EXTRACTION OF NONTYPE-SPECIFIC GROUP A STREPTOCOCCAL ANTIGENS WITH POTASSIUM THIOCYANATE

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Several common protein antigens, known as nontype-specific antigens (NTS antigens) have been found in the cell walls of different types of group A streptococci [12]. Among them some cross-reacting antigens (CR antigens) common with antigens of fibroblasts and sarcolemma of the myocardial muscle fiber have been found [5, 6]. A more detailed study of CR antigens and the search for a common protective antigen among cell wall proteins of group A streptococci are interesting [12]. Further research in this direction will require isolation of NTS antigens under conditions ruling out the possibility of protein degradation.

In the previous studies the sources of antigens were HCl extracts and washings of cultures of streptococci (F fraction) [1, 12]. Both methods have substantial disadvantages. Treatment with hydrochloric acid (95°C, 10 min) may lead to disturbance of protein structure and, as a result, to the loss of individual antigenic determinants. Aqueous extraction is insufficiently effective.

In some investigations type-specific cell wall M proteins of streptococci were obtained successfully without any disturbance of their primary structure by means of guanidine chloride and nonpolar detergents [9, 14]. In affinity chromatography, potassium thiocyanate is used to elute antibodies from immunosorbents, along with guanidine chloride, and it also has a reversible denaturing action on proteins [4].

This paper describes a method of extraction of surface NTS antigens of streptococci from the whole microbial cell with the aid of potassium thiocyanate.

EXPERIMENTAL METHOD

Strains of group A streptococci of the following types were used: 1-M (No. 2/49)*, 29-M (No. 62/59)*, 3 (T + M) (No. 4/59)*, 17 (T + M) (No. 10/55)* and 10 (NY-5), and group strain J17A4 (No. 6/49*).*

To extract antigens the mass of streptococci was washed three times with 0.15 M NaCl solution, pH 7.0, and treated with 3.5 M KCNS, made up in 0.015 M phosphate buffer, pH 7.0, with 0.15 M NaCl (3PS) at the rate of 3 ml of solution to 1 ml of solid residue. The suspension was mixed on a magnetic mixer at 4°C for 16 h. The residue was separated by centrifugation at 20,000 g and suspended in KCNS solution for re-extraction under the same conditions. Intensive dialysis of the extracts against large volumes of 3PS was carried out until all CNS^- ions had been completely separated. Freeze-dried solutions were described as unpurified KCNS extracts.

For precipitation with ammonium sulfate, the latter was added to the solution of the KCNS extract up to 70% saturation. The precipitate was dissolved in 0.15 M NaCl, pH 7.0, desalted on a column with Sephadex G-25, and freeze-dried (partially purified KCNS extract).

To prepare HCl extracts the streptococcal mass was treated with 0.05 N HCl at 95°C for 10 min [10]. Washings of cultures of streptococcus (F-fraction) were obtained at pH 6.8-7.4

*Asterisk indicates serial numbers of strains of Prague Collection.

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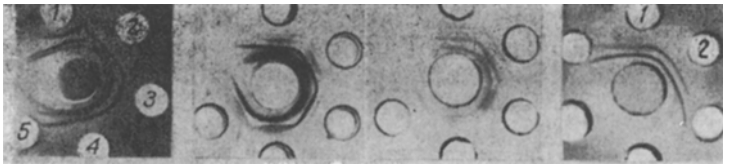

a b

Fig. 1 Fig. 2 Fig. 3

Fig. 1. Comparison of PEF, KCNS, and HCl extracts and fraction F by double immunodiffusion test in gel with serum against PEF. Central well contains serum No. 240 obtained by immunization with PEF (group A streptococcus, type 29). Peripheral wells contain: 1) PEF (type 1); 2, 4) partially purified KCNS extract (type 1); 3) HCl extract (type 3); 5) F fraction (type 17).

Fig. 2. Testing of KCNS and HCl extracts with serum against KCNS extract by double immunodiffusion method in gel. Central wells contain serum No. 14 against partially purified KCNS extract (group A streptococcus, type 29). Peripheral wells contain: a) KCNS extract (type 1); b) HCl extract (type 1).

Fig. 3. Comparison of partially purified KCNS and HCl extracts in double immunodiffusion test in gel with serum against KCNS extract. Central well contains serum No. 14 against partially purified KCNS extract (group A streptococcus, type 29). Peripheral wells contain: 1) KCNS extract (type 1); 2) HCl extract (type 1).

and at room temperature [1]. By preparative electrophoresis fractions were isolated from the HCl extracts in the zone of mobility of serum albumin (PEF), containing NTS antigens [12]. The thermostable fraction (TSF) was prepared from the culture medium of streptococci of strain NY-5 type 10 [2].

To obtain immune sera against partially purified KCNS extract, each rabbit was given an injection of the extract in a dose of 1 mg protein in Freund's complete adjuvant into the lymph nodes of the hind limbs, followed by monthly stimulation intravenously with the same dose of antigen [8]. Sera against PEF, TSF, and F fractions were obtained by immunization of rabbits with the corresponding preparations in Freund's incomplete adjuvant by the same method. Some of the animals were immunized intravenously with a culture of streptococcus treated with pepsin [13].

Protein was determined by Lowry's method [11]. Inorganic phosphate in digests of KCNS extracts was measured by the molybdate method [15].

The method of double immunodiffusion in agar gel in the micromodification [3] was used in the work; the immune sera were concentrated fivefold. To abolish the reactions with teichoic acid the plate with the agar gel was washed with 10% NaCl solution [12].

EXPERIMENTAL RESULTS

The protein concentration in the KCNS extracts of streptococci depended on the culture used in the experiment. For type 1 this value was about 18 mg to 1 ml of wet residue, but for type 29 it was 9-10 mg. Between 60 and 80% of the protein transfers to the primary extract and the rest during repeated processing of the microbial cells.

Streptococci washed to remove the denaturing agent preserved their structure, as confirmed by staining the preparations by Gram's method but they lost their ability to grow and multiply when seeded on blood agar.

Sera against PEF, when tested in the immunodiffusion test in gel, revealed two or three identical NTS antigens in the KCNS- and HCl-extracts (Fig. 1). These antigens were digested by trypsin. The content of nontype-specific protein antigens in the KCNS- and HCl-extracts

was considerably greater than in the F-fraction, although their concentrations as protein were equal (Fig. 1). Sera against the partially purified KCNS extract revealed from 2 to 5 protein antigens in HCl- and KCNS-extracts obtained from cultures of a streptococcus of heterologous type. These antigens were present in large quantities in the KCNS-extracts; in addition antigens not present in HCl extracts of the same type were found (Figs. 2 and 3). NTS antigens of unknown nature, not digested by pepsin or trypsin, were present in the KCNS-extracts. Unpurified KCNS-extracts, when tested by immunodiffusion in gel, reacted with sera and with F-fraction-containing antibodies against glyceroteichoic acid (the precipitation line was washed with 10% NaCl solution). The content of glyceroteichoic acid in partially purified KCNS extracts, as shown by determination of inorganic phosphate in digests, fell from 10 to 1.1 mg/mg protein. A large proportion of nontype-specific protein antigens still remained. The group polysaccharide was not found in the immunodiffusion test in gel with sera against a pepsinized culture of group A streptococcus in the KCNS extracts, unlike in HCl extracts. Tests on extracts with sera against TSF-fraction by this method showed that the KCNS extracts contained much less of the extracellular products than washings of the cultures with water. For instance, unpurified KCNS extracts of the first type gave a weak reaction (+—) with only one of the nine sera against TSF fraction. Meanwhile more than half of these sera reacted strongly (++) and formed three or four lines with the F fraction.

Treatment of streptococci with potassium thiocyanate thus enables extraction of nontype-specific protein antigens of the cell wall. The action of KCNS on streptococci inhibits vital processes in the cells but does not cause their lysis, as shown by preservation of their ability to stain by Gram's method. This suggests that antigens of membranes and cytoplasm are not present in the extracts.

The suggested method of obtaining surface antigens is more effective than mild extraction with saline solutions, in which the content of NTS antigens is very low [1]. Considerable quantities of extracellular streptococcal products, present as impurities in the F fraction, complicate the solution to the problem whether cross reactions with human and animal tissue antigens are observed on account of cell wall antigens or of extracellular products [5].

The mild conditions of isolation of NTS antigens by thiocyanate extraction preserve the covalent structure of the surface proteins of the streptococcus. This evidently explains the fact that besides common antigens, the KCNS extracts also contain antigens not present in HCl extracts (Fig. 3). The possibility cannot be ruled out that difficulties with the discovery of the common protective antigen [12] are connected with destruction of some of the antigenic determinants through the action of HCl, and the method described above thus offers fresh prospects for the solution of this problem.

Thiocyanate extracts contained several protein and nonprotein antigens. The object of a future study will be to separate them and to isolate NTS antigens in a pure form. This is necessary for the solution of problems regarding the nature of NTS antigens cross-reacting with mammalian tissues, and their exact localization on the surface of the microbial cell, and also to explain their role in the pathogenesis of streptococcal infections.

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MECHANISM OF LYSIS OF TARGET CELLS INFECTED WITH MYCOPLASMAS BY NATURAL KILLERS

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The important role of natural killer (NK) lymphocytes in antitumor surveillance has been repeatedly confirmed [1, 2, 9, 12]. Despite the large flow of information in the field of the study of NK lymphocytes there is as yet no general agreement on the structures which are the target for these killer cells [3, 13-15]. There is likewise no unanimity on the mechanism by which NK lymphocytes destroy target cells (TC) [10, 16, 18]. Reports were recently published that mycoplasmas, on the one hand, may act as agents stimulating blast transformation of lymphoid cells [6] and, on the other hand, may cause increased sensitivity of TC to the lytic action of NK lymphocytes [4].

This paper confirms observations on more intensive death of mycoplasma-infected TC in the cytotoxic test *in vitro*. However, the cause of the increased mortality of these cells is the cytotoxic action of the mycoplasma, whose weight increases rapidly on account of intensive proliferation of these organisms on effector cells.

EXPERIMENTAL METHOD

The cytotoxic test was carried out by the method described previously [12]; TC were cultured with syngeneic effector cells or with lytic factor in microplates for 48 and 20 h, respectively. The degree of lysis was assessed by the number of living TC remaining on the glass compared with the control. Splenocytes were freed from macrophages by passage through a column containing nylon wadding, as described in [11]. Cell-free lytic factor was obtained by filtering a suspension of syngeneic splenocytes with TC (after incubation for 48 h at 37°C in the ratio of 300 TC to 1.3×10^6 splenocytes in 1 ml of suspension) through a millipore filter with pore diameter of 2.5 μ. Activity of the factor was estimated by the intensity of lysis of L cells (fibroblasts from C3H mice subcultured *in vitro* for a long time). Infectivity of the cells by mycoplasmas was estimated by the difference in incorporation of tritiated uridine and uracil by the cells, i.e., by a method whose principle was described previously [17], and also by means of the dye Hoechst 33258 [5]. If the ratio of labeled uridine to uracil was over 100, the cells were considered to be free from mycoplasmas. Antiserum against mycoplasmas was obtained from a rabbit repeatedly immunized with a suspension of lymphoid cells infected by mycoplasmas. The serum was exhausted by healthy splenocytes, so that in the fluorescence test it caused luminescence only of mycoplasma particles.

EXPERIMENTAL RESULTS

A continuous culture (FC3H3) was obtained from embryonic fibroblasts of C3H mice as a result of spontaneous transformation *in vitro*, and its cells were selected for increased carcinogenicity by triple subculture *in vivo*. After these procedures cells of two tumors were returned to *in vitro* culture (FC3H3V1 and FC3H3V2). The FC3H3V1 cells were accidentally infected in the course of *in vitro* subculture by a mycoplasma (the species was not established), after which the infected subculture was conducted as an independent subline (FC3H3V1.1). As a result of intravenous inoculation of C3H mice with these cells a metastasis was obtained in the lung; its cells, on returning to *in vitro* culture, were found to be mycoplasma-free (FC3H3VM). Data illustrating the purity of the above-mentioned cell cultures, or their in-

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