Virchows Arch. Abt. A Path. Anat. 355, 220–226 (1972)

Persisting Streptococcal Cell-Wall Components in Murine Chronic Pyogenic Nephritis*

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Received August 9, 1971

Summary. Following intrarenal injection of live group A streptococci into mice, morphologically intact organisms were demonstrated in the centers of renal abscesses by Gram's stain and reaction with fluorescein-isothiocyanate-conjugated antibody to group A C-carbohydrate. Immunofluorescent staining also revealed cell-wall carbohydrate persisting within a small number of macrophages in the peripheries and capsules of renal abscesses, as well as in chronic inflammatory foci in kidneys atrophied in the course of pyogenic nephritis. The carbohydrate demonstrated in this study has previously been identified as a group-specific streptococcal cell-wall polymer chemically linked to mucopeptide in the intact cell wall. No conversion of group A to group A-variant C-carbohydrate was noted. The rapid breakdown of the streptococci in pyogenic inflammatory foci was attributed to enzymatic activity of the intensive granulocytic inflammation prevalent in the initial stages of the infection.

Introduction

Bacterial components persisting in tissues have been implicated in chronic inflammatory processes in which live microorganisms are no longer demonstrable (Dutz *et al.*, 1965; Aoki *et al.*, 1967; Cotran, 1969). For example, heat-killed group A streptococci persist in mouse liver for many weeks after phagocytosis by Kupffer cells and, without prior immunization of the host, can induce chronic, nonpurulent, granulomatous foci of inflammation consisting of macrophages, lymphocytes and a few plasma cells (Sellin *et al.*, 1970; Haferkamp *et al.*, 1970). Primarily, the reaction is induced by the cell wall of the killed bacteria. This wall is composed of mucopeptide and group-specific carbohydrate; it can be detected for weeks after injection of streptococci or cell walls within liver macrophages by immunofluorescence and immuno-electronmicroscopy (Schäfer *et al.*, 1970). Mucopeptide alone does not elicit these nodular tissue reactions (Haferkamp *et al.*, 1970). Group A streptococcal mucopeptide-carbohydrate complex remaining within the granulomas also reacts with antibody to group-specific carbohydrate of A-variant streptococci (Haferkamp *et al.*, 1970). Such alteration of antigen specificity within the living organism may be attributable to the action of the enzyme β-N-acetylglucosaminidase, which has been found in high concentrations in mouse liver (Conchie *et al.*, 1959). This enzyme splits β-N-acetylglucosamine, the antigenic determinant of group A carbohydrate, from its terminal position on rhamnose side chains, leaving rhamnose oligosaccharides, which are the antigenic determinant of group A-variant carbohydrate (McCarty, 1964). The simultaneous presence of mucopeptide has been demonstrated morphologically by electronmicroscopy (Schäfer *et al.*, 1970).

* Supported by a grant of the Deutsche Forschungsgemeinschaft.

The C-carbohydrate-mucopeptide polymer of the group A streptococcal cell wall has been found to elicit a number of other tissue reactions when injected into experimental animals. It was reported that this material produced chronic, remittent, nodular skin lesions (Schwab, 1965; Ohanian *et al.*, 1967), arthritis (Schwab *et al.*, 1967), and rheumatic-like carditis (Ohanian *et al.*, 1969). Recent observations suggest that the effects of streptococcal mucopeptide are due to intrinsic toxic properties and are not attributable to humoral or cellular hypersensitivity reactions (Heymer *et al.*, 1971).

These findings, obtained after injecting animals with killed streptococci or carbohydrate-mucopeptide complexes, led us to explore whether carbohydrate-mucopeptide complexes persist in tissues after the injection of live group A streptococci. Such persistence might account for the hitherto unexplained chronicity of inflammatory tissue changes frequently observed after experimental streptococcal bacterial infections. Since kidney abscesses readily develop in mice after intravenous injection of virulent group A streptococci (Smith *et al.*, 1966a and b), this particular animal and organ were chosen as an experimental model. Because immunofluorescent reactions of mucopeptide with homologous fluorescein-isothiocyanate—(FITC)—labeled antibody are of low intensity, they were not employed to demonstrate mucopeptide. The presence of mucopeptide-A carbohydrate complexes was inferred on the basis of previous studies (Schmidt, 1952; Haferkamp *et al.*, 1970) indicating that the uncomplexed carbohydrate or mucopeptide is rapidly excreted; only the conjugated complex was found to persist in cells.

Material and Methods

We used 30 mice (C57BL) of either sex, 55 to 60 days old, for these experiments. Each animal was injected intrarenally with 0.05 ml of an 18-hour culture of group A streptococci (strain B 196, type 17) in Todd-Hewitt broth as follows: The left kidney was exposed by lateral section during pentobarbital narcosis (0.3 ml of a 1:10 dilution injected intraperitoneally) and the streptococci were injected through the distal end of the kidney into the parenchyma, along the longitudinal axis of the organ. The incision was then clamped. Subsequently the mice were killed by decapitation at intervals of 1 to 98 days postinjection. The left kidney was dissected free and examined grossly for the presence of abscesses, pyonephrosis or hydronephrosis, or atrophy. Next the organ was halved longitudinally. One half was fixed in 4% formalin and embedded in paraffin; sections were prepared and stained with hematoxylin-eosin (HE) and Gram's stain. The other half was frozen in liquid nitrogen and stored at −70° C until further processing. The deep-frozen material was sectioned at 5 μ in the cryostat for immunofluorescence studies. The sections were air-dried, fixed in cold absolute ethanol for 5 minutes and rehydrated in cold phosphate-buffered saline (PBS), pH 7.2, for 30 minutes at room temperature. FITC-conjugated antibody was then layered onto the sections, followed by incubation for 30 minutes in a moist chamber at room temperature. Sections were washed and mounted by routine methods (Smith *et al.*, 1966a).

The two FITC conjugates employed had a molar F/P quotient of 4.6 to 4.8 (Wells *et al.*, 1966) and consisted of either monospecific rabbit antibody globulin to group A streptococcal C-carbohydrate (Smith *et al.*, 1966a; Sellin *et al.*, 1970) or corresponding antibodies to group A-variant C-carbohydrate. Both conjugates were used in dilutions of 1:5 to 1:40 which had proved effective in previous studies (Sellin *et al.*, 1970; Haferkamp *et al.*, 1970).

The specificity of the immunofluorescent stain observed was checked by "blocking" the reactions with the corresponding non-conjugated antibodies for 30 minutes prior to treatment with the homologous FITC conjugate. We confirmed the monospecificity of the reaction by demonstrating absence of staining with the heterologous anti-A-variant conjugate. To

achieve greater stability of the FITC-stained sections, we subsequently fixed some of the specimens for 30 minutes in absolute ethanol according to the method of Bienenstock and Dolezel (1970). Slides were examined with a Zeiss fluorescence microscope with an HBO-200 mercury bulb as the light source. Some of these were finally stained with HE and re-examined with a light microscope.

Results

Within a day after intrarenal injection of the mice, Gram's stain revealed streptococci surrounded by dense leukocytic infiltrates and undergoing phagocytosis. Streptococci stained with FITC-conjugated group A antibody showed characteristic strong yellow-green fluorescence of the cell wall, which is known to contain mucopeptide and group-specific carbohydrate (Fig. 1). Renal tubules draining these areas were frequently filled with granulocytes containing streptococci. Many of these organisms were intact, as demonstrated by Gram's stain and immunofluorescence (Fig. 1). During *days 1 to 15*, the purulent infiltrates became sharply demarcated from the surrounding healthy tissues, and an abscess membrane began to develop. Intact intracellular and extracellular streptococci remained identifiable in the center of the abscesses, decreasing in number towards the periphery. Whereas Gram's stain failed to reveal cocci in the abscess membrane, a few organisms were demonstrated by the more sensitive immunofluorescence method. Moreover, some of the macrophages in the peripheries of abscesses showed fine-grained fluorescent inclusions resembling those seen within the cells of liver granulomas (Sellin *et al.*, 1970). However, the majority of the macrophages in the kidney lesions contained no such fluorescent inclusions. Extracellular specific granular or diffuse fluorescence was not observed in the abscess membrane.

In 13 animals sacrificed from the *15th to the 98th day* after infection, HE staining disclosed histologic kidney changes of varying severity. The inflammatory process had progressed in six animals; in two cases, the abscess had ruptured into the kidney pelvis, with the development of pyonephrosis. Gram's stain and immunofluorescence staining revealed isolated intact streptococci within granulocytes and macrophages in the centers of the abscesses up to day 84. Whereas Gram's stain failed to show cocci in the abscess membrane, fine-grained fluorescent remnants of streptococcal cell walls were observed within some macrophages (Fig. 2). In five animals, the purulent inflammation had regressed spontaneously, and unilateral atrophy had developed between days 37 and 98. The atrophic inflammation consisted of macrophages, lymphocytes and isolated plasma cells. No streptococci were demonstrable by Gram's stain and immunofluorescence staining. However, fluorescent granules were present in the cytoplasm of a few macrophages (Fig. 2). As noted above, such granules were never seen extracellularly and were immunohistochemically demonstrated to contain group A

Fig. 1a–c. Mouse kidney 24 hours after intrarenal injection of live group A streptococci. a Early renal abscess with clumps of streptococci (arrows) in renal cortex (hematoxylin-eosin, ×64). b Streptococcal cell walls stained with FITC-labeled antibodies to group A C-carbohydrate. Note numerous morphologically intact streptococci in frozen section of early renal abscess (×500). c Myriad granulocytes in frozen section after hematoxylin-eosin staining (×400)

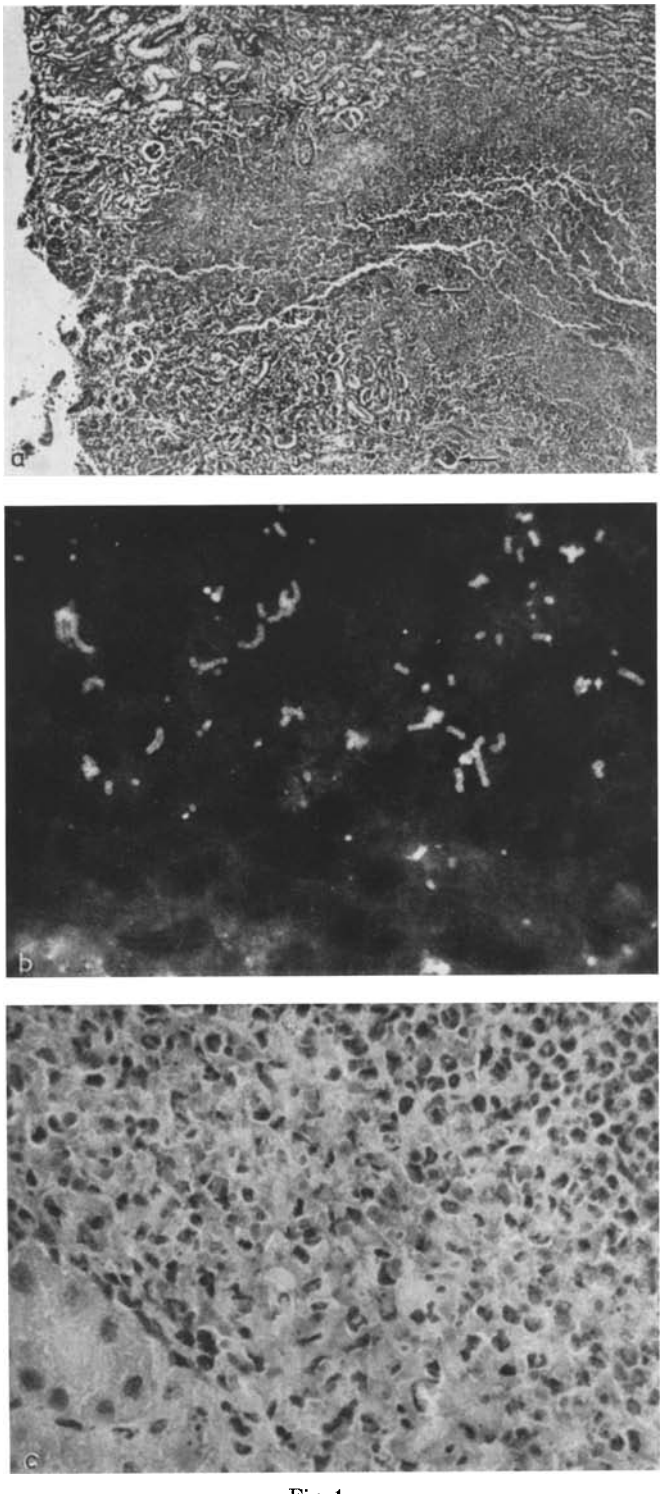

Fig. 1a–c

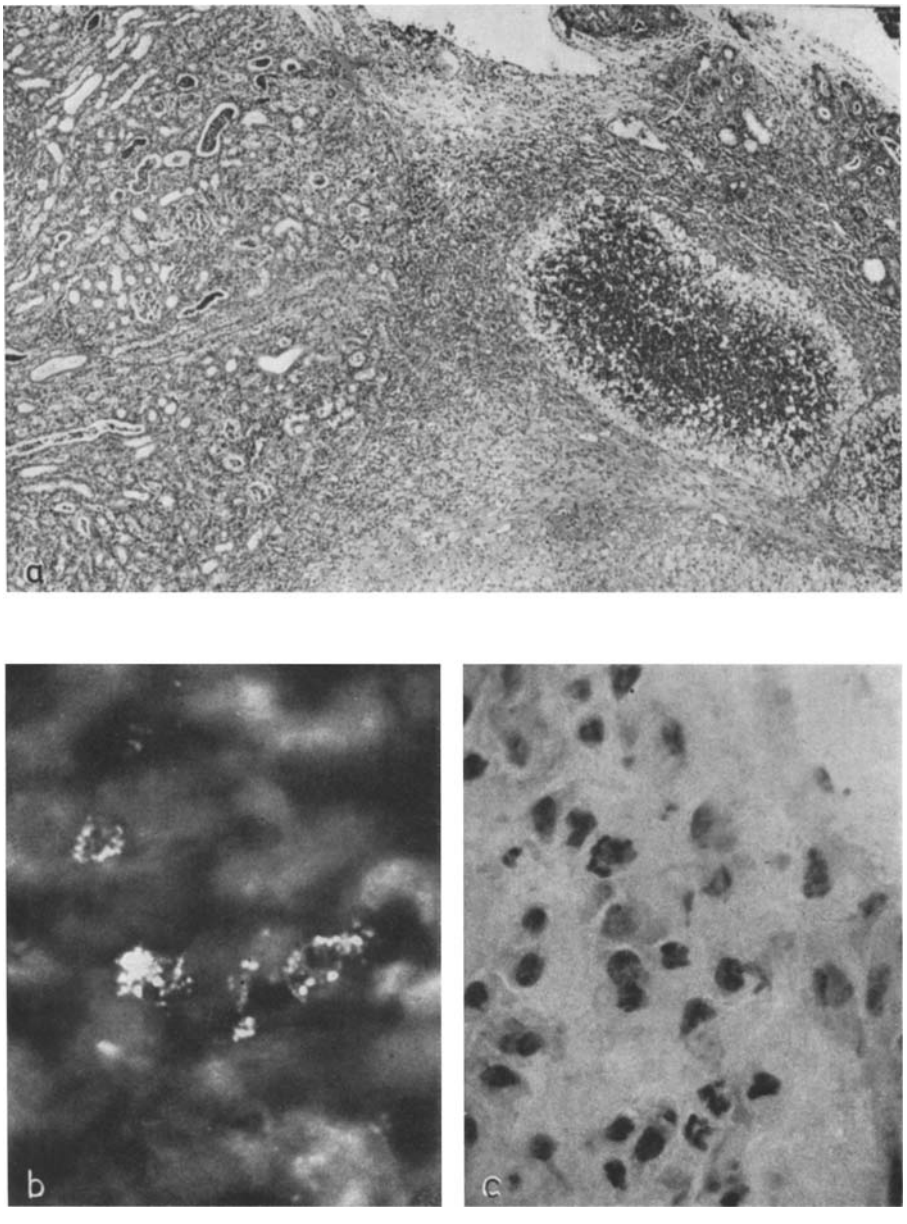

Fig. 2a–c. Mouse kidney 71 days after intrarenal injection of live group A streptococci. a A chronic pyogenic renal inflammation with old abscess, scarring, and infiltrating macrophages, lymphocytes and isolated plasma cells (hematoxylin-eosin, ×64). b Frozen section of kidney stained with FITC-labeled antibodies to group A carbohydrate. Immunofluorescence reveals fine-grained fluorescent remnants of streptococcal cell walls within macrophages of the area of chronic inflammation (×450). c Macrophages in frozen section after hematoxylin-eosin staining (×500)

carbohydrate. Reactions with the conjugated antibody to A-variant C-carbohydrate were consistently negative. Yellow-brown and blue-green fluorescence of cell contents was noted in some macrophages associated with chronic inflammatory foci in atrophic kidneys. This reaction, obtained with conjugated antibodies to both group A and A-variant C-carbohydrates, was not eliminated by blocking and was attributed to nonspecific binding of the conjugates to pigment stored by these cells, which also reacted positively to iron stain.

Discussion

Haferkamp *et al.* (1970) have identified streptococcal group A carbohydrate persisting as a mucopeptide-linked polymer within macrophages of murine liver granulomas induced by injection of killed streptococci. This group-specific cell-wall carbohydrate also persisted within macrophages associated with chronic pyogenic renal inflammation following the injection of live group A streptococci. Macrophages containing the carbohydrate were demonstrated in peripheral areas and membranes of kidney abscesses as well as in chronic inflammatory foci in kidneys atrophied as a sequel of streptococcal abscesses. However, the number of renal macrophages containing cell-wall components was considerably smaller than that observed in the hepatic granulomas (Sellin *et al.*, 1970). *In vivo* conversion of antigenicity from group A to group A-variant could not be demonstrated by the immunofluorescence method during the period of observation.

The smaller number of macrophages containing cell-wall residues found during and after renal abscess formation may be explained by the enzymatic activity of the intensive granulocytic response in the abscess (Cohn and Hirsch, 1960). This may lead to faster destruction of the bacterial cell wall than is the case in the lesser inflammatory response associated with the hepatic granulomas.

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