Persistence of Group A Streptococci Labeled with Fluorescein Isothiocyanate in Inflammatory Sites in the Heart and Muscle of Mice and Rabbits* (33917)

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(Introduced by T. N. Harris)

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Studies on the host parasite relationship in experimental streptococcal infections have been greatly aided by fluorescent antibody techniques (1). By such methods it is possible to follow the localization and persistence of group A streptococcal antigen in cells and tissues of laboratory animals (2–6). To detect such antigens by fluorescent techniques, frozen sections are often employed and the antisera to streptococcal antigens must be repeatedly absorbed with organ powders to remove nonspecific staining.

The present communication describes a simple method for applying a label of fluorescein isothiocyanate (FITC) to living group A streptococci, streptococcal cell-wall fragments, mucopeptides, and streptococcal L-forms. Such labeled streptococci and products were found to persist for long periods of time in inflammatory sites in the muscle and heart of mice and rabbits.

Materials and Methods. Bacterial strains: Group A streptococci (Type 6 and 12) were kindly supplied by Dr. S. Rabinowitz, of the Streptococcus Reference Laboratory, Government Central Laboratories, Ministry of Health, Jerusalem. The streptococci were cultivated in brain heart infusion broth (Difco), harvested at the end of the logarithmic phase of growth and washed with saline buffered with 0.05 *M* PO_4, pH 7.4 (PBS). The optical density (OD) of the suspensions was brought to 250 with a Klett photocolorimeter at 540 mμ.

Streptococcal products. Cell fragments were obtained from group A streptococci by disruption for 60 min in a 9-kc Raytheon sonic oscillator at 10°. The bacterial cell debris was then removed by centrifugation at 3000*g* for 10 min, washed in PBS and resuspended in the same buffer.

Lyophilized group A streptococcal L-forms (Strain GL-8) were kindly supplied by Dr. C. Panos of the Albert Einstein Medical Center, Philadelphia. The L-forms were suspended in buffer at 10 mg/ml. Group A streptococcal group-specific polysaccharide, and its insoluble residue, containing mucopeptide, were prepared from streptococcal cell walls by the formamide extraction method described by Heyman *et al.* (7).

Labeling of bacteria and products with FITC. To 5-ml aliquots of bacterial suspension, mucopeptide, cell debris, or L-forms FITC (N. B. C. Cleveland, Ohio) was added at a final concentration of 10, 25, 50, 100, and 250 μg/ml. After stirring for 30 min at room temperature with a magnetic stirrer the bacterial suspensions and products were washed 3 times with 50 ml of buffered saline, to remove unbound FITC, and were resuspended in the original volume of the buffer.

Determination of the viability of group A streptococci. Following labeling with FITC, the bacterial suspensions were plated in serial 10-fold dilutions and the number of viable bacteria was determined, using 10 ml of melted blood agar base (Difco) to which were added 1 ml of whole rabbit blood; and

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0.5 ml of dilutions of the bacterial colonies at the various dilutions was determined.

Determination of acid production. To determine the effect of FITC on the metabolism of the streptococci, 1-ml aliquots of unlabeled or FITC-labeled cells were incubated with 0.01 *M* glucose, 0.01 *M* $MgCl_2$ and 0.001 *M* cysteine (8). The volume of the reaction mixture was brought to 2.0 ml with 0.05 *M* PO_4 buffer, pH 7.4. The amount of acid produced by the streptococci after 60 min at 37° was determined by titration with 0.05 *N* NaOH.

Analytical methods. Streptococcal cell debris and mucopeptide were analyzed for their content of rhamnose by the method of Dische and Shettles (9), and for glucosamine by the method of Elson and Morgan (10). Cell-bound streptolysin S was assayed according to Ginsburg and Bentwich (8).

Injection of animals. Twenty Swiss mice weighing 20 g were injected intramuscularly in the thigh. Ten rabbits of a local stock weighing 2–3 kg were injected intramyocardially also, as described previously (11). The dose was in each case 0.1 ml of unlabeled or FITC-labeled streptococci products.

Histological technics. Animals were sacrificed 1–120 days following injection, and slices of tissues at the site of injection and portions of lung, liver, spleen, kidney, and lymph nodes were fixed in 10% neutral formalin. Paraffin sections of 4–6 μ were stained with hematoxylin, hematoxylin–eosin, and with the tissue gram stain (12). Both unstained and stained preparations were covered with a drop of glycerol and examined for fluorescence with ultraviolet light.

Fluorescent microscopy. An Ortholux phase fluorescence microscope with an automatic camera (Orthomat) (Leitz Co.) was used. The pass filter was a 3-mm BG12. A 1-mm UG5 pass filter was added to the system for ordinary light microscopy. The eye piece barriers were the Euphos UV filter and the source of light was an Osram HBO 200 lamp. In some experiments photographs were taken with a combination of visible and ultraviolet light (see below).

Results. Labeling of bacteria with FITC. Under the experimental conditions described the streptococci as well as group A streptococcal L-forms were labeled with as little as 10 μg/ml of FITC. The label could not be removed by repeated washings with PBS. It was also found that neither group A streptococci nor the mucopeptide lost their stainability with FITC on treatment with 0.1 *N* HCl, 0.1 *N* NaOH, ethanol, acetone, chloroform, ethyl acetate, fluorocarbon, trypsin, papain plus cysteine, ficin plus cysteine, pepsin, chymotrypsin, pronase, or by HCl followed by formamide extraction [Fuller (13)]. The results suggested that FITC was not bound to the streptococcal M protein (which is known to be destroyed by trypsin, and to be extracted by boiling with HCl) or to the group-specific polysaccharide, which is removed by formamide (13). To test this conclusion C-polysaccharide and mucopeptide were prepared from a type 6 streptococcus (7). The purity of these products were checked by quantitative determination of rhamnose and glucosamine. It was found that the C-polysaccharide preparation did not bind FITC. On the other hand the mucopeptide, which contained only 5% of rhamnose, but still contained cell membranes, took up FITC which could not be washed away with the buffers or solvents indicated above. It was also found that the viability of group A streptococci was unaffected by staining with FITC at 50 μg/ml. At 100 μg/ml the viable streptococcal count dropped by one log step, while at 250 μg/ml a 3–4 log step reduction of colony counts was obtained. Similar results were obtained by Grunberg and Cleeland (14) for *Proteus mirabilis* and *Staphylococcus aureus.*

In other experiments it was found that the production of acid from glucose by resting streptococci was unaffected by FITC at 100 μg/ml, and that this concentration of the dye did not affect the phosphate esterase and *N*-acetylglucosaminidase activities of washed streptococci. Cell-bound streptolysin S activity of the streptococci was reduced by 25% on exposure to FITC at this level.

Under the conditions studied, unstained histologic preparations were found suitable for the demonstration of fluorescence in tissues injected with FITC-labeled streptococci

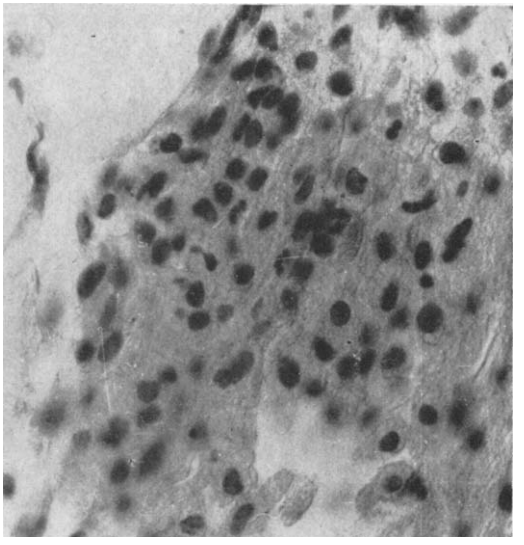

FIG. 1. Replacement of mouse muscle with histiocytes and inflammatory exudate 30 days following the injection of FITC-labeled group A streptococci; hematoxylin, ×420.

and products. Staining with hematoxylin for 1–2 sec did not quench fluorescence, but longer periods of time markedly diminished the intensity. Paraffin blocks as well as unstained histologic sections could be kept in a dark cool place for many weeks without a marked loss of fluorescence.

Localization of FITC-labeled Streptococci in tissues. (1) *Experiments with mice.* At the site in the thigh muscle, injected with group A streptococci, unlabeled or labeled with FITC, an abscess usually developed and spread along the muscle fibers. Figure 1 shows an inflammatory area in the muscle 30 days after the injection of FITC-labeled streptococci. The muscle fibers have been replaced by an inflammatory exudate consisting of histiocytes, lymphocytes, and fibroblasts. Many fluorescent granular clumps were found within histiocytes and some clumps were free in the interstitium (Fig. 2). Somewhat less intense fluorescence was observed 120 days after the intramuscular injection of FITC-labeled streptococci. Similar results were obtained with labeled group C streptococci and with *Streptococcus mitis*. Figure 3 shows the distribution of fluorescent particles in an inflammatory site in mouse muscle 4 days following the injection of FITC-labeled streptococcal cell debris. This photograph was taken with a combination of UV and visible light.

Attempts to culture streptococci from the injected tissues beyond 4–6 days failed. In a few cases, tissue gram stains performed on sections taken beyond 7 days after injection showed several gram-positive dots within histiocytes. In some experiments macrophages filled with fluorescent particles were found in the regional lymph node, 96 days following the injection of FITC-labeled streptococci, at a time when no trace of inflammation was evident in the muscle. Mice injected with labeled cell-wall debris or mucopeptide showed intracellular fluorescence in macrophages in inflammatory sites 60 days following injection.

On the other hand, following the injection of FITC-labeled streptococcal L-forms, no trace of fluorescence could be detected beyond 3 days.

(2) *Experiments with rabbits.* At the site of injection of unlabeled or FITC-labeled streptococci or mucopeptide in the thigh muscle or in the myocardium there were a number of large oval areas of myofiber destruction, as described elsewhere (15), sur-

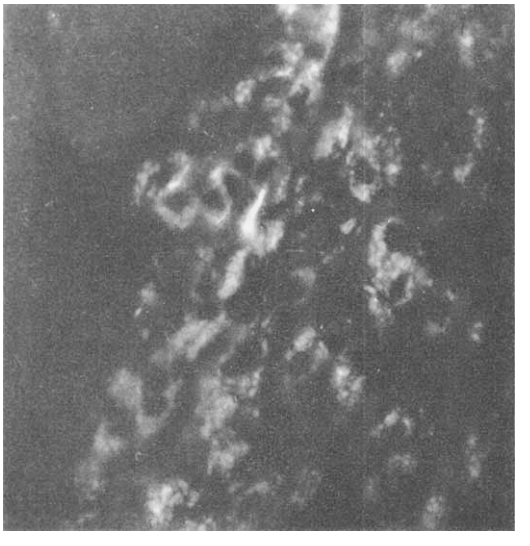

FIG. 2. Same field as Fig. 1, photographed with a fluorescence microscope. Note the bright fluorescent particles located perinuclearly; ×420.

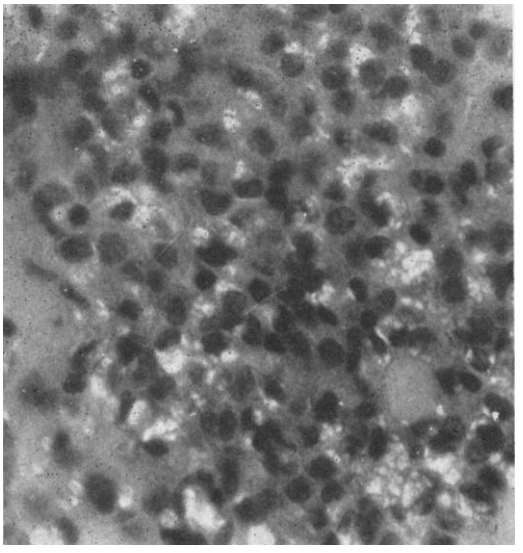

FIG. 3. Inflammatory area in the mouse muscle 4 days following the injection of FITC-labeled streptococcal cell fragments derived by sonication. Photographs were taken with a combination of ultraviolet and visible light. Note bright fluorescent clumps within the cytoplasm of histiocytic cells; Hematoxylin, × 640.

rounded by inflammatory and granulomatous zones (11). The centers of these zones consisted of cellular debris and ghost-like remnants of necrotic myofibers. While at the periphery there was much inflammatory exudate, consisting largely of granulocytes and of groups of large sharply demarcated polyhedral histiocytes with clear, finely granulated cytoplasm. The cytoplasm of these polyhedral histiocytes (Fig. 4) contained large amounts of finely granular specifically fluorescing material, similar to that illustrated in Fig. 2. The tissue response to injections of FITC-labeled streptococci products were identical with that occurring following the injection of the unlabeled material. Macrophages loaded with fluorescent particles were detected in heart lesions up to 90 days following the intracardiac or intramuscular injection with labeled streptococci.

In rabbits injected intratonsilarly (16) with FITC-labeled viable type 6 streptococci and sacrificed 48 hr later, fluorescence was mostly concentrated at the site of injection. Histiocytes containing fluorescent particles were found rarely in the cervical lymph nodes, in the Kupffer cells of the liver, and in macrophages of the spleen.

Discussion. A variety of bacterial species were labeled directly with FITC and at the low concentration of FITC required neither the viability of the streptococci so labeled nor their capacity to form acid from glucose was markedly affected. These results are similar to the findings of Grunberg and Cleeland (14) and of Pital *et al.* (17) for a variety of gram-positive and gram-negative organisms. Since the labeling of group A streptococci with FITC was not prevented by proteolytic enzymes, by organic solvents or by removing the C-polysaccharide by formamide, the binding sites in group A streptococci for FITC are probably associated with the mucopeptide of the cell wall.

Also, since group A streptococcal L-forms which are devoid of mucopeptide, took up FITC, it is possible that some of the staining seen in the mucopeptide preparation was due to the presence of streptococcal membranes. The site of attachment of FITC in the bacterial cells is, however, still unknown, and further studies concerning the binding sites

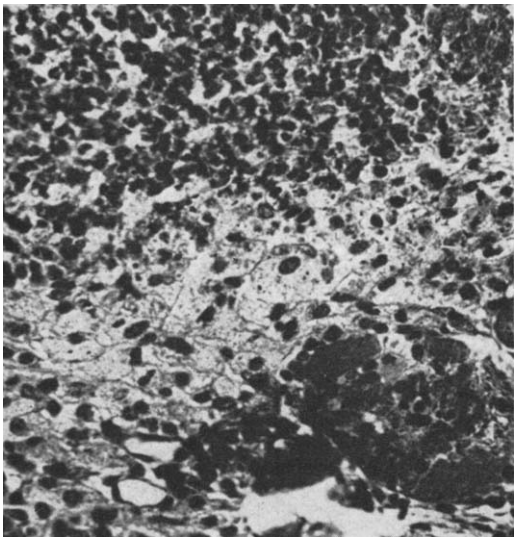

Fig. 4. Rabbit heart 14 days following the injection of FITC-labeled type 6 streptococci. Note phagocytosis of necrotic myofibers and the accumulation of large polyhedral cells. Fluorescent particles were found to fill only the cytoplasm of these cells; hematoxylin–eosin, ×480.

in streptococci for FITC are in progress. The strong complex which forms with the streptococcal cell components and the fact that the dye affects neither the viability nor the metabolism of the organisms (see also 14, 17) suggest that the fluorescent particles seen within macrophages are in fact persistent bacterial components. It may therefore be concluded that shortly after the injection of the FITC-labeled organisms into the muscle or heart, the labeled cells are taken up by phagocytic cells but while the organisms are readily killed intracellularly the cell wall components are not degraded to a large extent. On the other hand the L-forms, which lack a rigid cell wall, are readily digested by the host's enzymes. This conclusion is supported by the findings of Ohanian and Schwab (6, 18), who demonstrated with fluorescent antibody technic that 2 major antigenic components, mucopeptide and C-polysaccharide, prersisted together in skin lesions in rabbits for at least 8 weeks.

The persistence of fluorescent streptococcal products within macrophages for long periods of time may be due either to the lack of enzyme systems in mammals capable of degrading cell wall components, or to the modification by FITC of cell-wall components that otherwise would have been degraded by tissue enzymes. With respect to the first point, it was recently shown (19) that although rabbit and human macrophages possess an *N*-acetylglucosaminidase capable of a partial degradation of C-polysaccharide this enzyme did not lyse streptococcal cell walls. Moreover, Schwab and Ohanian (18) indicated that the presence of rhamnose in the mucopeptide preparations masks the latter and protects it from being split by tissue lysozyme. On the other hand, enzymes capable of lysing the cell walls of group A streptococci have been isolated from phage lysates of group C streptococci and from *Streptomyes albus* cultures (21) but not from mammalian sources, which may explain the prolonged persistence of FITC-labeled streptococci and products in the tissues of the mouse and the rabbit.

The second possibility, that FITC labeling interfered with cell-wall lysis by tissue enzymes must also be considered. However, the inability at the present time to demonstrate such enzymes in mammalian tissues precludes the performance of such an experiment. Preliminary experiments have, however, shown that 2 muralytic enzymes (group C streptococcal phage lysin and lysozyme) were not inhibited by 100 μg/ml of FITC. Also, both group A streptococci and *Micrococcus lysodeikticus* labeled with 100 μg/ml of FITC were as susceptible to lysis by muralysins as were the unlabeled organisms. Also, no trace of fluorescence was found after 3 days in the muscle of mice injected with FITC-labeled *M. lysodeikticus*. These results suggest that the activity of muralysins of tissue origin if present may also not be affected by FITC labeling of the substrate. The long periods of observation of intracellular fluorescent particles thus suggest the absence of enzyme systems capable of degrading streptococcal cell-wall components.

The tissue response of mouse and rabbit muscle to the injection of streptococci and mucopeptide differ to a large extent. While the mouse muscle responds with nonspecific inflammation, rabbit muscle always responds with the development of a granulomatous lesion (Fig. 4) (11, 15, 16). The very long persistence of streptococci and mucopeptides in the tissues visualized by the direct labeling technic and by electron microscopy in current experiments may shed light on the possible role of streptococcal cell-wall components in the pathogenesis of poststreptococcal sequelae and also may open a new approach to the study of host–parasite interrelations.

Summary. A simple method of labeling living group A streptococci with fluorescein isothiocyanate (FITC) is described. Low concentrations of FITC (10–50 μg/ml) labeled group A streptococci but affected neither the viability of the cells nor the capacity of the streptococci to produce acid from glucose or to form cell-bound streptolysin S. Mucopeptide prepared from group A streptococci by the formamide method and group A streptococcal L-forms were readily labeled with FITC. On the other hand C-polysaccharide could not be labeled with

FITC when in solution. Group A streptococci, streptococcal cell debris, and mucopeptide labeled with FITC injected into the thigh muscle of mice and rabbits and intramyocardially into rabbits persisted intracellularly in histiocytes for several months. Since FITC-labeled group A L-forms, which are devoid of cell walls, disappeared from the site of injection within 24–72 hr it is suggested that the long persistence of streptococci and products in the tissues is probably due to the inability of the host enzymes to degrade cell-wall components of streptococci. The direct method of labeling streptococci and products with fluorescent material may prove useful to the study of host–parasite relationships.

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