bone marrow lymphocytes carrying immunoglobulin receptors, identified by the immunoperoxidase method, on their cytoplasmic outgrowths, consist of a population of B lymphocytes. It must be recalled that the presence of receptors for Fc-fragments on other cells besides B lymphocytes [6, 8] reduces the specificity of detection of the B cells. To prevent the antibody molecule from binding with the Fc-receptors of the cells the horseradish peroxidase must be conjugated with $F(ab')_2$-fragments of the antibodies, and this is a matter for special study. Despite this shortcoming, the method of detection of immunoglobulin receptors on the surface of lymphocytes by means of monospecific antisera conjugated with horseradish peroxidase is perfectly suitable for the identification of B lymphocytes at the light-optical and ultrastructural levels. The results obtained show that lymphocytes carrying membrane-associated immunoglobulin receptors (B lymphocytes) constitute a morphologically heterogeneous population, and this fact must be borne in mind when cells of this type are identified.

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ULTRASTRUCTURAL LOCALIZATION OF THE TYPE-SPECIFIC ANTIGEN OF *Legionella pneumophila*

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The discovery of a new genus of bacteria, *Legionella* (the etiologic agent of a hitherto unknown group of serious acute respiratory diseases in man) has generated a considerable volume of research aimed at studying the cultural, morphological, and biochemical properties of this agent [4, 6, 7, 9]. In particular, the study of the cellular antigens of the microorganism and their role in the mechanism of pathogenesis and immunity in experimental infection is most interesting [4, 8, 10]. Several type-specific and group-specific antigens have been isolated from avirulent strains of *L. pneumophila* and it has been shown that they can be used for experimental vaccination of laboratory animals; their biochemical nature has been studied [18].

A type-specific thermolabile antigen III was isolated previously from a virulent culture of *L. pneumophila* [1]. This antigen, unlike other antigens isolated from avirulent strains described previously, was toxic for a culture of guinea pig peritoneal macrophages *in vitro*,

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and for guinea pigs and AKR/J mice [1]. The antigen is bound with the bacterial cell and is easily separated from it, but its cellular localization is not known. The present investigation was devoted to an electron-microscopic study of the localization of antigen III of *L. pneumophila* by immunoferritin and immunoperoxidase methods.

EXPERIMENTAL METHOD

A virulent culture of *L. pneumophila*, strain Philadelphia 1 (LD_{50} for guinea pigs 3.2×10^2–3.5×10^2 CFU) was grown on carbon-yeast agar for 72 h at 37°C, washed off with physiological saline (pH 7.2), and incubated for 1 h at 28°C. The supernatant, separated by centrifugation, was filtered through Millipore filters with a pore diameter of 0.45 μ, and concentrated on a PM 10 filter in Amicon cells to 0.1 of the initial volume. After fractionation on a column with Sepharose 6B fractions corresponding to the first peak were identified by the double radial immunodiffusion test in 1% agarose with 0.02 M phosphate buffer, containing 3% of polyethylene-glycol (mol. wt. 6000). The toxicity of the antigen was tested on a culture of guinea pig peritoneal macrophages and on AKR/J mice. Rabbit antiserum against antigen III was obtained after triple immunization with 0.5 ml antigen, mixed with an equal volume of Freund's complete adjuvant, intramuscularly. Globulin fractions were isolated from the antiserum by the sulfate-rivanol method [16] and the total globulin fraction was labeled with peroxidase with the aid of glutaraldehyde [5]. Unfixed bacteria were treated with the labeled conjugate for 1 h at 37°C. Peroxidase was detected by the cytochemical reaction with 3,3'-diaminobenzidine tetrahydrochloride (DAB) [3]. Donkey antirabbit serum labeled with ferritin [15] was used in the indirect immunoferritin method. Immunocytochemical reactions also were carried out on suspensions of bacteria fixed with 1% glutaraldehyde in phosphate-buffered salt solution (PBS), pH 7.2-7.4, or a mixture of paraformaldehyde and picric acid in PBS by Zamboni's method [17]. Bacteria treated with heterologous serum (O serum of *Vibrio cholerae*), and a suspension of bacteria incubated with DAB without preliminary treatment with antiserum (endogenous peroxidase control) were used as the controls.

After incubation with labeled sera and washing the bacteria were fixed with 1% glutaraldehyde in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Araldite M. The reaction for acid mucopolysaccharides with ruthenium red was carried out by the method in [12]. Ultrathin sections were cut on the LKB 8800 Ultrotome, stained with uranyl acetate, and studied in the JEM 100B electron microscope.

EXPERIMENTAL RESULTS

The study of the localization of antigen III of *L. pneumophila* by the indirect immunoferritin method in cells fixed with glutaraldehyde revealed label bound with the outer layer of a formation of microcapsule type on the surface of the bacteria (Figs. 1 and 2). A "microcapsule" was regularly found in all bacteria in the section and it surrounded the bacterial cells in a uniform layer 12-20 nm thick. Glutaraldehyde is known to inhibit interaction between labeled antibodies and cells [11], and for that reason the localization of the label was tested in separate experiments either in unfixed bacteria followed by fixation or in bacteria fixed with a mixture of formaldehyde and picric acid by Zamboni's method [17] which, as has been shown [11], does not affect binding of labeled antibodies. In unfixed preparations a fibrillary layer of varied thickness was found at the perimeter of the bacterium, outside the cell wall membrane (Fig. 3). Often this layer could form fairly large bundles closer to the poles of the cell. Ferritin granules were bound only with the outer part of this layer. Prefixation of the bacteria by Zamboni's method also clearly revealed a fibrillary layer of microcapsule type and the ferritin granules were bound exclusively with its outer part (Fig. 4).

On treatment of bacterial suspensions with antiserum against antigen III labeled with peroxidase in the direct immunoperoxidase method the reaction product was located in the form of amorphous globules on the surface of the cell wall membrane. Individual globules were in close contact with each other (Fig. 5). However, after prefixation with glutaraldehyde not all cells in the preparation contained the reaction product. When the test was carried out on unfixed bacteria the number of cells giving a positive reaction was greater and the globules on the surface of the bacteria were more widely separated and were larger. A similar distribution of type-specific high-molecular-weight antigen (F-I) isolated from avirulent strains, and free from toxic properties, has been described in legionellas of serologic group 1 [8].

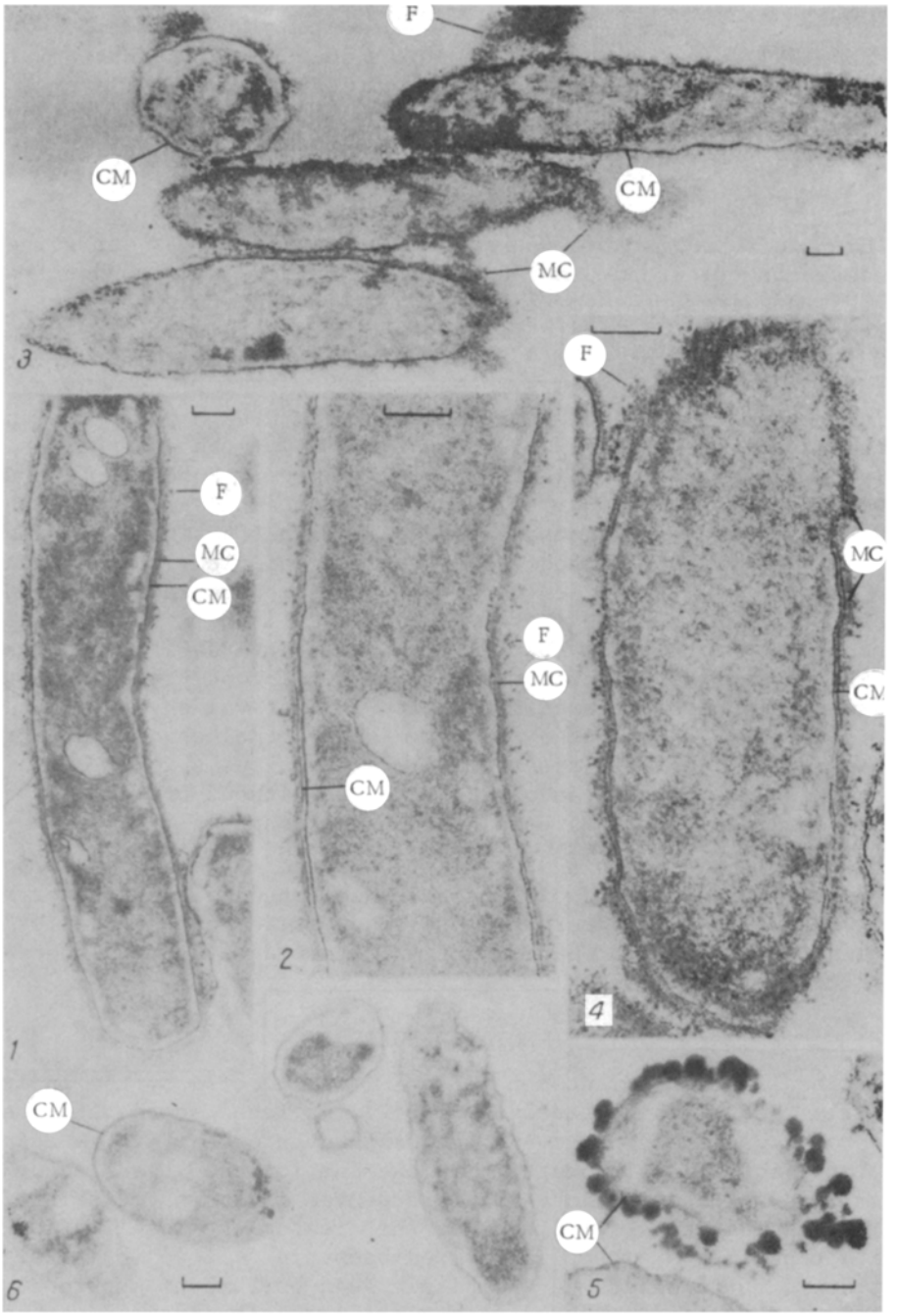

Fig. 1. Cells prefixed with glutaraldehyde. Indirect immunoferritin method. 70,000 ×. Here and in Fig. 2, scale line corresponds to 0.1 μ. MC) Layer of microcapsule type, F) ferritin granules, CM) cell wall membrane.

Fig. 2. Fragment of Fig. 1. Localization of ferritin granules on surface of fibrillary layer of microcapsule type clearly visible. 120,000 ×.

Fig. 3. Unfixed cells of *L. pneumophila*. Indirect immunoferritin method. 60,000 ×.

Fig. 4. Prefixation with mixture of formaldehyde and picric acid, indirect immunoferritin method. 125,000 ×.

Fig. 5. Bacteria prefixed with glutaraldehyde. Direct immunoperoxidase method. 90,000 ×.

Fig. 6. Control preparation, prefixed with glutaraldehyde. Treatment with heterologous immune serum in indirect immunoferritin method. Label and fibrillary layer of microcapsule type are absent. 60,000 ×.

On treatment of the control culture with heterologous immune antiserum against *Vibrio cholerae* O antigen, the surface fibrillary layer of the legionellas could not be seen. The label was absent (Fig. 6). No endogenous peroxidase was found in the bacteria tested. After ordinary fixation as used to study the ultrastructure of bacteria, no microcapsule could be found on the legionellas either in culture or in material from patients [7, 9]. The reaction for acid mucopolysaccharides with ruthenium red was negative both in our own experiments and in those of other workers who have studied legionellas [13]; no surface structures could be found outside the cell wall membrane.

The type-specific thermolabile antigen III of *L. pneumophila* is thus located on the surface of the bacterial cell in the outer layer of a fibrillary formation of microcapsule type, which surrounds the bacterium in a uniform layer 12-20 nm thick. In its chemical composition this formation evidently differs from the microcapsules of other bacteria, for it is not detected in the reaction with ruthenium red, evidence that its composition does not include acid mucopolysaccharides, which are characteristic of bacterial microcapsules, including the analogous structure of rickettsias [14]. Binding of the surface fibrillary layer to the cell wall of the legionellas is very labile and for that reason antigen III is easily separated from the cell, but the microcapsule cannot be revealed in cultures treated by ordinary electron-microscopic fixatives.

Since the microcapsules of bacteria are formed chiefly of mucopolysaccharides, firmly bound with the cell surface and detectable only in ultrathin sections or when dyes specific for polysaccharides are used [2], the fibrillary formation described on the surface of legionellas is not a true microcapsule, but something more similar to the capsule-like or mucous cover detection of which is facilitated by treatment with homologous antibodies [4], and it is evidently a secretion product of antigen III (and also, possibly, of other antigens).

The further study of antigen III located on the surface of legionella cells is interesting not only as a factor largely responsible for the virulence of this microorganism [1], but also as a sensitizing agent in the delayed-type hypersensitivity reaction. In our own experiments intradermal injection of antigen III into guinea pigs immunized with a sublethal dose of a virulent culture revealed the infection in the early stages (skin test after 5-6 days). Single subcutaneous immunization of guinea pigs with antigen III followed by infection with a lethal dose of the agent revealed the high immunogenicity of this antigen.

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