that "the infection with betacoronaviruses induces multi-specific and long-lasting T cell immunity against the structural N protein"[14].

Viral entry is based on a fine interplay between the virus and the host cell. Enveloped viruses can entry directly at the cell surface after binding to a receptor or enter in the endosomal compartment through endocytosis. Infection is initiated by the interaction between specific proteins on the cell surface with the viral particles. After initial binding on the receptor, the virus needs to fuse its envelope with host cell membranes in order to deliver its nucleocapsid to the host cytosol. Spike glycoprotein (S) mediates the cellular receptor binding and the fusion to the membrane. During the fusion process, conformational changes of the S protein take place, which can be initiated by receptor binding or by additional trigger factors, such as pH acidification or proteolytic activation. The result of these processes is the delivery of SARS-CoV-2 proteins and genomic information into the host cells cytoplasm, the place of SARS-CoV-2 replication. Unlike other coronaviruses, SARS-CoV-2 does not use aminopeptidase N (APN) and dipeptidyl peptidase 4 (DPP4) as a receptor[13], but a novel metallocarboxyl peptidase angiotensin receptor 2 to penetrate into human cells[30-32]. ACE2 has been identified to be the SARS-CoV-2 receptor for viral entry and is established that it is expressed on type II pneumocytes and enterocytes[6,33-35]. SARS spike protein (SARS-S) is classified as a large type I transmembrane protein (class I fusion protein). For activation of SARS-S, host cells cathepsins L and G (proteases) are essential, being responsible for SARS-CoV-2 infectivity[36]. In order to transit in active state, viral glycoproteins, termed class I fusion

proteins, depend on cleavage by host cell proteases[37]. S protein is the most immunodominant antigen, having maximum on B and T-cells epitopes[38]. Receptor engagement and membrane fusion are accomplished by two separate subunits of SARS-S: N-terminal surface (NTD) unit S1 as receptor binding and C-terminal (CTD) transmembrane unit S2 responsible for membrane fusion. S1 receptor-binding domain (RBD) binds directly to the peptidase domain (PD) of ACE 2 in order to access the host cells[34,39,40], this interaction representing the pivotal determinant for SARS-CoV-2 to infect a host species. S2 does not interact directly with the receptor, at its level being expressed functional elements required for membrane fusion of the virion. Binding of SARS-CoV-2 to ACE2 is thought to induce receptor-mediated endocytosis and virion transport in the endosomes of host cells[13,41]. Despite the great similarity of RBD in SARS-CoV, SARS-CoV-2 presents a high affinity to bind to ACE2 more effectively[42]. ACE receptors are expressed in almost all human tissues, whilst ACE2 is present predominantly in alveolar epithelial cells and capillary endothelial cells. ACE2 is highly expressed in highly vascular organs, such as lungs and kidneys the brain and gut. ACE and ACE2 exhibit 42% amino acid homology[30,43] but have different physiological functions. It is not impossible this homology to cause ACE to act as an alternative receptor in certain patients (patients' genetic background). The activity of the ACE receptor regulates the renin-angiotensin-aldosterone system (RAAS). The physiologic effects of RAAS activation are an increase in total body sodium and water and an increase in the vascular tone.

ACE2 counterbalances the deleterious effect of the ACE/RAAS pathway through its downstream ACE2/Angiotensin-(1-7)/MAS axis. ACE is expressed primarily in the vascular endothelium of the kidneys and lungs, but also on the epithelium of the lungs and the upper respiratory tract. The most important role of RAAS is associated with the pathogenesis of metabolic inflammatory diseases. The attachment of angiotensin II (Ang II) to type I and II AT receptors (angiotensin receptors) causes vasoconstriction, intravascular thrombosis, and increased blood volume. Elevated Ang II activates the coagulation cascade (thrombin and platelets), causing hypertension and increased thrombosis in the arterioles, activates neutrophils and macrophages flux to affected tissues and inhibits the production of nitric oxide (NO). Ang II is a mediator in cytokines activation (such as IL-6, TNF-α), to determine oxidative injury by reactive oxygen species (ROS) production, endothelial injury by inhibiting NO synthesis and vasoconstriction. The conversion of Ang I to Ang II is produced under the influence of various proteases, such as cathepsin G, tonin, trypsin, kallikrein, neutral endopeptidase and chymase[44]. ACE2 is a monocarboxypeptidase, which cleaves Ang I into a nonapeptide, Ang 1-9, and Ang II into a heptapeptide, Ang 1-7. Both peptides have vasodilatory, antiproliferative and protective functions by activating the MAS/G receptor (G-protein complete receptor MAS)[45]. ACE2 protects the lungs from damage by degrading Ang II and by producing the peptide Ang 1-7, and binding to the MAS receptor inhibits the actions of Ang II.

The coronavirus is multifaceted and can attack many tissues and organs. The crucial role of the spike protein in cell tropism has been investigated and demonstrated with chimeric viruses. Coronaviruses are able to adhere to many cell surface molecules in order to gain access in target cells. Calcium-dependent C-type lectins of the host play an important role in infections from SARS-CoV, IBV and FcoV. Lectin-specific intercellular adhesion molecule-3-grabing non-integrin (L-SIGN), present on the endothelial cells of the lungs and the liver, may represent an option for SARS-CoV receptor. Dendritic cell-specific intercellular adhesion molecule-3-grabing non-integrin (DC-SIGN) is a C-type lectin expressed on macrophages and dendritic cells. In trans transmission of SARS-CoV by dendritic cells to susceptible target cells has been documented, the spike protein being heavily glycosylated[46-48].

OUR HYPOTHESIS

Until the 21st century, coronavirus diseases were mainly of veterinary interest. Coronaviruses infect a broad range of mammalian and avian hosts and may affect multiple organs, producing respiratory, enteric, hepatic and/or neurological diseases, with a predominance of mild intestinal and respiratory diseases in animals and humans[32]. Based on studies performed on animals and our observations, we consider it appropriate to subdivide the coronavirus infection, according to the predominant symptoms, into mainly pulmonary, gastrointestinal and neurological forms. Many researchers postulated the possibility of interspecies viral transmission[49-51]. Coronaviruses are found in wild, farm and domestic animals as well as in humans. A literature review published in 2020 suggested the zoonotic links (bat-human) and spillover events as possible initial origin of SARS-CoV-2/COVID-19[50]. Based on genomic sequences, it has been established that all known human CoVs are derived from animal

sources[52]. SARS-CoV-2 is of animal origin (zoonosis), with further human-to-human transmission. It is transmitted from person to person by airborne droplets, airway secretions and even direct contact with the host. The possibility of transmission through the gastrointestinal tract cannot be ruled out without further investigation.

In our study, we consider the possibility of using components of our daily diet and the vaccination calendar of the Republic of Bulgaria to provide protection for the majority of the population until the availability of a treatment for COVID-19 or worldwide vaccination. The mechanism of airborne transmission of SARS-CoV-2 is very well studied, but, considering the zoonotic origin of this type of virus, we believe that the possibility of transmitting the infection through other mechanisms cannot be ruled out. In our hypothetical model, we believe it is possible that the spread of SARS-CoV-2 from wild, farm and domestic species to humans can occur through the gastrointestinal tract. This is possible when patients with COVID-19 have consumed meat products that are not well cooked, fruits and vegetables contaminated with animal faeces. About 70-80 percent of our immune tissue is located in the digestive system (mucosa-associated lymphoid tissue), which also raises the issue of personal hygiene to limit the spread of infection. The air passes through the nasopharynx, which means that some of the contaminated secretions are swallowed. Our hypothesis is based on studies examining the potential for competitive inhibition of viruses and bacteria by entrapment to the surface of enterocytes.

Apoptosis is an innate host defence mechanism that disrupt viral or bacterial replication by eliminating infected cells[53,54].

The genus Lactobacillus is a common inhabitant of the intestinal tracts of humans and animals[55]. The ability of Lactobacillus to adhere to the epithelial surfaces is extremely important for maintaining persistent colonization in mammalian intestines and other tissues. The peptidoglycan layer of Lactobacillus cell wall is covered with a variety of substances – lipoteichoic acids, neutral and acidic polysaccharides, and surface protein. Many Lactobacillus species are reported to present surface layer (S-layers) proteins (SLAP). SLAP is a single protein, non-covalently bound, which forms the outermost cell envelope. This protein is composed of crystalline arrays of protein subunits. One of the major characteristics of Lactobacillus S-layer proteins is the high isoelectric point (pI), showing them as highly basic. SLAP was identified as being involved in a process of adhesion[56]. Lactobacillus acidophilus is well studied. It is one of the strains of the genus Lactobacillus found in the human gut and, due to their probiotic characteristics, are generally recognized as safe. In the gastro-intestinal tract (GIT), L. acidophilus regularly encounters many dendritic cells (DCs) antigens[20], which express DC-specific ICAM-3-grabbing nonintegrin protein (DC-SIGN), a cell-surface receptor expressed mainly in DCs, and recognize mannose and fructose glycans present on microbial and viral surfaces. It can be said that DCs play an important role in the adaptive innate immune response[57]. DC-SIGN has been shown to increase viral entry of different viruses, such as HIV type 1, hepatitis C, Ebola, Dengue and SARS[58]. In addition to the interaction with DC-SIGN, DCs also interact with the S-layer presented on the bacterial cell-envelope, causing the induction of a number of cytokines involved in cellular immune regulation[59,60]. Other members of the genus Lactobacillus, the properties of which have also been studied, are Lactobacillus paracasei subsp. paracasei M5-L and Lactobacillus casei Q8-L, isolated from koumiss. Their S-layer participates in the adherence to the human intestinal cells. Analysis of adhesion forces revealed that, if the initial adhesion forces were of 1.41 and 1.28 nN, after the selective extraction of surface S-layer protein (SLAP) the forces were of 0.70 and 0.48 nN (a secondary weakening of the adhesion process)[61].

Another member is Lactobacillus delbrueckii subsp. bulgaricus which, by means of its surface enzymes (cell surface proteinase PrtB), mediates Lactobacillus adhesion to mucin and human epithelial cells[62]. It is one of the lactic acid bacteria used in the industrial milk fermentation. Lactobacillus delbrueckii subsp. bulgaricus (until 1984 known as Lactobacillus bulgaricus) is one of the bacteria used to produce the yogurt Le “Kissélo mléko” de Bulgarie. The bacterium was first described in 1905 by the Bulgarian student Stamen Grigorov in a report presented at the Pasteur Institute, Paris. Thanks to Lactobacillus bulgaricus, yogurt is a natural probiotic that has a great beneficial effect on humans. One of the first probiotics in the world was developed by Prof. Nikola Alexandrov in the 80s on the basis of Lactobacillus bulgaricus. The yogurt (Kissélo mléko) is a widespread food among the Bulgarian population. Probiotics are live microorganisms contained in food, the ingestion of which in sufficient quantities playing an important role in the control of the intestinal microbiota and in the modulation of the human immune response. S-layers are thought to function as protective coatings, maintain cell shape and ion exchange, and participate in adhesion to biotic and abiotic surfaces. Many studies have shown that the S-surface

protein layer of some Lactobacillus species has antimicrobial and antiviral activity[63,64].

The viral life cycle consists of a viral attachment, entry and replication. Our hypothesis is based on the ability to protect patients against SARS-CoV-2 infection by inhibiting the viral life cycle using Lactobacillus surface S-layer protein and surface enzymes (cell surface proteinase PrtB). Virus-induced apoptosis is critical for SARS-CoV-2 pathogenesis and replication, and the antiapoptotic approach may be an appropriate strategy in developing targeted therapy to combat COVID-19. We hypothesize that, as with SARS-CoV infection, calcium-dependent (C-type) lectins play the role in SARS-CoV-2 infection as alternative pathway. To clarify our thesis, we will use studies conducted in mammals to explain the probable mechanism in humans. Xiaoyan Zhang et al. investigated the ability of Lactobacillus acidophilus S-layer protein to inhibit PEDV - induced apoptosis of Vero cells[11]. To demonstrate the effect of the S-layer protein on the binding of PEDV to Vero cells, they were pretreated with S-layer protein and then PEDV was added, allowing the viruses to bind to the surface of the cells, but not enter. The authors evaluated the antiviral efficacy by analysis of viral loads. The authors studied the activity of apoptotic factors caspase-3 and caspase-8 that are activated by extrinsic and intrinsic pathways and are responsible for morphological features of apoptosis[65]. The results revealed that in PEDV-infected Vero cells treated with S-layer, the apoptosis rate was decreased and cell damage reduced[66]. The virus may induce caspase-independent apoptosis through activation of the mitochondrial apoptosis-inducing factor[67]. The interesting thing that can be seen when considering the studies on the inhibitory effect of the S-surface layer is the different mechanism of inhibition in bacteria and viruses. For example, Lactobacillus acidophilus S-layer protein has significant antagonistic activity against Salmonella enterica serovar Typhimurium infection of Caco-2 cells[20,68]. The suggested antimicrobial mechanism of Lactobacillus acidophilus S-layer protein is represented by the competition for binding sites located on host epithelial cells surface and direct interaction between S-layer protein and Salmonella Typhimurium cell surface[69]. Unlike bacteria, Lactobacillus acidophilus S-layer protein can counteract the entry and replication, but not when attaching PEDV to Vero cells and only after pretreatment with S-layer protein. Antiviral activity of S-layer protein is not based on competition with PEDV for binding sites on the surface of host cells. The S-layer protein has been shown to bind to the lectin-specific C-type intercellular adhesion molecule-3, which grabs non-integrin (DC-SIGN, CD209)[70]. In practice, the S-layer protein of the genus Lactobacillus is also used in the inhibition of JUNV (Argentine Hemorrhagic Fever Virus) infection by direct interaction with the DC-SIGN receptor[62]. Inhibition of JUNV infection has been demonstrated when the Vero cells were treated with purified S-layer protein from L. acidophilus before infection.

Bacillus Calmette-Guérin (BCG) vaccine is an integral part of the vaccination calendar in the Republic of Bulgaria, being listed by World Health Organization as an essential vaccine. Mycobacterium bovis BCG is a live attenuated tuberculosis vaccine that protects us from disseminated tuberculosis. BCG exhibits heterogeneous protective effects against unrelated viral and bacterial infections, which have been extensively studied and summarized by Pramod Gupta[71]. The underlying mechanism of protective effects of BCG vaccine is not fully understood. It is thought to induce the expression of genes involved in the antiviral innate immune response against viral infections. Long-term maintenance of BCG-activated cellular immunity has been observed by inducing memory in innate immune cells such as monocytes (Mo), natural killer cells (NK) and macrophages (Ma). BCG vaccination gives broad protection against respiratory infections. A study performed by Wardhana et al. observed an important decrease in acute upper respiratory tract infection in elderly people after BCG vaccination, administered once a month, for three consecutive months[72]. A randomised controlled trial involving trivalent influenza vaccine reported enhanced induction of functional antibody response against AH1N1 strain in those cases with BCG vaccination prior to influenza vaccination[73].

Many studies have shown that recombinant BCG strains expressing SARS-CoV-2 antigens may offer protection against the virus, due to the activation of both the innate and the specific adaptive immune response. Epidemiological studies indicate that the Bacillus Calmette-Guérin (BCG) vaccine may have protective effects against coronavirus disease 2019, but there is no evidence of the mechanism by which BCG vaccination can cause T-cell response to SARS-CoV-2. The spread of COVID-19 varies from country to country. Some studies have shown a correlation between BCG vaccination and COVID-19 infection and fewer COVID-19 cases in the universally vaccinated countries populations. An epidemiological study by Miller et al. attributes the variations in morbidity and mortality from COVID-19 between different countries according

to the national BCG vaccination program[74]. Another meta-analysis suggests that BCG is associated with a reduction in COVID-19 infections if the scope of BCG vaccination is above 70% of the population in the country concerned[75].

We hypothesize that BCG may have a protective effect against SARS-CoV-2 infection, but it is unlikely that the BCG vaccine will be able to provide complete protection against SARS-CoV-2 infection, due to the lack of specific immunity. Recombinant BCG strains expressing SARS-CoV-2 antigens may be able to provide protection against COVID-19 due to the activation of both innate and specific adaptive immune responses. This might be useful in preventing coronavirus infection in the human population.

Several mechanisms of action of the BCG vaccine are considered as possible for its antiviral immunomodulatory effect[76]: a) enhancement of the body's immune response to unrelated viral and bacterial infections, determining increased production of interferon gamma (IFNγ) by CD^+4 cells and induction of a heterologous lymphocyte response[77,78]; b) modulation of cytotoxic T-lymphocytes subsets, such Th1, Th17 and Th22 responses, via CD^+4 and CD^+8 cells activation[79,80]; c) trained/nonspecific immunity: on any secondary infection with the same viral type, an effective heterologous response is initiated due to the immunological memory in the natural killer (NK) cells, myeloid cells (monocytes and macrophages) that form part of innate immunity and increased proinflammatory cytokine-mediated responses[81]; d) increase in proinflammatory cytokines such as IL-1β and tumor necrosis factor-α (TNF-α) may lead to better outcomes with viral infection[82].

Based on our observations in the treatment of recurrent respiratory papillomatosis of the larynx using BCG (Calgevax) according to the scheme and studies of Mohd Israr et al.[83], we suggest that BCG vaccination may limit the spread of SARS-CoV-2. Like the above-mentioned authors, during the long-term research for treatment of recurrent respiratory papillomatosis or at least a real dilution of recurrences, we were impressed that it occurs in a very small part of the population in Bulgaria. This led us to proceed to immunological tests of patients in order to look for changes in the immune response. We found that it was a chronic viral infection and that the effectiveness of the antiviral response depended on the cytokine response. Administration of BCG increases the effectiveness of the antiviral T-cell response by restoring the Th1 / Th2 / Th17 cytokine balance and inducing Treg differentiation. The latter prevent the depletion of effector clones in conditions of chronic infection and promote the development of a protective immune response of T cells. It should not be ruled out that patients with COVID-19 may also have genetically determined changes in innate and adaptive immunity or even a primary immunodeficiency disease that determines the susceptibility of part of the human population to this disease.

In an absence of definitive and approved treatment, the protection and prevention are utmost important.

CONCLUSIONS

Many drugs, alone or in combination, have been tried to treat COVID-19 disease, but none have been approved yet. The use of foods containing Lactobacillus bulgaricus may protect patients from SARS-CoV-2 infection, as its surface proteins and enzymes in the S-layer can inhibit the life cycle of the virus, preventing its entry and replication. In this case, the antiviral activity of the S-layer protein is not based on competition with the viruses for the binding sites on the surface of host cells. This is due to its binding to DC-SIGN, which is an adhesive factor on the cell surface, that facilitates the entry of viruses. We hypothesize that BCG alone is unlikely to provide us with complete protection against SARS-CoV-2 due to a lack of specific immunity, but may play a significant role in reducing coronavirus infection in the human population. Its action is applicable to the majority of the human population. In the presence of genetically determined changes in congenital and acquired immunity or even primary immunodeficiency disease, the susceptibility of this part of the human population to COVID-19 is probably different. In permanently infected hosts (chronic viral infection) with genetically determined changes in innate and acquired immunity, mutations can lead to the virus becoming a virulent strain or cause variations in its virulence. The modification of SARS-S can alter the tropism of cells and tissues and, when combined with other viral factors, can alter the pathogenicity of the virus. It is possible that SARS-CoV-2 infection in patients with genetic changes occurs as in animals, causing the targeting of immune cells (monocytes and macrophages) to achieve systemic spread. Many studies are pending to determine whether SARS-CoV-2 has the potential to cause fatal immune-mediated disease in some patients with genetic changes, like some coronavirus infections in animals.

Lactobacillus bulgaricus and bacillus Calmette-Guérin may reduce the possibility of transmission

by competitively inhibiting and activating the immune response. In the absence of definitively approved treatment and vaccination for all, protection and prevention are paramount. The use of components of the diet and vaccination calendar for protection against coronavirus-related diseases is fully applicable. We hypothesize that Lactobacillus and BCG may have a protective effect against SARS-CoV-2 infection alone or in combination in healthy individuals.

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