

Cell wall deficient forms of mycobacteria: a review

V. BERAN¹, M. HAVELKOVA², J. KAUSTOVA³, L. DVORSKA¹, I. PAVLIK¹

¹Veterinary Research Institute, Brno, Czech Republic

²National Institute of Public Health, Prague, Czech Republic

³Regional Institute of Public Health, Ostrava, Czech Republic

ABSTRACT: Cell wall deficient (CWD)-forms or L-forms of bacteria are characterized by a complete or partial loss of cell wall components and by the change of cellular shape. CWD-forms (spheroplasts) of bacteria are commonly prepared *in vitro* and are used for various practical applications. However, very little is known about the conditions required for the formation of CWD-forms of bacteria and mycobacteria *in vivo* and their significance in aetiology of various chronic diseases of humans (tuberculosis, sarcoidosis, and Crohn's disease) and animals (paratuberculosis). It is quite difficult to detect CWD-forms of mycobacteria in biological material and the possibilities of their detection by microscopy, culture, DNA hybridization techniques and other methods are limited. This obviously leads to a relatively small amount of published data on the detection and the isolation of CWD-forms of mycobacteria both from human or veterinary biological material, which can lead to the conclusion of a non-infectious origin of the diseases. The present review also includes studies performed by authors from the former Soviet Union and likely represents the first complete summarization of their knowledge in this sphere. However, certain results may be viewed as somewhat controversial. Accordingly, more attention should be paid to the research of cell wall deficient forms, not only in association with chronic and latent mycobacterial infections.

Keywords: protoplasts; antituberculars; reversion; intestine; respiratory tract; basilar tuberculosis; skin tuberculosis; lymph node; Johne's disease, *Mycobacterium tuberculosis* complex; *Mycobacterium avium* complex; zoonoses; food safety

List of abbreviations: AFR = acid-fast rods; AG = arabinogalactan; AT = antituberculars; BCG = Bacillus Calmette-Guerin; CFU = colony forming units; CWD-forms = cell wall deficient forms; DNA = deoxyribonucleic acid; E = ethambutol; ELISA = enzyme-linked immunosorbent assay; EM = electron microscopy; GPL = glycopeptidolipids; H = isoniazid; HEYM = Herrold's egg yolk medium; HPC = hexadecylpyridinium chloride (N-cetylpyridinium chloride monohydrate); LAM = lipoarabinomannan; LOS = extractable glycolipids; M. = *Mycobacterium*; MIC = minimum inhibition concentration; M7H9 = Middlebrook 7H9; PAS = *para*-amino-salicylic acid; PCR = polymerase chain reaction; PGL = phenolic glycolipids; pv. = pseudovar; R = rifampicin; RFLP = restriction fragment length polymorphism; SDS PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis; S = streptomycin; TSB = tryptic soy broth; Z = pyrazinamide; ZN = Ziehl-Neelsen

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1. Introduction

Cell wall deficient forms (CWD-forms) of bacteria were described for the first time in 1883 for *Mycobacterium tuberculosis* (Malassez and Vignal, 1883 as quoted by Imaeda, 1984). It was revealed that animals had been infected with these typical "coccoid bodies" surrounded with a viscous matter. The reversion to rods is typical for the bacillary form of tuberculosis that has occurred in animal organisms. Protoplasts or spheroplasts capable of growth and propagation in a medium with appropriate osmotic qualities have been designated as CWD-forms, or L-forms of bacteria (Kaprlek, 2000).

The term "L-forms" of bacteria was used for the first time by Klieneberger in 1935 (Klieneberger, 1935 as quoted by Domingue and Woody, 1997). This designation ("L" before the word "forms") was used according to the Lister Institute in London where the above mentioned investigator worked.

The etymology of this designation of the "L-forms" is not widely known and it is possible to encounter the opinion that the term has been derived from the roll- or L-shape cells. L-forms of bacteria are characterized by a complete or partial loss of cell wall components and accordingly, the term "CWD-forms" started to be used in recent literature; that is more general and gives a true picture of them (Domingue and Woody, 1997; Markova et al., 1997; Mattman, 2001; Huber, 2002; Hines and Styer, 2003). We will therefore use this latter term in the text below.

At the beginning of 20th century, a number of phenotypically different forms of *M. tuberculosis* had been described in literature and some of them stained negatively according to Ziehl-Neelsen (ZN) (Much, 1907; Seiler et al., 2003). Microscopy was primarily involved in the initial research of shape and structure of CWD-forms of bacteria (Much, 1907; Csillag, 1964). Studies dealing with culture

and other detection methods of CWD-forms appeared later (Belsheim et al. 1983; Graham et al. 1987; El-Zaatari et al., 1996). Recently, the interest in CWD-forms of bacteria has increased, particularly in medicine. The reason is likely to be the significant role of CWD-forms, i.e. bacteria in a dormant stage, during pathogenesis of some chronic diseases of both humans and animals (Wayne, 1994; Domingue and Woody, 1997; Ulrichs and Kaufmann, 2002).

The purpose of the present study is to review the essential literature on the structure and origin, detection methods and occurrence of CWD-forms from an aspect of etiopathogenesis, above all those of consequential mycobacterial species. Firstly, basic classification of CWD-forms of bacteria and their consequences in medicine, agriculture and industry are described briefly.

2. Basic classification of CWD-forms of bacteria

According to their ability to spontaneously revert to cells with a standard cell wall, CWD-forms have been classified as follows: (i) stable (spontaneously non-reverting) and (ii) non-stable (spontaneously reverting). According to the degree of loss of cell wall components, CWD-forms have been described as two basic types (Kapralek, 2000; Mattman, 2001):

1. protoplasts and
2. spheroplasts.

2.1. Protoplasts

Protoplasts have completely lost the cell wall and therefore, they are lined only with the surface membrane that separates them from external environment. Protoplasts of bacteria can be confused with mycoplasmas as they also have no cell wall (Krieg and Holt, 1984). From the reason of complete absence of all constituents of the cell wall, bacterial or yeast protoplasts cannot regenerate to the cells with a normally developed wall and subsequently divide (Mattman, 2001).

2.2. Spheroplasts

The cell wall is usually partly maintained in spheroplasts. Their surface is considerably disturbed

and therefore allows increased penetration of substances into the cells in comparison with cells with a normal cell wall (Cover et al., 1991). Spheroplasts may divide and they are more likely to form and survive under both *in vivo* and *in vitro* conditions than protoplasts (Murty and Venkitasubramanian, 1983).

3. Importance of CWD-forms of bacteria

Recently, CWD-forms of bacteria have attracted the attention of medical research, above all in relation to chronic and persistent infections. Nowadays, CWD-forms are usually viewed as a product of interaction between an infectious agent and the defence system of the host (Mattman, 2001). They can subsequently survive for a long time in a dormant stage inside a macroorganism. CWD-forms can induce manifestation of a disease after being activated by various stress factors (Domingue and Woody, 1997).

3.1. CWD-forms of bacteria in medicine

In the 1990s, experiments were conducted on animals that were infected with CWD-forms of bacteria. The results of these experiments indicated a general association between CWD-forms and chronic infection causing agents such as *Yersinia pseudotuberculosis* (Isachkova et al., 1993), coagulase-negative staphylococci (Goksu et al., 1996) and *Listeria monocytogenes* (Markova et al., 1997).

Aetiology of a number of diseases of humans is assumed to be related to CWD-forms of bacteria. A potential role of CWD-forms of *Helicobacter pylori* is considered in patients suffering from peptic ulcers (Wang et al., 2003). In concert with the *Bartonella* genus, CWD-forms of the *Proteus* genus may act as activating factors for HIV/AIDS disease (Sood et al., 1997). CWD-forms have been also described for spirochetes. A relationship between CWD-forms of bacteria (spirochetes) and development of certain autoimmune diseases have also been discussed (Koch, 2003). The disease is usually considered to be of non-infectious origin due to the fact that an infectious agent could not usually be isolated. However, a generally low likelihood to detect CWD-forms by culture supports this presumption.

3.2. CWD-forms of bacteria in agriculture and industry

CWD-forms of bacteria may be important to agriculture. CWD-forms of *Pseudomonas syringae* pv. *phaseolicola* that are pathogenic for plants may cause increased resistance of affected plants against fungus *Botrytis cinerea* (Hammerschmidt, 2003). Accordingly, they do not always adversely affect a host macroorganism.

The significance of protoplasts in biotechnology should also be mentioned. Genetic engineering takes the advantage of the loss of the natural surface barrier in protoplasts, resulting in an increased permeability, for vector transformation, fusion of protoplasts, cloning and production of proteins that are important for both the industry and therapy of various diseases. A certain success in the production of vaccines was achieved using protoplasts for the prevention of some fish diseases such as furunculosis caused by *Aeromonas salmonicida*. CWD-forms of certain bacteria may also stimulate the immune response of mammals by a controlled production of tumour necrotizing factor α (TNF- α) or by increasing the numbers of macrophages (Grichko and Glick, 1999).

4. CWD-forms of mycobacteria

As described in previous chapters, CWD-forms are characterized in that they have completely or partly lost the cell wall components; that is presented by altered cell shape, chemical composition and enzymatic activity. Cell wall composition of CWD-forms of mycobacteria and factors that may cause changes in the structure will be described for a better understanding of their origin.

4.1. Mycobacterial cell wall

The cell wall of mycobacteria is characterized by a unique structure which is caused by a partly distinct chemical composition in comparison with the cell wall of other bacteria. This variation causes a marked resistance of mycobacteria to the immune system of the host and antibiotics (Kolattukudy et al., 1997). Resistance to antimicrobial substances is usually ascribed to a very low permeability of mycobacterial cell wall (Brennan and Nikaido, 1995). Its low permeability is mainly

due to a high content of unique lipids that cause a high hydrophobicity.

4.1.1. Mycobacterial cell wall composition

The mycobacterial cell wall, in principle, contains the following components (Brennan and Nikaido, 1995):

1. Peptidoglycan (contains L-alanyl-D-isoglutaminyl-*meso*-diaminopimelyl-D-alanine)
2. Arabinogalactan (AG)
3. Mycolic acids
4. Lipoarabinomannan (LAM)
5. Extractable lipids including:
 - a. Extractable glycolipids (LOS)
 - b. Phenolic glycolipids (PGL)
 - c. Glycopeptidolipids (GPL)
 - d. Waxes, acylated trehaloses and sulfolipids
 - e. Glycerophospholipids
6. Proteins

4.1.2. Mycobacterial cell wall and plasma membrane structures

The studies of Rastogi (1991) and Brennan and Nikaido (1995) were focused on the architecture of mycobacterial cell wall in detail. The plasma membrane structure is the first layer of the cell envelope from the inner side of the cell evenly as in other bacteria and may also contain other substances such as carotenoids; which produce yellow to orange pigmentation in some non-tuberculous mycobacteria (*M. goodii*, *M. kansasii*, etc.). A layer of peptidoglycan with a chemically bound polysaccharide arabinogalactan (AG) is found above the plasma membrane. Outer terminuses of AG are esterified by taxonomically specific mycolic acids.

The other above mentioned components are linked with these constituents; many of them act as antigens or are toxic (e.g. cord factor in *M. tuberculosis*, which consists of trehalose 6,6'-dimycolate). Rastogi and Helliö (1990) observed mycobacterial cells phagocytized by macrophages. These were surrounded by capsules, most likely of lipid origin, which obviously protected them from lysosomal activity. As mentioned, the mycobacterial cell wall contains a number of antigens (glycolipids or proteins), that may be used in preliminary serologic diagnosis (Brennan, 1989). LAM is a dominant liposaccharidic antigen of *M. tuberculosis*. Phenolic

glycolipids (PGL) in *M. leprae* and *M. bovis*, glycopeptidolipids in *M. avium* complex or glycolipids (GPL) that contain acylated trehaloses in *M. kansasii*, *M. szulgai*, and *M. malmoense* species are also specific antigens. A source of protein antigens is primarily the peptidoglycan layer.

Mycobacterial biosynthesis of lipids has been well described in the study of Kolattukudy et al. (1997). The principle of targeted mutagenesis with the use of transposons, which put the gene out of the function, is used for the investigation of gene functions that might be responsible for the cell wall component synthesis. Provided a transposon binds to a site containing a gene or regulatory domain that plays a key role in the synthesis of a certain constituent of the cell wall, its inactivation occurs. It is represented by an altered phenotype, such as the shape of colonies (Laurent et al., 2003).

4.1.3. The effects of antituberculous, antibiotic and chemotherapeutic agents on mycobacteria and their cell wall

High resistance of mycobacteria to antituberculous and other antibacterial substances is associated mainly with the high complexity of their cell wall. So called “first-line antituberculous drugs” used in clinical practice for the treatment of human tuberculosis caused by *M. tuberculosis* are as follows (Zhang and Telenti, 2000): isoniazid (H), rifampicin (R), pyrazinamide (Z), ethambutol (E) and streptomycin (S). Therapy of *M. avium* infections may often be more complicated by the fact that its resistance is higher than that of *M. tuberculosis* (Bartos et al., 2004). An overview of *M. avium* complex members’ sensitivities to antituberculous (AT) is presented by authors Heifets (1996) and Dvorska et al. (2002). *In vitro* antimicrobial susceptibility of *Mycobacterium* spp. isolated from patients with Crohn’s disease was investigated by Chiodini et al. (1984c).

Some antituberculous drugs can cause changes in the structure of cell wall. The following antituberculous (AT) are effective on the mycobacterial cell wall: isoniazid, ethambutol, cycloserine, and probably also ethionamide (Zhang and Telenti, 2000). Isoniazid is characterized in a wide range of effects on the mycobacterial cells; the most important and best recognized is mycolic acids

synthesis inhibition. Takayama et al. (1973) studied the processes of cell destruction in *M. tuberculosis* exposed to isoniazid by scanning electron microscopy. The effect of isoniazid to the destruction *M. tuberculosis* cells is high; however, some atypical mycobacteria, especially *M. avium* complex members, are resistant to this drug (Bartos et al., 2004). Ethambutol inhibits polymerization of arabinogalactan and lipoarabinomannan. This chemotherapeutic agent also has a high minimum inhibition concentration (MIC) for *M. avium* complex (Heifets, 1996; Dvorska et al., 2002). Ethionamide, whose structure is similar to that of isoniazid, also likely inhibits mycolic acid synthesis (Zhang and Telenti, 2000). Cycloserine inhibits peptidoglycan synthesis by blocking the functions of the following two enzymes: D-alanine racemase and D-alanyl-D-alanine synthetase (Rastogi and David, 1981).

4.2. Characteristics of CWD-forms of mycobacteria

4.2.1. Morphology of CWD-forms of mycobacteria

Cell morphology is most usually analysed by transmission or scanning electron microscopy. CWD-forms of mycobacteria may be highly pleomorphic, although round forms are most common. Due to their small sizes and pleomorphy, CWD-forms can penetrate through standard bacteriological filters with a pore size of 220 nm (Sweany, 1961; Imaeda, 1984).

Special round virulent forms of *M. tuberculosis* non-stainable according to ZN were observed at the beginning of 20th century (Much, 1907). Round Gram positive mycobacterial shapes were later described and designated as “mycococcus form” (Csillag, 1964). Imaeda (1975) described large “amoeba-like cells” in aged cultures of *M. phlei*. Koch (2003) described giant round non-cellular structures in his study, so called “budding yeast-like structures” that contained clusters of CWD-forms of bacteria. “Elementary bodies and filament structures” have been described in association with CWD-forms of mycobacteria (Merkal et al., 1973; Konstantinova et al., 1982). Michailova et al. (2005) observed atypical granular CWD-forms of *M. tuberculosis* resistant to streptomycin and isoniazid. So called “membrane vesicles” have been prepared *in vitro* from the cells of *M. aurum*; those are round forms deprived of

the cell wall and cytoplasmic material (Rastogi and David, 1981).

Mycobacteria have long been considered to be associated with sarcoidosis. Distinct morphological subtypes of cells with different stainability have been found in the tissues from sarcoidosis patients (so called “pleomorphic chromogens”): yeast-like structure, multivariate pigmented elements, vacuolated forms (spheroplast stage) and round bodies detectable in granulomas (Alavi and Moscovic, 1996). Various morphological forms of *M. tuberculosis* were observed also during experimental infection of murine macrophages and some of them lacked the cell wall (Gerasimov et al., 2003).

4.2.2. Chemical composition and enzymatic activity of CWD-forms of mycobacteria

Altered cell qualities are concurrent to altered structures. Those are primarily quantitative changes in chemical composition or changes in enzymatic activity such as increased urea level and increased Tween hydrolysis in the *in vitro* produced spheroplasts of *M. aurum*, which is likely associated with increased penetration of substrates into the cells partly deprived of outer barrier (Rastogi and David, 1981). Hines and Styer (2003) also observed differences in the investigated phenotypic properties between CWD-forms and non-treated cells of *M. avium* subsp. *paratuberculosis* (*M. a. paratuberculosis*).

4.3. Formation of CWD-forms of mycobacteria

Nowadays, mycobacterial spheroplasts may be prepared *in vitro* using antibiotics or enzymes (Udou et al., 1983; Naser et al., 1993; Hines and Styer, 2003). However, formation of CWD-forms of mycobacteria *in vivo* is not known in detail and is often discussed.

4.3.1. Formation of CWD-forms of mycobacteria *in vivo*

As mentioned under item 3, CWD-forms may be products of the interaction between infecting microorganisms and the host immune system, so that

mycobacterial cells are not completely destroyed. These dormant forms of mycobacteria are able to persist for a long period in macroorganism and resist its immune response (Rastogi, 1990; Wayne, 1994; Domingue and Woody, 1997; Ulrichs and Kaufmann, 2002; Zhang, 2004). Reversion to acid-fast rods (AFR) may be associated with the disease reactivation.

The effects of antibiotics, enzymes and other substances. The most likely mechanism of CWD-forms formation seems to be the lytic activity of lysozyme within phagolysosomes after phagocytosis of mycobacteria by macrophages (Willett and Thacore, 1966; Gerasimov et al., 2003).

Pathogenic/wild type strains of *M. a. paratuberculosis* block phagosomal acidification and this leads to the replication pathway within bacteriophorous vacuoles (Cheville et al., 2001). *M. a. paratuberculosis* can also induce macrophage apoptosis. Studies carried out with other bacteria *in vitro* suggest that the formation of CWD-forms is caused by antibody-complement-lysozyme interaction (Mattman, 2001).

Cirillo et al. (1997) found that *M. avium* enters and replicates in the protozoan host *Acanthamoeba castellanii*. In addition, similar to that shown for mycobacteria within macrophages, *M. avium* inhibits lysosomal fusion and replicates in vacuoles that are tightly juxtaposed to the bacterial surfaces within amoebae. It follows that mycobacteria are able to survive inside macrophages or other host cells under certain circumstances. Accordingly, they may survive there in part or CWD-deficient forms.

CWD-forms may also appear after antibiotic therapy targeted to the mycobacterial cell-wall (Dorozhkova and Volk, 1973c; Imaeda, 1984; Wang and Chen, 2001). The effects of dihydrostreptomycin (Dorozhkova and Volk, 1972a), cycloserine (Dorozhkova and Volk, 1972b), ethambutol and ethionamide (Dorozhkova and Volk, 1973a) and rifampicin (Dorozhkova and Volk, 1973b; Karachunskii et al., 1986) have been studied. Gaiginschi et al. (1964) detected CWD-forms of mycobacteria after the treatment with trichloriodopyridine. Mycobacteria have not been eliminated from the host organism in any of these cases. The cell wall is only destroyed if suboptimal doses of antibiotics are administered or if the antimicrobial agent is not sufficiently active in the target site.

The genetic mechanism of the formation of CWD-forms of mycobacteria. Another hypothesis

for the formation of CWD-forms is that based on genetic mechanisms such as mutation. However, this hypothesis has not been confirmed yet, and no mutations in the investigated genes or differences in restriction profiles between CWD-forms and “normal” mycobacterial cells were found (Imaeda, 1984; Chiodini et al., 1986).

4.3.2. Preparation of CWD-forms of mycobacteria *in vitro*

The investigation of the properties of CWD-forms of mycobacteria and their formation in a host macroorganism cannot be done without model studies of CWD-forms of mycobacteria prepared under *in vitro* conditions. Their preparation also has a practical impact, particularly to the pharmaceutical industry and biotechnological procedures where the prepared spheroplasts or protoplasts are used for fusion and cloning (Grichko and Glick, 1999). A variety of protocols and procedures for the preparation of CWD-forms of mycobacteria have been published to date; the method using glycine and lysozyme is most common. These substances affect biosynthesis of peptidoglycan, which is one of the key components of cell wall of all bacteria (Hammes et al., 1973).

Rastogi and David (1981) used a combined treatment with D-cycloserine, L-glycine and lysozyme for the preparation of CWD-forms of *M. aurum*; lysozyme and other substances were used for final conversion to spheroplasts. The preparation of membrane vesicles is also described in the study; these can be obtained by subsequent lysis of spheroplasts by nucleases followed by osmotic shock. Udou et al. (1983) also prepared spheroplasts of *M. smegmatis* using glycine and lysozyme. Their method can be generally applied for the preparation of spheroplasts of fast growing mycobacteria; exact concentration of glycine is different for each species or strain.

Naser et al. (1993) also prepared spheroplasts of *M. smegmatis* using glycine and lysozyme; they used them for transformation of a mycobacteriophage DNA. Adamek et al. (1969) investigated the effect of glycine and lysozyme on the formation of *M. smegmatis* spheroplasts, targeting the comparison of the effect of each of these substances separately. Hines and Styer (2003) modified the method according to Naser et al. (1993) for preparation of CWD-forms of *M. a. paratuberculosis*. Tryptic soy

broth (TSB) medium was replaced by Middlebrook 7H9 (M7H9) medium and the incubation time increased with respect to a longer generation period of *M. a. paratuberculosis*.

4.4. Reversion of CWD-forms of mycobacteria to acid-fast rods

The reversion to AFR is possible due to the maintained ability of CWD-forms of mycobacteria to synthesize peptidoglycan and other macromolecular components of the cell wall (Murty and Venkitasubramanian, 1984). Udou et al. (1982) studied mechanisms of reversion of spheroplasts to bacillary forms (AFR) in detail, mainly from an aspect of morphology and physiology. This reversion is a process consisting of two morphologically distinct stages; the first is a budding of the stalks from the spheroplasts and the second is a formation of intracellular daughter cells in the stalks.

The second stage may be explained by either of the following two hypotheses:

1. Bacillary forms originate from elementary bodies, formed in cytoplasm of stalks and spheroplasts.
2. Bacillary forms originate from initial bodies as a result of fragmentation of stalks and spheroplasts.

Extrinsic factors that influence transformation of CWD-forms to AFR have not been well recognized yet. A certain role is played by the culture conditions. The frequency of spheroplasts regeneration may be affected by different concentrations of Mg^{2+} and Ca^{2+} ions in culture medium (Udou et al., 1983). The above mentioned authors found frequency of spheroplasts reversion to range between 0.1 and 30.0%. Graham et al. (1987) isolated CWD-forms of mycobacteria from tissues of patients suffering from Crohn's disease and documented frequency of their reversion in 33.3% of cases.

4.5. Storage and collection of CWD-forms of mycobacteria

Due to the fact that CWD-forms of mycobacteria are rather sensitive, their storage is difficult. Their outer cell barrier is disturbed and therefore non-favourable osmotic conditions are lethal for them. Accordingly, a considerable percentage of cells die and others then revert to AFR. CWD-forms can be

stored in a sucrose containing protoplast buffer or media containing various salts with osmotic activity (Udou et al., 1982; Hulten et al., 2000b). Takahashi et al. (1980) investigated survival CWD-forms of bacteria (staphylococci, streptococci and *Escherichia coli*) after freezing in special solutions with the aim to define the conditions of their storage. They recorded only a minor decline in CFU per ml counts at the temperature of -80°C after three years, in comparison with the storage at -20°C where the survival rate was impaired.

4.6. Methods of investigation of CWD-forms of mycobacteria

4.6.1. Microscopy

Samples of patients' tissues positive by culture or PCR for mycobacteria may be ZN-negative. This may be caused either by (i) a small number of mycobacteria present in tissues not detectable by microscopy or (ii) the mycobacteria are in a dormant stage (CWD-forms) and they are difficult to stain by this technique or do not stain at all (Greenstein, 2003; Seiler et al., 2003). CWD-forms and bacillary forms of bacteria can be differentiated by the Hucker Gram staining technique (Allan et al., 1992). Puzanov and Nikolaeva (1994) tested various staining techniques in different mycobacterial strains (*M. tuberculosis* strains Erdman TMC 107 and Academia, *M. bovis* strain Bovinus 8, *M. bovis* BCG strain Russian, *M. avium* strain Avium-53): ZN, Boy (fluorescent staining), Gram, and Giemsa-Romanovsky. Non-conventional Gram and Giemsa-Romanovsky techniques were able to identify CWD granular balls and non-acid-fast coccus-like forms.

Light and fluorescence microscopy. ZN bright-field and fluorescent microscopy may approximately define the cell shape, i.e. differentiate between rod bacillary forms and coccoid CWD-forms. Among fluorescent methods, acridine orange or auramin-rhodamin staining are usually used (Chattman et al., 1969; Langdale-Brown and Haqqani, 1989); these can be also combined with ZN staining (Lavallee, 1973). Fluorescent microscopic methods using specific antibodies appeared to be a convenient tool for the detection of CWD-forms of mycobacteria (Imaeda, 1984; Dorozhkova et al., 1985).

Kovalenko and Dorozhkova (1986) documented that CWD-forms of mycobacteria may conserve

genus and species specific antigens, which are less serologically active. The response of indirect immunofluorescence appeared to be most convenient for species identification of CWD-forms of mycobacteria and was able to differentiate *M. avium* and *M. kansasii* from two members of *M. tuberculosis* complex (strain H37Rv *M. tuberculosis* and strain *M. bovis* 8). However, these two members of *M. tuberculosis* complex could not be differentiated because they are closely related.

Electron microscopy. Conventional light microscopy is not a sufficient tool for the study of the shape and structure of cells in detail. The only relevant method for detailed observation of CWD-forms of mycobacteria is electron microscopy (EM), either conventional transmission EM, or scanning EM, or transmission EM of ultra-thin sections, which can help detection of changes in the cell wall ultrastructure. Bykov et al. (1972) examined *M. tuberculosis* strain 551 and observed formation of small vacuoles (from 800 Å to the size of the cell diameter) in cytoplasm of the *in vitro* prepared CWD-forms of mycobacteria.

Applicability of microscopic techniques. A disadvantage of all microscopic techniques is presence of various artefacts in preparations that may be confused with cells, especially in CWD-forms, which may be round or pleomorphic (Imaeda, 1984).

4.6.2. Culture

The low detection rate of persistent mycobacteria by culture methods is likely to be associated with low oxygen content in granulomatous processes and consequent incapability of cells to grow *in vitro* under standard culture conditions (Ulrichs and Kaufmann, 2002). *M. tuberculosis* adapts to the low oxygen content within the lung granuloma by decreasing metabolic activity and increasing expression of certain enzymes (Wayne, 1994). Mycobacteria may survive in these tissues as CWD-forms (Golanov, 1986; Samillo, 1986; Bobchenok et al., 2002). The very long time necessary for culture is likely to be conditioned by a low capability of CWD-forms to divide. It may also be explained by a slow reversion of CWD-forms to bacillary forms and this may be the reason for the prolonged time to detect them.

Decontamination. With respect to the sensitivity of CWD-forms of mycobacteria against the

factors of the external environment, it is essential to select a good decontamination method before culture. Various modifications of conventional decontamination techniques applied on tissue samples before the culture of mycobacteria have been described in different studies. Graham et al. (1987) exposed human tissue samples for one day to 0.1% hexadecylpyridinium chloride (HPC; N-cetylpyridinium chloride monohydrate) commonly used for the isolation of *M. a. paratuberculosis* (Pavlik et al., 2000). Chiodini et al. (1986) applied on tissue samples 0.1% benzalkonium chloride for one day. Schwartz et al. (2000) employed the method with NaOH-N-acetyl-L-cysteine for 20 min.

An alternative method is decontamination by a filtration through 220 nm filter; that may remove not only contamination, but also bacillary forms of bacteria (Belsheim et al., 1983). Also, it is possible to culture CWD-forms of mycobacteria from blood on veal infusion medium with yeast extract without previous use of a decontamination agent (Almenoff et al., 1996).

Culture media. Culture media for isolation of CWD-forms of mycobacteria should meet the specific nutritional requirements of these sensitive microorganisms. CWD-forms can be most likely detected in liquid media supplemented with glycerol or Tween 80, serum (equine, porcine, ovine or human) and osmotic stabilizers (sucrose, salts, and others), which allow spheroplasts to survive (Chiodini et al., 1986; Markesich et al., 1988). Graham et al. (1987) isolated CWD-forms of mycobacteria on standard solid Herrold's egg yolk medium (HEYM) during 2 to 12 months. However, solid media do not provide optimum conditions for the culture of CWD-forms of mycobacteria. During conventional culture examination, contamination of samples was decreased from 35.0% to 4.5% after medium supplementation with malachite green. 1.0% sterile aqueous solution in the amount of 11 ml per 1 l of Dorozkhova nutrient medium was used (Golyshevskaja and Popesku, 1989).

Properties of isolated CWD-forms of mycobacteria. Chiodini et al. (1986) isolated CWD-forms of mycobacteria from tissues of patients suffering from Crohn's disease on HEYM after 18 to 30 months. These were then closer non-determined unique fastidious mycobacteria relative to *M. a. paratuberculosis*, showing CWD-forms-like properties. Van Kruiningen et al. (1991) conducted biological experiments on chickens using one of these strains (strain Linda). The isolate

was obtained from biological material from a patient with Crohn's disease and was proved to be *M. a. paratuberculosis*. It was later detected as IS900 RFLP type B-C5, typical of *M. a. paratuberculosis* (Pavlik et al., 1999).

Automated culture systems. These systems are useful for a rapid (usually within 2 weeks) isolation of mycobacteria and may be used for the detection of CWD-forms of mycobacteria including *M. a. paratuberculosis*. The time necessary for culture of the isolates from tissues of patients with Crohn's disease in liquid M7H9 medium ranges between 10 to 12 weeks in the case of resection and up to 40 weeks in the case of biopsy (Schwartz et al., 2000).

4.6.3. Isolation of CWD-forms of mycobacteria on chicken embryos

Balan (1991) developed a technique of isolation of CWD-forms of mycobacteria on 4 to 14 days-old embryos. They detected that CWD-forms of *M. tuberculosis* propagated well after 2 to 3 days incubation at 37°C in allantoic and amniotic fluids. They started to grow as soon as 6 hrs after the infection of these fluids. In contrast, no growth was observed in egg yolk.

4.6.4. Methods of molecular biology

As mentioned under item 4.3.1., no differences between genotype of bacillary forms and CWD-forms of mycobacteria have been found. Due to this fact, no specific PCR method able to distinguish between them has been developed. In the study of Wall et al. (1993) entitled "Identification of spheroplasts-like agents isolated from tissues of patients with Crohn's disease and control tissues by polymerase chain reaction" only conventional identification systems of *M. a. paratuberculosis* have been used. It is not possible to differentiate between these two physiological forms of bacteria by means of IS900 detection. These were considered as CWD-forms on the basis of culture (unidentified growth) and microscopy (ZN negative or slightly stainable) and not of the polymerase chain reaction (PCR).

Vishnevskaja et al. (2001) used the PCR method intended for the detection of a non-specified sequence, which should be specific for all

M. tuberculosis complex members, for the identification of CWD-forms of *M. tuberculosis* isolates. PCR was used to verify the tuberculous origin of 60 mycobacterial isolates different in the morphology of colonies and the rate of growth from clinical non-respiratory samples from patients with extrapulmonary tuberculosis. PCR was positive in 51 (85.0%) CWD-forms of *M. tuberculosis* isolates.

Quantitative nucleic acid determination by spectrophotometry is one of the molecular-biological methods capable of distinguishing between CWD-forms and bacillary forms of mycobacteria. Increased DNA content in CWD-form cells can be detected in contrast to mycobacteria with a fully preserved cell wall (Chattman et al., 1969). Hence, it is not a difference in genotype, but a difference in the amount of cellular DNA.

Recently, possibilities of detection have been extended by *in situ* hybridization methodology. It is possible to detect respective mycobacterial species by the use of species-specific hybridization probes. If insertion sequence IS900 specific for *M. a. paratuberculosis* is used as a hybridization probe, it is possible to detect CWD-forms of *M. a. paratuberculosis* only, and not bacillary forms, directly in tissue sections (Hulten et al., 2000a,b). Specificity of the method was confirmed by a testing system using relative and non-relative species of bacteria and bacillary forms of *M. a. paratuberculosis*.

4.6.5. Immunofluorescence and serology

Immunofluorescence. Differences in phenotype (in total protein composition) between CWD-forms and bacillary forms of *M. a. paratuberculosis* have been detected by SDS PAGE and Western blot (Hines and Styer, 2003). This represents potential for their immunological differentiation, e.g. indirect fluorescent antibody analysis was used for the detection of CWD-forms of *M. tuberculosis* (Almenoff et al., 1996) or for differentiation between CWD-forms of some species of mycobacteria (Kovalenko and Dorozhkova, 1986). The CWD-forms of mycobacteria were confirmed by microscopic examination of shape alterations after fluorescent staining.

Serology. Brunello et al. (1991) used a protoplasm antigen for the detection of antibodies against *M. a. paratuberculosis* in human patients affected by tuberculosis and Crohn's disease. This antigen may be potentially used for the detection of antibod-

ies against CWD-forms of *M. a. paratuberculosis* that lose cell wall structures, and where the presence of antibodies against cell wall antigens could be limited. Because a number of authors assumed occurrence of CWD-forms or bacillary forms of *M. a. paratuberculosis* in patients with Crohn's disease (Chiodini et al., 1984b; Greenstein, 2003), p35 and p36 antigens specific for *M. a. paratuberculosis* were used for the diagnosis (Shafran et al., 2002). A close relationship between *M. a. paratuberculosis* and CWD-forms of mycobacteria isolated from the intestinal tract of some patients suffering from Crohn's disease was confirmed by the enzyme-linked immunosorbent assay (ELISA) in early 1980s (Thayer et al., 1984).

4.6.6. Biological experiments on animals

Virulence of CWD-forms of *M. tuberculosis* for experimental animals. Experiments on guinea-pigs showed that spheroplasts of *M. tuberculosis* do not acquire pathogenicity before their reversion to bacillary forms (Ratnam and Chandrasekhar, 1976). Sibirnaia a Snitinskaia (1990) documented that a specific cell-mediated response was induced after infection of guinea pigs with CWD-forms and ultra fine forms of *M. tuberculosis*. The intensity of cell immunity was directly related to the extent of gross changes found after the infection. Some CWD-forms of *M. tuberculosis* might have reverted to bacillary forms during the experiment.

Inoculation of CWD-forms of *M. tuberculosis* on experimental animals and their persistence in host organisms. CWD-forms of *M. tuberculosis* of the strain H37Rv have been observed by EM in the organs of guinea pigs three weeks after intraperitoneal injection and intranasal infection (Li, 1990). The numbers of the CWD-form colonies induced by two infection passages were highest in the eighth week after the infection. The findings suggest that the CWD-forms are easily induced and may persist for a long time *in vivo*.

Belianin et al. (1997) infected CBA mice with two strains of *M. tuberculosis* (strain Erdman and polyresistant clinical isolate 2255) which died within one month. Dissolved ozone in therapeutic doses that increased resistance of experimental animals to the infection was added to strains administered intravenously. All "infected with ozone non-treated mice" died within one month. Their organs contained a great deal of bacillary *M. tuberculosis* both

outside and inside the macrophages. On the other hand in “infected ozone-treated mice” surviving by five months, no bacillary forms of *M. tuberculosis* were found. Many granular and CWD-forms of *M. tuberculosis* and a large number of highly vacuolated (foamy) alveolar macrophages were observed in the lungs.

Conventional diagnosis using experimental animals. Dorozhkova et al. (1987) compared various diagnostic methods for the detection of tuberculosis in resected tuberculomas from the lungs. They found biological experiments on guinea pigs to be most convenient. *M. tuberculosis* was detected in the highest percentage of patients with tuberculosis by this obsolete and nowadays only occasionally used method. This fact was explained by the occurrence of CWD-forms of *M. tuberculosis* that have been formed after the damage of the cells induced by a therapeutical agent and immune response of the host. Dykhno et al. (1974) investigated antibodies formation against bacillary forms, CWD-forms and reverting cells of *M. tuberculosis* by means of biological experiments on animals.

Snitinskaia et al. (1990) performed biological experiments on animals with CWD-forms of *M. tuberculosis* to confirm the diagnosis of tuberculosis. They found that all the isolates of CWD-forms of *M. tuberculosis* induced infection in guinea pigs and their immunological response to the causative agent of human tuberculosis was evident.

5. Clinical significance of CWD-forms of mycobacteria in certain human diseases

Mycobacterial infections in humans still remain serious diseases despite considerable progress in their diagnosis, treatment and prevention was reached. Accordingly, the following section of the present review was focused on the clinical signifi-

cance of CWD-forms of mycobacteria and also bacteria potentially associated with human chronic diseases, which have been long known to be caused or are considered to be caused by mycobacterial aetiological agents. Tuberculosis is still viewed as the most serious disease and occurrence of CWD-forms of *M. tuberculosis* has been studied for a long time (Imaeda, 1984).

5.1. Intestinal tract diseases

5.1.1. Isolation of CWD-forms of mycobacteria

Isolation of CWD-forms of bacteria. Belsheim et al. (1983) isolated bacterial CWD-forms of a variety of species from inflammatory bowel disease patients (Table 1). However, they did not isolate CWD-forms of mycobacteria in any of the cases. Isolates of CWD-forms of *Escherichia coli* and *Enterococcus faecalis*, which are common in the intestinal tract, were present.

Isolation of CWD-forms of mycobacteria. From four patients with Crohn's disease, *Mycobacterium* spp. with unique characteristics representing either a biovariant of *M. a. paratuberculosis* or a new species of *Mycobacterium*, which showed qualities of CWD-forms, was isolated (Chiodini et al., 1984b). Van Kruiningen et al. (1991) later detected that that was *M. a. paratuberculosis*. Chiodini et al. (1986) isolated spheroplasts that were not studied in detail from 61.0% of patients with Crohn's disease. Burnham et al. (1978) isolated CWD-forms of mycobacteria from intestinal lymph nodes from 23 of 27 patients suffering from Crohn's disease; only one of these isolates was classified as *M. kansasii* species. These authors also isolated CWD-forms of bacteria from 7 of 13 patients with ulcerative colitis and from 1 of 11 humans from a control group. Due

Table 1. Detection of cell wall deficient (CWD) forms of bacteria* by culture from clinical material**

Detection results	Crohn's disease		Ulcerative colitis		Controls	
	biopsy	patients	biopsy	patients	biopsy	patients
No. of examined patients	71	44	121	69	140	75
Cultured spheroplasts (%)	33.8	45.4	42.1	53.6	1.4	2.7
Cultured protoplasts (%)	11.3	13.6	16.5	20.3	8.6	12.0
Total of detected CWD-forms of bacteria (%)	45.1	59.1	58.7	73.9	10.0	14.7

*bacterial species other than mycobacteria were isolated

**according to Belsheim et al. (1983)

Table 2. Detection of cell wall deficient (CWD) forms of mycobacteria by culture from clinical material

Patients	Sample origin	Graham et al. (1987)		Markesich et al. (1988)	
		No.	%	No.	%
Crohn's disease	resection	37	27.1	55	23.6
	biopsy	22	9.1	32	15.6
Ulcerative colitis	resection	2	50.0	3	33.3
	biopsy	17	11.8	22	9.1
Controls	resection	8	0	16	18.8
	biopsy	19	0	25	8.0

to this fact, they might be considered as a causative agent of this inflammatory bowel disease.

Graham et al. (1987) most frequently isolated CWD-forms of mycobacteria from surgically resected colon of patients with ulcerative colitis (Table 2). The time to detection of the spheroplasts in patients with Crohn's disease, ulcerative colitis and controls ranged between 2 and 12 months; a reversion to bacillary forms was detected in 33.3% of the cases. Frequencies of culture detection of CWD-forms of mycobacteria from tissues of patients suffering from Crohn's disease, ulcerative colitis and tissues of healthy patients have been also described by Markesich et al. (1988). They did not observe any mycobacteria in healthy patients, but the frequencies of detection in other tissues were similar to the previous authors (Table 2). The reversion of spheroplasts was recorded in 53.8% of cases.

Isolation of CWD-forms of *M. a. paratuberculosis*. Recently, data on the detection of CWD-forms of mycobacteria from the digestive tract of humans, particularly in association with *M. a. paratuberculosis* have been published. Hulten et al. (2001) used a specific method of *in situ* hybridization for the detection of CWD-forms of *M. a. paratuberculosis*; they found differences in percentages between patients suffering from Crohn's disease (granulomatous versus non-granulomatous form), ulcerative colitis and healthy humans. Sechi et al. (2001) recorded high detection rates of CWD-forms of *M. a. paratuberculosis* in more than 70% of patients with Crohn's disease. However, in contrast to the previous authors, they did not reveal significant differences in their detection from the granulomatous and nongranulomatous forms of Crohn's disease. Schwartz et al. (2000) described isolation of "prespheroplasts" of *M. a. paratuberculosis* from tissues

of patients suffering from Crohn's disease; however they do not mention the number of isolates.

5.1.2. Relationship between CWD-forms of *M. a. paratuberculosis* and Crohn's disease

Crohn's disease is a systemic chronic inflammation of the human intestine of multifactorial aetiology. The terminal ileum and colon are most frequently affected. According to Greenstein (2003), *M. a. paratuberculosis* is the most likely etiological agent of Crohn's disease. This species causes paratuberculosis in domestic and wild ruminants. It has also been detected in pasteurized cow milk showing that it cannot be reliably eliminated by pasteurization (Ayele et al., 2005), and consequently could be transmitted to humans. High sensitization to Crohn's disease, but not to ulcerative colitis, has been associated with a defective NOD2 gene, responsible for the immune response to bacterial infection (Greenstein, 2003).

The above mentioned data support the hypothesis of mycobacterial aetiology of Crohn's disease. However, comparison of studies of different authors revealed that *M. a. paratuberculosis* was detected by culture in only 5% of cases of the Crohn's disease (Hermon-Taylor and Bull; 2002). Accordingly, this mycobacterial species was not proven to be the causative agent of this disease in many of the cases. Unsuccessful attempts to detect *M. a. paratuberculosis* from tissues of patients suffering from Crohn's disease may be explained by presence of CWD-forms that are very difficult to culture.

The etiological agent of Crohn's disease has not been recognized yet according to many authors

(Brunello et al., 1991; Quirke, 2001). The attitude of Van Kruiningen (1999) is rather negative due to the fact no clear relationship between Crohn's disease and *M. a. paratuberculosis* has been documented. He critically reviewed the results of *M. a. paratuberculosis* detection from tissues of patients suffering from Crohn's disease obtained by various methods according to respective authors (culture, animal experiments, serology, PCR, immunocytochemistry, response to antibiotic treatment). It follows from this study that percentages of positive detections vary among the authors. The above author also disputed the appropriateness of culture methods for spheroplast isolation.

5.2. Respiratory tract diseases in men

5.2.1. Sarcoidosis

Detection of CWD-forms of mycobacteria. Mycobacteria and particularly their CWD-forms have been long associated with another system granulomatous disease "sarcoidosis" (Rutherford and Gilmartin, 2004). Cantwell (1981b) detected mycobacteria of various shapes that he considered to be CWD-forms in one patient with sarcoidosis and coexisting "scleroderma-like cutaneous disease" (hypodermatitis sclerodermiformis). One year later, Cantwell (1982b) detected different acid-fast cocco-bacillary forms, coccoid forms along with larger forms resembling CWD-forms "large bodies" and short AFR by histology in three patients. Based on this finding, he considered CWD-forms to potentially participate in the aetiology of sarcoidosis. Mitchell (1996) obtained similar results. In contrast, Milman et al. (2004) failed to demonstrate with prolonged culture the presence of mycobacteria in sarcoid tissue and did not suggest a direct role of mycobacteria in pathogenesis of pulmonary sarcoidosis.

CWD-forms of mycobacteria were isolated from the blood of 19 out of 20 patients suffering from sarcoidosis and concurrently they were not isolated from blood samples from any control individuals (Almenoff et al., 1996). In contrast, Brown et al. (2003) deny a relationship between CWD-forms of mycobacteria isolation from blood and sarcoidosis. They documented their attitude by analysis of a high number of patients from various regions in the USA with negative results. Besides blood, CWD-forms of mycobacteria were also detected in other biologi-

cal materials. Alavi and Moscovic (1996) detected CWD-forms of *M. tuberculosis* complex in lymph nodes of patients with sarcoidosis. Cell wall-defective mycobacteria were isolated from skin lesions and cerebrospinal fluid of patients with sarcoidosis and identified to be *M. a. paratuberculosis* or other *M. avium-intracellulare* complex members (El-Zaatari et al., 1996).

Detection of CWD-forms of bacteria. Johnson et al. (1996) described the so called mycoplasma-like organisms as a cause of pulmonary sarcoidosis. They mentioned the obligate intracellular CWD-forms of bacteria, but not in association with mycobacteria.

5.2.2. Pulmonary tuberculosis

CWD-forms of *M. tuberculosis* have been described as pleomorphic cells of various sizes, completely or partly free of the cell wall, and so called "vacuolated forms" (Bykov et al., 1970). Presence of CWD-forms of mycobacteria was documented both in caverns and in sputum from patients with pulmonary tuberculosis (Tsybulkina, 1979), likewise in patients with chronic pleural emphysema tuberculosis (Bogush et al., 1981), in patients with chronic destructive pulmonary tuberculosis (Karachunskii et al., 1983b), in patients with cirrhotic lung tuberculosis (Zhangireev, 1991) and also in patients with pulmonary and pleural tuberculosis (Osiiskii and Kotliar, 1989). Snitinskaia et al. (1990) recorded that CWD-forms of *M. tuberculosis* were isolated from 42% patients with freshly or previously diagnosed pulmonary tuberculosis with different clinical forms of the disease.

Occurrence of CWD-forms of *M. tuberculosis* during therapy. Shedding of CWD-forms of mycobacteria was also recorded during therapy (Karachunskii et al., 1978; Khomenko et al., 1980). During treatment of cavernous pulmonary tuberculosis, Khomenko (1980) confirmed shedding of *M. tuberculosis* through sputum in various growth variants (slow and fast growing isolates and CWD-forms) by culture. These detected that treatment with isoniazid and rifampicin combined with protionamide or ethambutol had a rapid bacteriostatic effect. In contrast, treatment with isoniazide, streptomycin, and *para*-amino-salicylic acid (PAS) limited shedding of fast growing isolates of *M. tuberculosis*. The other growth forms (slow growing isolates and CWD-forms) were shed during treatment in incident cases of cavernous tuber-

culosis before 4, 6, and 9 months in 73.9%, 93.0%, and 96.0% patients, respectively.

Clinical impact of the occurrence of CWD-forms of *M. tuberculosis*. A total of 117 patients with extended and neglected destructive tuberculosis were monitored for 5 to 7 years with the purpose of assessing the clinical impact of CWD-forms of *M. tuberculosis* during the course of the disease, using microbiological, clinical, and roentgenological methods. CWD-forms of mycobacteria were detected in 46.6% of patients who were assessed as abacillary based on conventional culture examination, but had remaining destructive cavities in the lung aggravations and relapses (Dorozhkova et al., 1989).

Bacillary forms of *M. tuberculosis* were much more frequently detected in the elderly than in young patients as follows: in 67.6% of 142 patients at the age of 60 to 89 years and in 42.9% of 132 patients at the age of 17 to 40 years. In contrast, CWD-forms of *M. tuberculosis* were less frequently (28.0% positive) isolated from the group of elderly patients in comparison with 69.7% of positive young patients (Dorozhkova et al., 1990a). Based on these results, the authors assumed that CWD-forms of *M. tuberculosis* play an important role particularly in the reactivation process of the infection.

In 142 incident cases of pulmonary tuberculosis in "elderly and senile" patients, the clinical status was assessed during treatment. According to the shedding of bacillary and/or CWD-forms of mycobacteria the patients were divided into three groups. The treatment was most successful in the first group of patients with a detected bacillary form of *M. tuberculosis*, despite this the highest death rate was recorded in this group. In the second group with both growth forms of *M. tuberculosis* detected, the clinical course of the disease after the treatment was similar as in the first group. The time necessary for treatment was longer and less successful than in the first group. In the third group, where only CWD-forms of *M. tuberculosis* were isolated, the treatment was least successful (Kochetkova, 1989).

CWD-forms of *M. tuberculosis* were also detected in 66.3% of 166 patients with pneumoconiosis combined with pulmonary tuberculosis (Gurenko et al., 1990): in the first group, in 97.3% of 37 patients with active tuberculosis, in the second group, in 56.1% of 41 patients with non-active tuberculosis and in the third group, in 57.9% of 88 patients with tuberculosis-free of pneumoconiosis.

In the patients from the second and third group, minute amounts of monophora-like vacuolated bodies of CWD-forms were observed. Noreiko and Gurenko (1990) observed the same CWD-forms of *M. tuberculosis* in patients with inactive form of anthracotic tuberculosis. In the group of patients with the active forms of anthracotic tuberculosis, massive shedding of CWD-forms of *M. tuberculosis* with various morphology was recorded: dominated giant cells, cord-factor, heterogeneous granulation and homogeneous mass as a consequence of overall destruction of the cells.

A total of 103 patients were allocated into two groups according to their ability of social adaptation: the first group consisted of 66 patients with asocial behaviour and the second group of 37 patients with a normal way of life. In the first group of patients, the resistance and multiresistance to antituberculous agents increased and mortality was 4.2 times higher. Higher occurrence of CWD-forms of *M. tuberculosis* in these patients was most likely associated with poor adaptability to treatment (Bondin, 1992).

Shedding of the bacillary form of *M. tuberculosis* by patients with infiltrative pulmonary tuberculosis was stopped within three months following chemotherapy. In contrast, isolation of CWD-forms remained for a longer period of time; at month six of therapy there was an increase in the number of patients who were found to have CWD-forms of the bacteria (Litvinov et al., 1994). Potential endogenous reactivation of tuberculosis due to reversion of persistent CWD-forms of mycobacteria is viewed as very serious from a clinical aspect (Dorozhkova et al., 1995).

Occurrence of CWD-forms of *M. tuberculosis* after completion of treatment. Occurrence of CWD-forms of mycobacteria was above all documented in patients after a successful treatment (Karachunskii et al., 1980). Berezovskii and Golanov (1981) confirmed the presence of CWD-forms of mycobacteria in patients with abacillary lung cavities. These forms may evidently reactivate cured tuberculosis (Berezovskii and Salobai, 1988).

Sputum from 1 651 patients was examined by culture with the objective to clarify the role of CWD-forms of *M. tuberculosis* in patients with residual tuberculous lesions in the lungs. Typical bacillary carriers were detected in 3.1% of cases. Among them, active tuberculosis was detected in 86.3% of patients. In contrast, CWD-forms of *M. tuberculosis* were detected in 5.0% of patients; active tuberculosis

was detected in only 42.7% of them (Dorozhkova et al., 1990b). Accordingly, the authors drew attention to the fact that CWD-forms of *M. tuberculosis* may be detected in patients with abacillary tuberculosis after completed treatment. Krudu and Dorozhkova (1992) examined a total of 2 412 patients hospitalized for various reasons; among them, bacillary and CWD-forms of *M. tuberculosis* were detected by culture in 9% patients. Detection of both the forms of *M. tuberculosis* is viewed by the authors as a potential risk of process reactivation. Golanov et al. (1994) draw the same conclusions after the examination of 268 patients with pulmonary tuberculosis.

The impact of CWD-forms of *M. tuberculosis* on the development of resistance to antituberculous. The current tuberculosis chemotherapy while effective in killing growing bacilli is ineffective in killing persistent or dormant bacilli, which are highly phenotypically resistant to various AT (Zhang, 2004). A total of 101 patients with pulmonary tuberculosis were divided into two groups according to shedding of various forms of *M. tuberculosis* in sputum (Golanov et al., 1991): Sixty-one patients shed bacillary and CWD-forms of *M. tuberculosis* and 40 patients shed only bacillary forms of *M. tuberculosis*. Resistance to AT was statistically significantly ($P = 0.01$) more frequently detected in patients of the first group (63.8%) in comparison with patients of the second group (40.0%).

Infectiveness of patients shedding CWD-forms of *M. tuberculosis* for their surroundings. Khomenko and Muratov (1993) investigated dangerousness of patients shedding CWD-forms of *M. tuberculosis* for their surroundings. They detected that evidently non-stable CWD-forms of *M. tuberculosis* were capable of reversion to bacillary *M. tuberculosis* and subsequently infected other individuals who were in contact with such patients. Clinical signs in these “fresh patients” (contacts) were as follows: scarce predominance of respiratory infiltration and solitary destructive lesions, and slowly regressive inflammation.

Detection of CWD-forms of *M. tuberculosis* in the peripheral blood of pulmonary tuberculosis patients. Zhu et al. (2000) examined biological material, particularly sputum and blood, from 65 patients with pulmonary tuberculosis for presence of bacillary and CWD-forms of *M. tuberculosis*. Positive rates of bacillary and CWD-forms of *M. tuberculosis* in the cultured blood samples were

15% and 26%, respectively (total isolation rate was 32%) and in the hemolysed-centrifuged blood the positive rate was much higher than direct cultural method. In sputum the positive rates of bacillary and CWD-forms of *M. tuberculosis* were 38% and 20%, respectively (total isolation rate was 52%). The total positive rate of combinative detection of blood and sputum samples was 65%. It was possible to conclude, that bacillary and mainly CWD-forms of *M. tuberculosis* exist in peripheral blood of patients with pulmonary tuberculosis. Culture with hemolysed-centrifuged blood in liquid media and staining with immunoenzyme techniques are valuable in routine detection of mycobacteria and their CWD-forms in peripheral blood. The positive rate of bacterial culture of pulmonary tuberculosis could be increased in combination with blood culture for the presence of mycobacteria and their CWD-forms.

Diagnostic significance of the detection of CWD-forms of *M. tuberculosis*. Diagnostic significance of the detection of all forms of the causative agent of tuberculosis may be demonstrated by the following results of Sakhelashvili et al. (1998). Comprehensive clinical and roentgenological evaluation was done in 495 patients with primary tuberculosis over the age of 18. The clinical pattern of primary forms of the specific process was characterized by predominance of tuberculosis of intrathoracic lymph nodes (tumorous form), infiltrative tuberculosis and exudative pleurisy. Primary tuberculosis was diagnosed in 56.4% patients. Examination of sputum for CWD-forms of *M. tuberculosis* increased the frequency of verification of diagnosis by 16.0% in comparison with conventional microbiological assays.

5.3. Skin and lymph node diseases

CWD-forms of mycobacteria have been described in a variety of skin lesions. Cantwell et al. (1980) used histological methods to observe non-acid-fast coccoid forms in rare form of scleroderma associated with multiple, elevated, dermal nodules, which were considered as CWD-forms of mycobacteria according to their shape. A year later, Cantwell (1981a) observed variably acid-fast and variably sized coccoid forms, suggestive of CWD bacteria and mycobacteria in tissues of two dead patients with Hodgkin's disease during the post-mortem diagnosis.

The following year Cantwell (1982a) used histology to document that a 74-year-old woman with evidence of coexistent cutaneous sarcoid-like granulomas, malignant lymphocytic lymphoma, and multiple basal cell carcinomas had variably acid-fast coccoid forms present in the sections of the cutaneous, non-caseating granulomas. Variably acid-fast, extra and intracellular coccoid forms and granular bodies were seen in the lymph nodes showing lymphoma. The possible relationship between sarcoid-like granulomas, sarcoidosis, and malignancy were discussed, as well as the possible role of cell wall deficient forms (L-forms) of mycobacteria in the pathogenesis of these diseases. Karachunskii et al. (1983b) also described occurrence of CWD-forms of *M. tuberculosis* in peripheral lymph nodes.

5.4. Meningeal tuberculosis

CWD-forms of mycobacteria concurrent to meningeal tuberculosis were also detected (Kudriavtsev et al., 1974). From a clinical aspect, Insanov et al. (1990) documented that the disease was accompanied by a relatively rapid development of inflammatory changes in children with detected bacillary forms of *M. tuberculosis* in cerebrospinal fluid. In contrast, insidious onset and slow accumulation of pathologic lesions were typical for detection of CWD-forms of *M. tuberculosis* in cerebrospinal fluid. Treatment of these patients was less successful than in the previous cases.

Gadzhiev (1990) drew similar conclusions. This author describes examination of cerebrospinal fluid from 128 children with tuberculous meningitis. During the chemotherapy a quick disappearance of the bacillary forms of *M. tuberculosis* and presence of persistent CWD-forms of the agent in the cerebrospinal fluid were observed. Accordingly, the author supposed that presence of CWD-forms of *M. tuberculosis* was indicating the possibility of chronic development and the danger of relapses.

Six years later, Insanov et al. (1996) published the results of the examination of 133 children and 89 adults, aged 3 to 60 years, who had been admitted for suspected tuberculous meningitis. The diagnosis of tuberculous meningitis was established in 113 children and 79 adults. After puncture, spinal fluid was examined by three methods: microscopy, culture, and biological assay. Microscopy detected mycobacterial tuberculosis in 5.3% and 2.5% of

children and adults, respectively. Biological assays confirmed human tuberculosis in 39 (34.5%) children and in 15 (30.0%) adults. Bacillary form of *M. tuberculosis* was isolated more frequently in children (58.4%) than in adults (27.9%). The spinal fluid much more frequently displayed changed CWD-forms of *M. tuberculosis* than bacillary ones. CWD-forms were isolated by culture in 62 (54.9%) children and 59 (74.6%) adults. In total, spinal fluid culture for CWD-forms of *M. tuberculosis* improved the diagnosis of tuberculous meningitis which was confirmed in 87.6% children and 87.3% adult patients.

Gadzhiev and Alieva (1996) studied the composition of the cerebrospinal fluid in the 113 children with tuberculous meningitis mentioned above. They have found that the changes in the cerebrospinal fluid were most pronounced in bacillary form of *M. tuberculosis* infection and were minimally changed in the absence of both bacillary and CWD-forms of *M. tuberculosis*. In the case of the occurrence of CWD-forms, the changes in cerebrospinal fluid were lower than in bacillary form. It follows from the above results that CWD-forms of *M. tuberculosis* are less clinically significant, which may be supported by investigated infections of other organs (lungs, lymph nodes, and skin) described in the above chapters.

5.5. Other forms of the disease

The occurrence of CWD-forms of *M. scrofulaceum* was described in one case of generalized infection (Korsak, 1975) and the presence of CWD-forms of *M. tuberculosis* (Zemskova et al., 1985) was recorded in one child with generalized tuberculosis. Presence of CWD-forms of mycobacteria was also detected in patients with pyelonephritis (Higuchi, 1970), in urine from patients with renal tuberculosis (Kochemasova et al., 1975), concurrent to congenital diseases (Dorozhkova et al., 1972; Ezerskii et al., 1987) and osteoarticular tuberculosis (Stanislavleva et al., 1979; Krivtsova and Mazhenova, 1986; Chenskikh and Mazhenova, 1987).

Examination of 162 samples from the periodontal foci of infections revealed CWD-forms of *M. tuberculosis* in 71.6% cases (Avdonina et al., 1992). In the following study, the results obtained by various available methods in 113 patients with pulmonary tuberculosis and with periodontal foci of infection were analyzed (Avdonina et al., 1993).

Persistence of CWD-forms of *M. tuberculosis* in 59.3% of patients suggests that the periodontal foci of infection could be a form of extrapulmonary tuberculosis.

5.6. Effects causing higher prevalence of CWD-forms of *M. tuberculosis* in the human population

5.6.1. Effect of HLA-genotype of humans on *M. tuberculosis* infection

Pospelov and Mikhailova (1993) studied the effect of the HLA-genotype of 150 patients with infiltrative tuberculosis and 135 healthy subjects. Clinical-immunogenetic correlations provided evidence for significantly higher incidence of antigens A11, B12, Cw2, DR2, and DR5 in patients with infiltrative tuberculosis versus healthy subjects. Antigens Cw2 and DR2 occur more frequently in progressive disease. Patients with infiltrative tuberculosis disseminating *M. tuberculosis* carry more frequently antigens DR2, those with CWD-forms of *M. tuberculosis* antigens DR5, and those with antigenemia antigens DR2 and DR4.

5.6.2. Effect of prolonged exposure to low-dose radiation after the Chernobyl accident on *M. tuberculosis* infection

A total of 31 *M. tuberculosis* isolates from tuberculous patients exposed to low-dose radiation after the Chernobyl accident were compared *in vitro* to those obtained from tuberculous non-irradiated controls for CWD-forms incidence, L-transformation, and virulence. In response to L-transforming preparations, the frequency and rate of CWD-forms induction in the test strains were much higher than those in the controls. Higher frequency of CWD-forms virulence was also registered in the case of isolates from irradiated patients (Gurevich et al., 1993).

6. Isolation of CWD-forms of mycobacteria from tissues of naturally infected animals

On one hand, mycobacterial infections of animals in Central Europe have been successfully controlled (bovine and avian tuberculosis) on the other hand, they represent an increasing danger. That applies

above all to paratuberculosis (Ayele et al., 2001). Two types of complications may be encountered during bacteriological diagnosis of paratuberculosis. The first one is the occurrence of relatively slow growing strains found in small and wild (particularly sheep and mouflons) ruminants (Pavlik et al., 1999; Machackova et al., 2004).

Another complication is the occurrence of paucibacillary cases of paratuberculosis in animals with clinical signs of the disease. In such cases, AFR are difficult to demonstrate by microscopy in imprinted specimens and only isolated CFU of the causative agent of paratuberculosis can be detected by culture (Chiodini et al., 1984a). This phenomenon may be explained as an autoimmune response of intestinal mucosa to the presence of a minute amount of highly immunogenic cells of *M. a. paratuberculosis* (Kazda, 2000). Another possible explanation is the presence of CWD-forms of mycobacteria, which may also explain occurrence of slow growing strains in certain animal species (Pavlik et al., 1999). Occurrence of CWD-forms of mycobacteria is also assumed in some animals with bovine tuberculosis; that may play a certain role in recurrent outbreak of the disease in controlled herds (Baiteriakova et al., 1982).

One interesting hypothesis, that CWD-forms of mycobacteria can be related to spongiform encephalopathies in animals and humans, was postulated by Broxmeyer (2004). He suggests that, virus-like, CWD-forms of *M. bovis* can penetrate through blood-brain barrier and cause meningitis similarly to mad cow disease. Mad cow disease is normally considered to be caused by prions. The author supports his assertion by the highest incidence of bovine tuberculosis caused by *M. bovis* in the same area of Southwest England which also has the highest occurrence of bovine spongiform encephalopathy (BSE).

Information on the detection of CWD-forms of mycobacteria in humans is scarce and isolation of CWD-forms of mycobacteria in animals, namely in cattle affected with paratuberculosis, is even scarcer. In the literature available, only two studies from the same research team have been found (Hulten et al., 2000a,b). These authors localized CWD-forms of *M. a. paratuberculosis* in tissue sections from 4 of 5 tested cows using *in situ* hybridization technique. One of them was experimentally infected with a suspension of *M. a. paratuberculosis* and paratuberculosis was diagnosed in the others.

Specificity of the method for the detection of CWD-forms of *M. a. paratuberculosis* was confirmed using other mycobacterial species and AFR of *M. a. paratuberculosis* as controls, where negative results were obtained in all cases.

7. Conclusions

CWD-forms (spheroplasts) of bacteria are commonly prepared *in vitro* and are used for various practical applications. However, very little is known about the conditions required for the formation of CWD-forms of bacteria and mycobacteria *in vivo* and their significance in aetiology of various chronic diseases of humans (tuberculosis, sarcoidosis, and Crohn's disease) and animals (paratuberculosis). It is quite difficult to detect CWD-forms of mycobacteria and the possibilities of their detection by microscopy, culture, DNA hybridization and other methods are limited. This obviously leads to a relatively small amount of published data on the detection and isolation of CWD-forms of mycobacteria both from human or veterinary biological material. The present review also includes studies performed by authors of the former Soviet Union and likely represents the first complete summarization of their knowledge in this sphere. However, certain results may be viewed as somewhat controversial. Accordingly, more attention should be paid to the research of cell wall deficient forms of mycobacteria, not only in association with chronic and latent mycobacterial infections.

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Corresponding Author:

Professor MVDr. Ivo Pavlik, CSc., Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic
Tel. +420 533 331 601, fax +420 541 211 229, e-mail: pavlik@vri.cz; URL: <http://www.vri.cz>
