LOCALIZATION OF STREPTOCOCCAL MEMBRANES IN MOUSE TISSUES BY AN IMMUNOFLUORESCENCE METHOD

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The object of the present investigation was to study the localization, and the duration of persistence and of the pathogenic action of antigens of the streptococcal membrane, and especially of the M protein, following subcutaneous and intravenous injection into mice. Such an investigation is made necessary by the fact that attempts have been made to carry out prophylactic immunization of patients with rheumatism and persons suffering from frequent recurrences of tonsilitis and pharyngitis with streptococcal membrane containing M antigen [3, 4, 6]. Meanwhile, the biological action of this preparation has received inadequate study, and the pattern of its distribution and the duration of its stay in the tissues have not been investigated.

EXPERIMENTAL METHOD

To prepare the streptococcal membranes (SM) a 24 h culture of Streptococcus haemolyticus type 12 (a strain containing M antigen) and grown on Todd-Hewitt dialyzed broth was used. The mass of streptococci contained in 1 liter of broth was precipitated by centrifugation, washed three times with pyrogen-free water (after each washing the streptococci were precipitated by centrifugation), and suspended in 100 ml of pyrogen-free water. The streptococci were broken up in a Mikkl disintegrator (50 cps) for 30 min in separate batches containing 10 ml of streptococcal suspension (the degree of destruction of the bacterial cells was verified by means of the electron microscope). After the end of this process, the suspension containing the disintegrated streptococci was centrifuged for 60 min at 9000 rpm. The residue consisting of SM was washed in 5 changes of pyrogen-free water (after each washing the SM were precipitated by centrifugation for 30 min at 4500 rpm) and suspended in 75 ml pyrogen-free water, to which Merthiolate (1 : 10,000) was added. The nitrogen content in the SM suspension was 1.33 mg/ml of suspension.

Experiments were carried out on 57 albino mice weighing about 20 g. The animals of group 1 (24 mice) received a single subcutaneous injection of 0.5 ml of SM into the paratracheal cellular tissues of the neck. The animals of group 2 (18 mice) received a single intravenous injection of the same dose of antigen. The animals of group 3, the control group (5 mice), received an injection of 0.3 ml of a killed culture of Salmonella typhi 6S containing 3 billion bacterial cells per ml into the paratracheal cellular tissue of the neck. The animals of group 4, also controls (10 mice), were intact. The experimental animals were sacrificed in pairs 1, 2, 3, 5, 7, 10, 15, 20, and 30 days after injection of SM (in the case of the paratracheal injection of the antigen, the mice were sacrificed at the above times and also on the 25th, 45th, and 60th days after injection of the antigen). The animals of group 3 were sacrificed on the 5th and 7th days after injection of the killed culture of S. typhi 6S. Pieces of the internal organs and tissues (heart, lungs, liver, kidneys, spleen, paratracheal cellular tissue), not more than 5 mm in thickness, were fixed in ethanol cooled to 4°. Embedding in paraffin wax, preparation of serial sections, and removal of the paraffin wax were carried out by Sainte-Marie's method [5]. An immune antistreptococcal serum was obtained by immunization of rabbits with heated (56°, 30 min) streptococcal vaccine prepared from an M-antigenic strain of type 12 streptococcus. The titer of antibodies in the serum was determined by the precipitation reaction in capil-

lary T. The antistreptococcal serum thus obtained was exhausted with a streptococcus of heterologous type, after which it reacted only with an extract of streptococcus of homologous type. The prepared serum was sterilized by passage through a Seitz filter and preserved with Merthiolate.

The globulin faction of the antistreptococcal serum isolated from the serum by addition of ammonium sulfate (to 50% saturation) was conjugated with fluorescein isothiocyanate by the method of Coons and Kaplan as modified by Marshall and co-workers [2]. To remove excess of dye not used in the reaction, the conjugate was dialyzed for 11 days against buffer salt solution (0.02 M solution of carbonate-bicarbonate buffer, pH 9.0), after which the labeled globulin faction of the antistreptococcal serum was poured into ampules and lyophilized. Immediately before use, the labeled globulins were dissolved in distilled water and adsorbed twice with powdered mouseliver.

The antigens of the streptococcal membrane were detected in the organs and tissues of the experimental mice by a direct immunofluorescence method. The deparaffinized sections were washed three times in buffered salt solution (0.01 M solution of phosphate buffer, pH 7.2), and then treated for 1 h in a moist chamber at room temperature with the globulin faction of the antistreptococcal serum, labeled with fluorescein isothiocyanate; the sections were then washed in 5 changes of buffered salt solution and mounted in buffered glycerol. The microscopic sections were investigated by means of the ML-2 luminescence microscope in blue-violet light (filters FS-1 and BS-8). Since exhausted type-specific serum was used, in fact the immunofluorescence method did not reveal streptococcal membranes, but the M protein which they contain.

The specificity of the reaction was verified by an inhibitory test, for which sections of the organs of the experimental mice were first treated with unlabeled, and then with labeled antistreptococcal serum, by treating sections of organs of unimmunized mice (group 4) and of animals immunized with the killed culture of S. typhi 6S (group 3), labeled with antistreptococcal serum and, finally, by treating sections of the organs of the experimental animals with labeled nonspecific (antirabbit) serum. In all the controls listed above, no luminescence was observed in the sections of the organs, with the exception of the first control (the inhibitory test), when luminescence was still present, but its intensity was greatly reduced.

Besides the immunofluorescence investigation, sections of the organs of all the animals (both experimental and control) were stained with hematoxylin-eosin, azocarmine, toluidine blue, and methyl green-pyronine (control with ribonuclease).

RESULTS

Subcutaneous (paratracheal) injection of SM. In the first two days after injection of SM, the bulk of the material was found among extensive groups of partially dying neutrophils, and also in the cytoplasm of surviving neutrophils (Fig. 1a, b). On the third day the intensity of the neutrophil reaction in the paratracheal cellular tissue fell sharply; among the clusters of cells only solitary neutrophils were found, and most of the cells containing SM in the cytoplasm were macrophages with an extensive vacuolated cytoplasm and mainly with an eccentrically situated nucleus. On the fifth-seventh day of the experiment, extensive collections of cells were found in the paratracheal cellular tissue, consisting entirely of macrophages, the cytoplasm of which was tightly packed with intensively fluorescent SM (Fig. 1c, d and Fig. 2a). Throughout the subsequent period of the experiment (two months) the injected SM were found regularly only in the cytoplasm of the macrophages, forming loose or compact groups in the paratracheal cellular tissue, and the intensity of the fluorescence of the cytoplasm of the macrophages was essentially unchanged. SM were found only at the place of their injection and none were found in the internal organs.

Histological investigation of the internal organs and tissues of the experimental animals showed that the fibroblastic reaction at the point of injection of the SM was only very slight throughout the duration of the experiment. In the cytoplasm of the macrophages ingesting the SM, collections of PAS-positive material appeared; no RNA was found in the cytoplasm of the macrophages. In the lungs, kidneys, and spleen, no abnormality was found. In the heart, stimulation and proliferation (focal and diffuse) of the histiocytic elements of the stroma were observed, and sometimes focal collections of histiocytes were seen around the blood vessels and in the region of the annulus fibrosus of the valves and the coronary fissure; in isolated cases some muscle fibers were destroyed and undergoing phagocytosis. In the liver of the animals killed on the 5th day after injection of SM or later, focal degenerative changes and the development of "histiocyte nodules" (next page) were observed in places where the degenerative changes were most marked.

Intravenous injection of SM. After intravenous injection of SM their localization in the internal organs of the experimental mice was identical throughout the experiment (30 days). In the lung tissues, SM were found in ex-

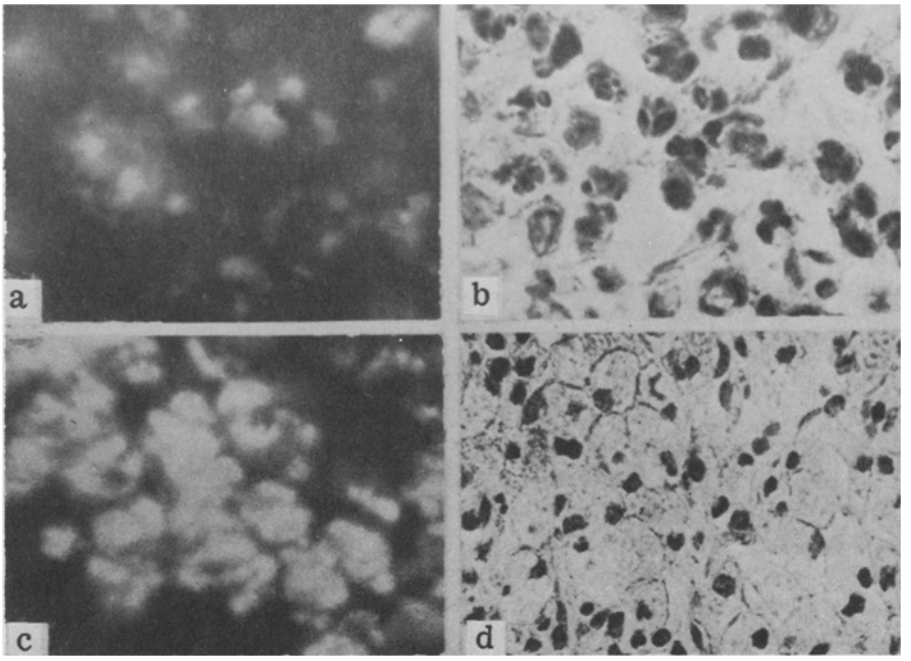

Fig. 1. Subcutaneous cellular tissue at the site of injection of SM on the 2nd (a and b) and 7th (c and d) days. a) Intensive fluorescence in the cytoplasm of neutrophils containing SM after treatment of the preparation with antistreptococcal serum labeled with fluorescein isothiocyanate; b) staining with hematoxylin-eosin; c) intensive fluorescence in the cytoplasm of macrophages containing SM after treatment of the preparation with antistreptococcal serum labeled with fluorescein isothiocyanate; d) staining with hematoxylin-eosin. Magnification 400x.

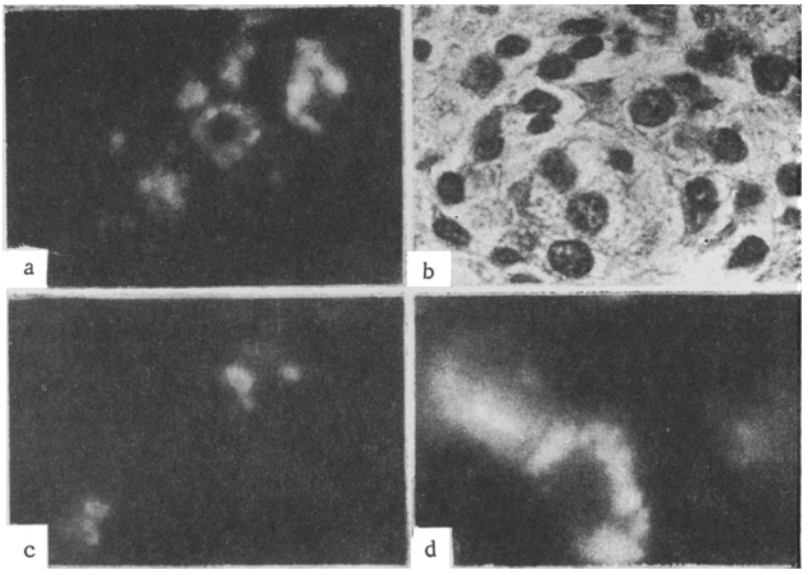

Fig. 2. Intravenous injection of SM. Intensive fluorescence of OS absorbed by histiocytic elements of the liver, forming a "histiocytic nodule" (a), and by histiocytic elements of the spleen after treatment of the preparations with antistreptococcal serum labeled with fluorescein isothiocyanate (c). Magnification 400 x ; b) "histiocytic nodule" in the liver. Stained with hematoxylin-eosin. Magnification 900 x.

tremely small quantities in only two mice. Cells containing SM in their cytoplasm were usually arranged in small groups in the alveolar septa of the subpleural portions of the lungs. In the liver, SM were found as a rule in small numbers. They occurred in the cytoplasm of the Kupffer cells and histiocytes; the latter formed multiple focal collections in the parenchyma of the liver (Fig. 2a, b), and also around the central vein (histiocyte nodules). In the liver cells there were no SM. In the spleen, in most cases far fewer SM were found, than in the liver. SM were present in the cytoplasm of the macrophages and histiocytes scattered throughout the red pulp (Fig. 2c); no SM were found in the cells of the lymphoid follicles. SM were absent from the tissues of the heart and kidneys.

Histological examination of the internal organs of the mice receiving SM by intravenous injection revealed no significant abnormalities in the heart, lungs, and kidneys. In the liver (in the parenchyma and close to the central vein) focal degenerative changes were found in the liver cells, sometimes reaching the stage of necrobiosis; in these areas, clusters of proliferating histiocytes were found among the dying liver cells, containing no glycogen or RNA in their cytoplasm (in some of these cells SM were detected by the immunofluorescence method). Since the histiocytes in the foci of degeneration were numerous and compactly arranged, the corresponding areas of the liver had the appearance of "histiocyte nodules." The maximal number of these histiocyte nodules in the liver tissue was observed in the mice sacrificed 7-30 days after injection of SM. In the spleen of the animals moderate reactive changes in the pale germ centers of the follicles starting on the 10th day after immunization (an increase in the number of immature cells of the lymphocyte series containing RNA in their cytoplasm), and also a slight increase in the number of reticulum cells, immature plasma cells, and blast cells in the red pulp.

To summarize the changes described above, it can be said that after subcutaneous (paratracheal) injection of SM, the latter were found only at their site of injection. During the first two days after injection they were ingested by neutrophils (the neutrophil phase), and starting with the 3rd day they appeared in the cytoplasm of the macrophages (the macrophage phase), where they persisted for a long time (over two months). After intravenous injection, the SM accumulated selectively in the reticulum cells and histiocytes of the liver and spleen and were not held up in the lungs and heart, although as the antigen was distributed throughout the body, the lungs and heart were the first organs it reached.

In both experiments degenerative changes were observed in the liver parenchyma, varying in severity. It may be considered that these changes resulted from the toxic action of the M protein or C polysaccharide contained in the SM [7, 8].

Hence, unlike the soluble free M protein, which persists only temporarily in the tissues and organs of mice [1], the M protein bound in SM may persist for long periods in the animal organism. During this long persistence of SM, M protein may be liberated either quickly or slowly. In the first case, conditions are provided for intensive antibody formation, and thus for the development of a lasting immunity. In the second case, antibody production is low, and it is possible for incomplete antibodies to be formed; these may be absorbed by the tissues, thus leading to sensitization of the organism.

SUMMARY

The direct immunofluorescent method was used to study the distribution and duration of persistence of the streptococcus membranes in the tissues of mice after their subcutaneous (paratracheal) and intravenous injection. It has been shown that in paratracheal injection of the streptococcus membranes the latter are discovered only in the cellular tissue at the site of their entry. At first they are absorbed by neutrophils and then accumulated in the cytoplasm of the macrophages where they are retained for more than 2 months. In the case of intravenous injection of streptococcus membranes one observes their selective absorption by the reticulo-histiocytic cells of the liver and spleen.

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