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FATE OF STREPTOCOCCAL M PROTEIN AFTER EXPOSURE TO PLASMIN AND HUMAN LEUKOCYTES**

Untreated group A streptococcal infections are followed by a durable type-specific immunity. Antibodies responsible for this immunity are directed against the M antigen and although late in appearance, have been reported to persist for 30 years.[1] Attempts to induce type-specific immunity in humans by means of bacterial vaccines and partially purified preparations of M protein[2] have met with limited success. The contrast of long lasting natural immunity with the weak response to purified antigens led to these studies on the disposition of M protein in host tissues and fluids.

In the present work M protein has been exposed to streptokinase-activated plasminogen, and to viable human leukocytes. Following such exposure the fate of M antigen, in purified form and intact on the bacterial cell wall, has been explored by precipitin and immunofluorescent techniques.

MATERIALS AND METHODS

One lyophilized strain of type 12 (SF42)† and two strains of type 1 streptococci were used. Strain T1/162/7 was obtained in the lyophilized state‡ and strain T1/2788 as phenolized bacterial cells.¶ After repeated serial mouse passage the LD50 of both T1/162/7 and SF42 for 30 gram white mice reached 10-50 organisms: daily subcultures from human blood agar plates were then employed and new cultures were taken from lyophilized stock whenever loss of virulence was shown by an inability to grow in normal human blood.[3]

Heterologous type strains were similarly maintained and were used in absorbing and testing specificity of antisera: they were 165/2 (type 3), 169/2 (type 6), and C203 (type 3).

Media: All streptococcal cultures were grown in Todd-Hewitt (Difco) media. Subcultures were plated on 5 per cent human blood agar.

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Preparation of M protein: Partially purified M protein was prepared from T1/2788 streptococcal cells by the method of Lancefield and Perlmann.[4] Boundary electrophoresis of this material at pH 8.6 revealed migration as a single peak but with sufficient boundary spreading to suggest some heterogeneity. No further purification was attempted.

Enzymes: Plasminogen (profibrinolysin) was prepared from Cohn's plasma Fraction III, by the method of Kline.[5] Starting with 10 gm. of Fraction III the final enzyme solution was concentrated by lyophilization and dissolved in 4 ml. of distilled water to which two drops of N HCl were added. Streptokinase was obtained as Varidase® (Lederle lot 2200-630A), a commercially available preparation also containing desoxyribonuclease. Dilutions were prepared in 0.01 M phosphate buffer at pH 7.6. In preliminary experiments casein was used as substrate; proteolysis was determined by an increase in optical density at 280 mμ of the filtrate following trichloracetic acid precipitation. The use of skimmed milk was adopted as a substrate to monitor proteolytic activity following the methods of Kramer[6] and Barnett.[7] The advantage of this system is that proteolysis of suspended casein leads to clearing of opalescent skimmed milk, an easily perceived end-point. Skimmed milk (Pet®) was obtained in the dried form, prepared as a 20 per cent solution, autoclaved for 10 minutes at 15 lb. pressure and tested for sterility.

Leukocytes: The same human donor was used for all leukocyte experiments. Blood was aseptically withdrawn from the antecubital vein into a syringe containing heparin (Liquaemin,® Organon, Inc.), in final concentration of 0.1 mg/ml. The syringe was placed vertically in the refrigerator and blood allowed to sediment for 1-2 hours; thereupon the plasma and buffy coat were removed and the leukocytes washed, at 4°C., three times in Tyrode's buffer containing .1 per cent gelatin (Knox). Leukocytes were counted in a Neubauer counting chamber using 1 per cent acetic acid as diluent. No attempt was made to further separate red cells from the leukocyte preparation.

Leukocyte "buttons" were prepared by a modification of a method originally described by Wright.[8] A drop of fingertip blood was allowed to clot on a clean glass slide in a moist Petri dish at 37° for 30 minutes. The clot was then covered with Gey's buffer solution containing 1 per cent human serum albumin and removed with a fine forceps. Leukocytes persisted as a disc, affixed to the surface of the slide, even after vigorous washing in Gey's albumin buffer. These leukocytes, largely polymorphonuclear, remained viable and phagocytic.

Antisera: Group and type-specific antistreptococcal sera were made available through the courtesy of Dr. John Winn, Diagnostic Reagent Section, Communicable Disease Center, in Chamblee, Georgia. Precipitin tests were performed with the capillary tube technique.[9]

Immunofluorescent methods: Fluorescein conjugates were prepared from antisera obtained by immunizing rabbits with repeated injections of whole heat-killed organisms for a five week course. Strain No. SF42 was used to prepare type 12 antisera, and strain No. T1/162/7 for type 1 antisera. Two weeks after the last injection rabbit sera were tested for the presence of type-specific antibody by direct and indirect bactericidal tests.[8] These were confirmed by production of long chains of homologous streptococci grown in the antiserum.[10] Gamma globulin prepared from antisera was conjugated with fluorescein isothiocyanate by the method of Riggs, *et al.*[11] After exhaustive dialysis the conjugate was purified on DEAE cellulose (Eastman) column.

Ion exchange material was first cycled three times with N HCl alternating with N NaOH. It was then washed with 0.005 M phosphate buffer at pH 8.3 in a 4-liter beaker and allowed to sediment for one hour. The supernate, which contained small fibers, was decanted and the washing repeated three times. The suspension of ion exchange material was then poured into a 20 mm. column and allowed to pack by gravity. Approximately one liter of 0.005 M buffer was run through the column by gravity alone. The effluent was then checked to confirm the pH of 8.3. Fluorescein conjugated gamma globulin was dialyzed against starting buffer and run into the column at six drops per minute. After loading with approximately 100 mg. of protein, 0.005 M buffer was again run through the column at the same rate. Little or none of the fluorescein conjugate appeared in the initial effluent. With change of buffer to 0.06 M phosphate at pH 6.3, a band of fluorescent material could be followed with a Wood's lamp as it traveled down the column. The effluent was collected in 150 ml. of buffer. After concentration to 20 ml. by flash evaporation, the conjugate was dialyzed against 0.01 M phosphate buffered saline at pH 7.2. It was then absorbed twice with equal volumes of packed bacterial cells of strain No. T6/169/7 (type 6) and C203 (type 3). Each absorption proceeded at 37° for two hours with mild agitation and was completed by overnight incubation at 4° C. To remove non-specific staining properties for human leukocytes, the conjugate was finally absorbed with ½ volume of packed human spleen homogenate prepared as follows: freshly obtained human spleen tissue weighing 20 gm. was minced in 40 ml. of saline and homogenized in a Waring blendor at 4° C. until a fine suspension was made. The resulting homogenate was frozen and thawed to effect lysis of the red cells, and washed five times with buffered saline to remove hemoglobin from the suspension. The washed homogenate was then centrifuged in conical tubes at 2,500 rpm for 30 minutes and the fluorescein conjugate added to packed tissue homogenate. Finally, the purified, absorbed conjugate was dispensed in 2 ml. screw-capped vials and frozen at -20°C. until used. No preservatives were added.

Fixation and staining: Smears of bacterial suspensions were heat fixed. Slides containing leukocyte discs were fixed by rapid drying in an air jet. To a dry slide, one drop of specific conjugate was added, and reaction allowed to occur for 50 minutes in a moist chamber. The slides were then washed in two changes of buffered saline, pH 7.2, for 10 minutes, with gentle agitation on a shaking machine. Wet mounts were prepared with glycerol buffer.

Specificity of staining: The following criteria were met to control specificity of staining: 1) conjugates were completely absorbed with homologous partially purified M protein; 2) fluorescent staining was inhibited by prior treatment of slides with unlabelled specific antisera; 3) heterologous conjugates failed to stain test organisms; 4) pepsin-treated bacterial cells did not stain.

For the last control bacterial cells were suspended in a 2 per cent solution of pepsin (Difco, 1:10,000) and the reaction lowered to pH 2. After incubation for two hours at 37° C. the suspension was neutralized and the cells washed twice in the cold. Controlled suspensions without pepsin were always included.

Microscopy and photography: The Leitz microscope was equipped with an Osram HBO 200 ultraviolet light source, a UGI 2 mm. exciter filter, and the D1.20 dark field condenser; a T-tube arrangement allowed alternate illumination of the same microscopic field with white light and ultraviolet light. Photographs were made

in color with Super Anscochrome film, daylight type, using 2-4 minute exposures for fields illuminated with ultraviolet light, and 10-30 second exposures for those illuminated with white light.

EXPERIMENTAL RESULTS

Effect of streptokinase-activated plasminogen on M protein

In preliminary experiments whole serum was used as a source of plasminogen; no effect on M protein could be demonstrated. A semi-quantitative system was devised so that proteolytic activity could be assayed.

TABLE 1. EFFECT OF TRYPSIN ON SKIM MILK AND "M" PROTEIN SUBSTRATES [24 HOURS AT 37°]

Dil. of Trypsin 0.8 ml.	*Substrate [0.2 ml.]*	
	Skim Milk	*"M" Protein*
10^{0}	0	0
10^{-1}	0	0
10^{-2}	0	0
10^{-3}	0	TR.
10^{-4}	0	+++
10^{-5}	++++	++++
10^{-6}	++++	++++
Control	++++	++++

Trypsin was selected as an enzyme reference because of its known ability to destroy M protein.[4]

Serial dilutions of crystalline trypsin (General Biochemicals, Inc.) were prepared from a 0.1 per cent solution of 0.01 M phosphate buffer at pH 7.6, and filtered through a Swinney filter. To 0.8 ml. of trypsin dilution, 0.2 ml. containing 500 gamma of type 1 protein was added. A similar amount of skimmed milk was added to duplicate trypsin tubes. After incubation at 37° for 24 hours, the tubes containing skimmed milk were read as follows: 0 — complete clearing; 1+ — slight opalescence; 2+ — moderate opalescence; 3+ — slight clearing; 4 + — no change from control tube. After incubation tubes containing M protein were placed in a boiling water bath for ten minutes to inactivate the trypsin. Capillary precipitin tests were performed using type-specific antiserum. A maximal precipitin reaction was recorded as 4+; no reaction was indicated by 0. Results of this experiment are presented in Table 1.

Approximately 10 to 100 times the concentration of trypsin needed to clear skimmed milk was required for hydrolysis of M protein. Under conditions of the experiment this relationship was constant. To determine whether streptokinase-activated plasminogen was capable of M protein hydrolysis, concentration of pro-enzyme was necessary so that in dilution of 10^{-3}, the proteolytic system would clear skimmed milk. Sufficient concentration was achieved by starting with 10 grams of Fraction III and dissolving the lyophilized pro-enzyme in a 4 ml. volume as described above. Activation of the concentrated plasminogen in the presence of substrate was optimal with five units per milliliter of streptokinase. In sub-

TABLE 2. EFFECT OF STREPTOKINASE-ACTIVATED PLASMINOGEN ON SKIM MILK AND "M" PROTEIN SUBSTRATES [24 HOURS AT 37° C]

Streptokinase [100 U/ml]	*Plasminogen [0.2 ml.] Dilution*	*Substrate [0.2 ml.]*	
		Skim Milk	*"M" Protein*
0.6 ml.	10^{0}	0	0
0.6 "	10^{-1}	0	0
0.6 "	10^{-2}	0	0
0.6 "	10^{-3}	0	++
0.6 "	10^{-4}	++++	++++
0.6 "	10^{-5}	++++	++++
0.6 "	Buffer	++++	++++
Buffer 0.8 ml.	None	++++	++++

sequent experiments streptokinase was always used at ten times the minimal concentration necessary for optimal activation.

Serial dilutions of plasminogen were prepared in sterile distilled water. To 0.2 ml. of pro-enzyme, 0.2 ml. of either skimmed milk or M. protein substrate was added. Streptokinase (100 units/ml.) prepared in 0.01 M phosphate buffer at pH 7.6 was pipetted into each tube in 0.6 ml. amounts. Contact with buffer altered reaction of undiluted plasminogen solutions to pH 7.6 and precipitation of the pro-enzyme occurred; however, plasminogen dilutions of 1:10 or higher appeared stable. After 24 hour incubation at 37° C. tubes containing skimmed milk were read as before; plasmin tubes were placed in a boiling water bath for ten minutes, cooled, and capillary precipitin tests performed with type-specific antiserum. The effect of streptokinase-activated plasminogen on skimmed milk and M protein substrate is illustrated in Table 2. With adequate concentration of pro-enzyme it was possible to demonstrate hydrolysis of M protein.

Effect of leukocytes on M protein

To determine the effect of fresh human leukocytes on M protein a similar system was used. Leukocytes were obtained from peripheral blood by concentrating the supernate after erythrocyte sedimentation. Cells were counted, and serial tenfold dilutions made in Tyrode's gelatin buffer; 0.8 ml. of the suspension was added to 0.2 ml. of skimmed milk or M protein substrate. Incubation at 37° C. proceeded for 24 hours. Although the suspended white cells initially contributed to the opalescence of the skimmed milk preparations, sedimentation of leukocytes had occurred at

TABLE 3. EFFECT OF HUMAN LEUKOCYTES ON SKIM MILK AND "M" PROTEIN SUBSTRATES

	Substrate [0.2 ml.]	
**WBC Dilution [0.8 ml.]*	*Skim Milk*	*"M" Protein*
10^{0}	0	0
10^{-1}	±	++
10^{-2}	++++	++++
10^{-3}	++++	++++
10^{-4}	++++	++++
10^{0} [Heated]	++++	++++

*WBC = 40,000/mm²

24 hours and clearing of the skimmed milk could easily be read. At the end of the incubation period tubes containing M protein were frozen and thawed to effect cellular disruption. The debris was removed by centrifugation at 15,000 *g* for 20 minutes and the supernate tested by the capillary precipitin technique for the presence of M substance. The results of this experiment are reported in Table 3. Fresh leukocyte suspensions completely destroyed reactivity of M protein with type-specific antiserum. Although some loss of M substance was apparent at 1:10 dilution of leukocytes, complete hydrolysis required 32 x 10^{6} cells. Disruption of leukocytes following the incubation period was included to detect intracellular M protein.

Fate of M protein after phagocytosis of viable streptococci

Type 1 and type 12 streptococci were grown overnight in Todd-Hewitt broth and washed three times in Gey's-albumin solution. Hyaluronidase (Worthington) was added to concentration of 1 TRU/ml., and the sus-

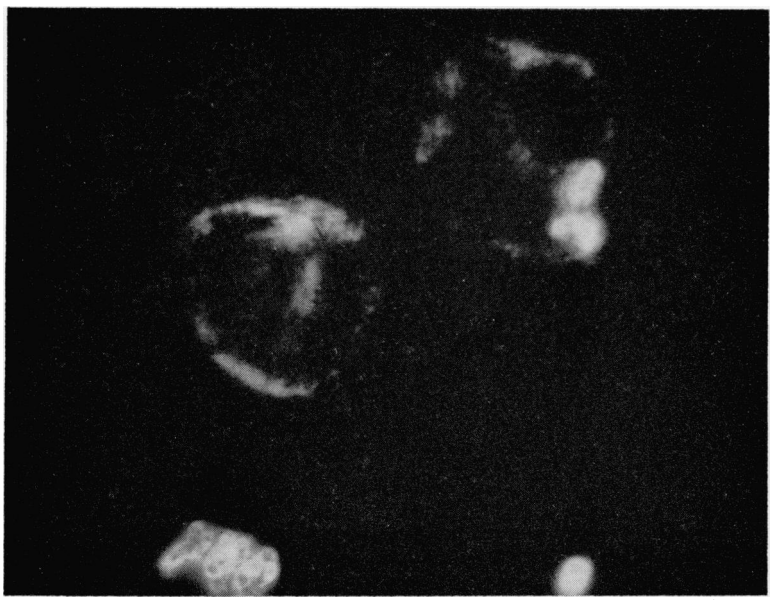

FIG. 1. Intracellular streptococci immediately after phagocytosis viewed with white light using the dark field condenser (x970). Note granules as well as cocci are refractile.

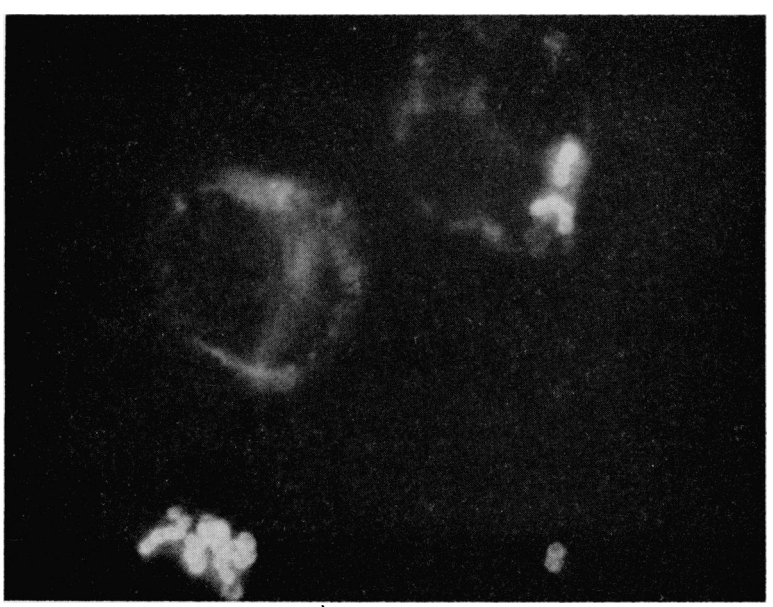

FIG. 2. Same field as above illuminated with ultraviolet light.

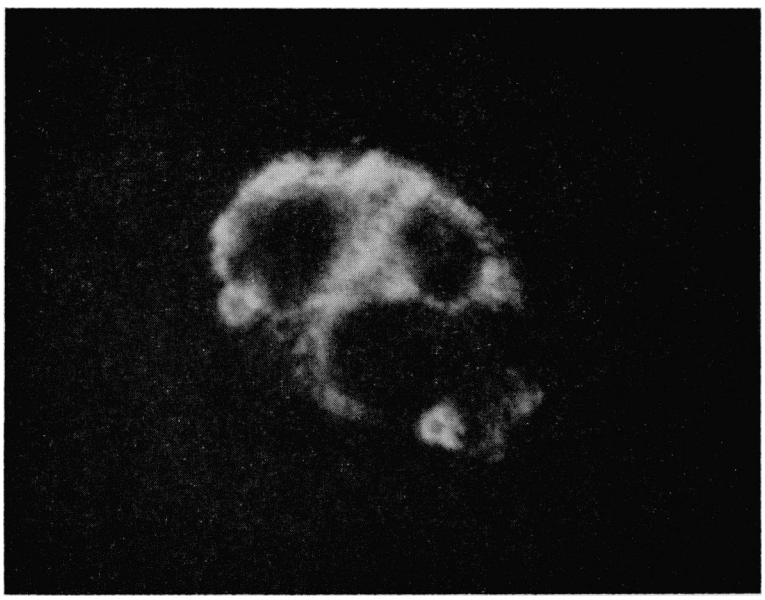

FIG. 3. Intracellular streptococci 10 minutes after phagocytosis viewed with white light (x970).

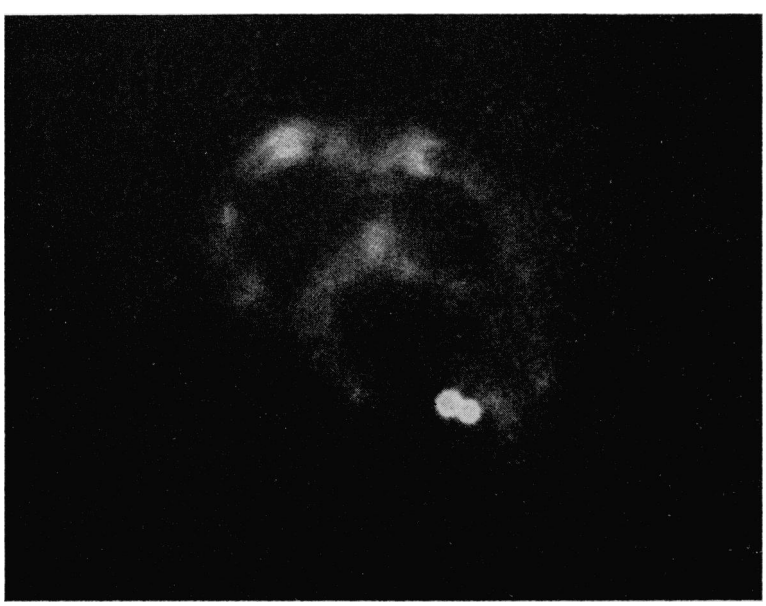

FIG. 4. Same field as above illuminated with ultraviolet light.

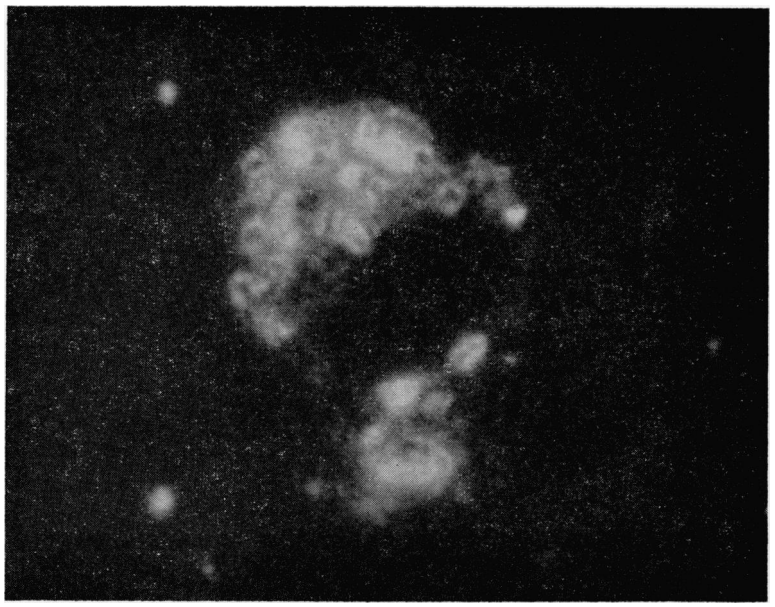

FIG. 5. Intracellular streptococci 65 minutes after phagocytosis viewed with white light (x970).

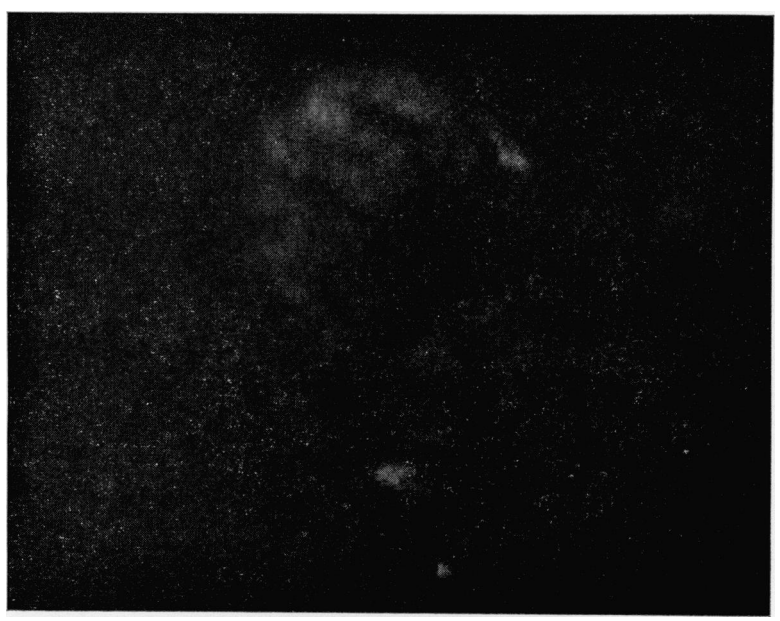

FIG. 6. Same field as above illuminated with ultraviolet light. Note absence of bacteria fluorescence.

pension incubated 15 minutes at 37° C. A dilution of the suspension equal to 1:10 of the overnight culture was made in Gey's-albumin solution containing 2 per cent guinea pig complement. Freshly prepared and washed leukocyte "buttons" were flooded with the bacterial suspension and then incubated from 5-7 minutes at 37° C. Slides were washed in four changes of Gey's-albumin buffer to remove extracellular streptococci, then flooded with fresh buffer and incubated at 37° C. in a moist Petri dish. Preparations were fixed by rapid drying in an air jet at 5-minute intervals for the first half hour and every 15 minutes thereafter for 4 hours. Duplicate slides were stained with Wright's stain and with type-specific fluorescein conjugated gamma globulin. Although exposure of phagocytes to bacteria was short, intracellular streptococci were common, many with surrounding vacuoles. Slides fixed immediately after removal of the bacterial suspension showed only occasional extracellular streptococci.

Preparations were viewed under oil immersion with white light using the dark field condenser (Fig. 1). Intracellular streptococci appeared highly refractile; other cellular particles such as granules, although moderately refractile, were easily differentiated from cocci. The same field was then illuminated with ultraviolet light. In those slides fixed immediately after phagocytosis, streptococci fluoresced a bright greenish-yellow color; the rest of the cell remained a dark blue (Fig. 2). After intracellular residence of ten minutes, bright fluorescence of intracellular streptococci was still apparent (Figures 3 and 4). Slides fixed after 15-75 minutes of incubation showed progressive irregular loss of fluorescence. At 65 minutes intracellular streptococci were clearly seen in fields illuminated with white light (Fig. 5). However, loss of M antigen was demonstrated by absence of fluorescence (Fig. 6). The cytoplasm and nucleus of these cells also were devoid of antigen. Occasional extracellular chains of streptococci which eluded initial phagocytosis continued to stain with specific fluorescein conjugate.

DISCUSSION

Exposure of partially purified streptococcal M protein to streptokinase-activated plasminogen or to human leukocytes resulted in rapid loss of reactivity with specific antibody. M antigen, *in situ,* on the bacterial cell wall, failed to stain with specific fluorescent conjugate after a short intracellular residence in the polymorphonuclear leukocyte.

These data are wholly consistent with the well-known ability of tryptic-like enzymes to destroy M protein.[4] Such proteases are associated with

cytoplasmic granules with polymorphonuclear leukocytes,[12] and it seems likely that rapid proteolysis of M protein occurs soon after phagocytic ingestion. The possibility exists, however, that following phagocytosis, M antigen is removed intact from the bacterial cell wall and diffuses throughout the cytoplasm of the phagocyte; reaction with specific fluorescent conjugate would not be apparent because of antigen dilution. This explanation seems less tenable since serial slides done at 5-minute intervals did not reveal diffusion of antigen away from bacteria. Furthermore M protein in serologically reactive form could never be recovered from disrupted leukocytes following exposure of partially purified M protein to leukocyte suspensions.

The progressive loss of M-antibody-staining material on the ingested bacteria was determined by serial preparations which led to an estimate of intracellular residence necessary to abolish the staining reaction. The time of disappearance of the M antigen, between 15 and 75 minutes, is consistent with the bactericidal effects of the leukocyte as measured by Wilson and Wiley.[13] In their studies, intracellular residence of about 8 minutes resulted in loss of viability of 50 per cent of streptococcal chains following disruption of the leukocyte by means of an electric current. The time lag from ingestion until destruction of M protein and bacterial death may be related to the kinetics of the enzyme-substrate systems involved. An alternate explanation for the lag period in M destruction may involve processes necessary for the enzyme-bearing granules to contact the phagocytic vacuole and deposit the appropriate proteases. Visualization of the phagocytic vacuole was not possible in these studies using dark field microscopy.

The mechanism of M-antibody production is unknown; however, three possibilities remain consistent with the data herein presented: 1) The M antigen, either as a separate extracellular product, or intact on the bacterium, is carried via lymph channels directly to regional lymphoid tissue and antibody production is initiated without prior passage of antigen through phagocytic cells. 2) Another cell such as a macrophage provides more favorable, i.e. less proteolytic, environment and is thereby suitable to transport the intact antigen to the antibody-producing apparatus or 3) shortly after ingestion by a phagocytic leukocyte, antigenic information is conveyed to the phagocyte so that the intact antigen is not required at the antibody-producing site. Some support for the third alternative is found in the studies of Fishman[14] demonstrating the necessity of prior in-

cubation of bacteriophage with macrophages before a primary antibody response could be obtained in lymph node cell cultures.

The technique described in the present report allows study of the fate of various antigens within diverse cell types of the same and different species.

SUMMARY

Partially purified type 1 streptococcal M protein was destroyed by exposure to streptokinase-activated plasminogen or to viable human leukocyte suspensions. After phagocytosis of intact streptococci by human polymorphonuclear leukocytes and a short intracellular residence, the bacteria fail to stain with specific fluorescent anti-M conjugates.

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