normal level, an abnormally high LD5 isoenzyme fraction may be an indication of the lack of total correction of the abnormalities present during the acute phase of the disease. As such, this may be a sign of the need of more active therapy.

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Detection of M Protein in Colonies of Streptococcal L Forms by Immunofluorescence.* (29796)

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A major difficulty in the study of L forms of any bacterial species concerns the problem of classification. For instance, the gross appearance of L-form colonies in agar medium is not sufficiently characteristic to identify a particular L-form colony as a derivative of a bacterial species. Freimer *et al*(1) indicated that a useful characteristic of L-form colonies of Group A streptococci is the elaboration of type-specific M protein identical to that of the streptococci from which the L forms were derived. While almost all of the M protein produced by intact streptococci is confined to the cell wall, a significant portion of this antigen elaborated by L-form colonies diffuses into the surrounding agar medium. Although this special feature of streptococcal L forms has afforded a method for serological identification, in practice, appreciable growth of a subculture of the L-form colony in question is required to perform a precipitin analysis. By use of a fluorescent-antibody technique which employs fluorescein-conjugated type-specific antisera, M protein can be directly detected in the substance of a L-form colony, thus permitting rapid identification on the initial culture.

Materials and methods. Streptococci. Group A streptococci T1/114/7 (type 1), B930/24/5 (type 3), B90/3 (type 5), S43/100 (type 6), T14/46/4 (type 14), and SS132 (nontypable) were obtained from Dr. R. C. Lancefield, Rockefeller Institute.

L forms of streptococci. Types 3 and 14 L forms were obtained from Dr. E. A. Mortimer, Cleveland Metropolitan General Hospital, Cleveland, Ohio. Types 1 and 5 L forms were isolated by previously described procedures(1). Type 6 L forms were isolated by the method of Mortimer(2).

Cultivation of L forms. L forms were grown on an agar medium prepared with

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Difco brain-heart infusion broth, supplemented to contain: 1.1% agar, 2% NaCl, 0.5% yeast extract, 10% horse serum and 1000 units of penicillin per ml.

Extraction of M protein from an L-form agar culture. M protein was extracted from L-form growth in agar medium by freezing and thawing according to the method of Freimer *et al*(1).

Serological identification. M protein was detected by the capillary precipitin method of Lancefield(3).

Preparation of fluorescein-labeled type-specific antibody. Types 1, 5 and 6 fluorescein-labeled type-specific antibodies (FA conjugate) were prepared by the method previously described(4). Types 3 and 14 FA conjugates were prepared from hyperimmune rabbit sera employed for identification of streptococci by the precipitin technique. A disadvantage of these sera, although suitable for precipitin analyses, is that they yield FA conjugates lacking type-specific immunofluorescence. Specificity was achieved, however, by absorption of the FA conjugate with a lyophilized preparation of a heterologous type of Group A streptococci, which had been disrupted in the Braun homogenizer(5). One hundred mg of the streptococcal powder was added to 1 ml of FA conjugate and the mixture incubated at 37°C for 30 minutes. The insoluble material was collected by centrifugation at 20,000 $\times$ *g* and the adsorbed FA conjugate dialyzed against buffered saline pH 7.4.

Fluorescent-antibody staining. L-form colonies of streptococci were removed from the agar plate with an inoculating needle and placed upon an alcohol-washed glass slide. After residual small clumps of agar were teased away with a needle, the colony was broken into small fragments and smeared onto glass slides. The smears were dried in air and fixed with heat. Direct FA staining and the 2-step inhibition test(6) were performed on the smears. After exposure to the FA conjugate for 10 minutes, the slides were washed in 2% saline for 10 minutes, and rinsed in buffered saline pH 7.4. The preparation was covered with a drop of buffered glycerol (9 parts glycerol and 1 part phosphate buffer pH 8.0) and a cover slip. Immunofluorescence of whole streptococci was performed by the method of Karakawa *et al* (4). All slides of either L forms or streptococci were examined under oil immersion with a Leitz ortholux microscope equipped with a HBO 200 mercury vapor lamp, a BG 12 exciter filter and a OG 1 barrier filter. Photographs were made with Kodak high-speed Ektachrome film exposed for 24 minutes. FA-staining reactions were recorded as follows: 4+, intensely bright staining; 3+, bright staining; 2+, moderate staining; 1+, weak staining. Large aggregates of L forms should not be employed, because they are difficult to wash free of excess dye, and may, therefore, give rise to false positive reactions. In practice, controls using heterologous FA conjugates facilitate interpretation.

Results. A subculture of the L-form colony to be identified was prepared so that sufficient material was available for examination by immunofluorescence and a standard precipitin technique. The original streptococcal culture from which the L-form colony was derived was also identified by these two techniques. Photomicrographs of specific immunofluorescence for types 6 and 14 L forms and streptococci are depicted in Fig. 1. It is

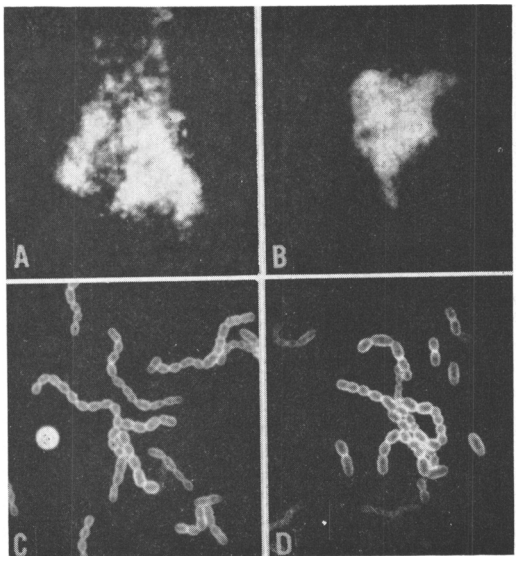

FIG. 1. Immunofluorescence of types 6 and 14 L forms and streptococci with homologous FA conjugate. A, type 6 L forms; B, type 14 L forms; C, type 6 streptococci; D, type 14 streptococci.

TABLE I. Precipitin Reactions Between M-protein Extracts of L-form Growth in Agar and Type-Specific Antisera.

L-form extracts	Type-specific antisera 1	3	5	6	14
1	+	—	—	—	Tr
3	—	++	—	—	—
5	—	—	++	—	Tr
6	—	—	—	+++	—
14	—	—	—	—	++

Tr = Trace.

clear that the spherical forms of an L-form colony exhibit a diffuse fluorescence whereas the intact streptococci demonstrate an intense fluorescence confined to the periphery of the cell. It was not feasible to obtain photomicrographs of the minimal fluorescence exhibited by an L form or a streptococcus and heterologous FA conjugates. These results indicate that M protein is associated with the L-form colony. The following data emphasize the type-specificity of the fluorescent-antibody method.

Depicted in Table I are the results of the precipitin test on L-form colonies of types 1, 3, 5, 6, and 14. Although types 6 and 14 were characterized by strong precipitin reactions with homologous antisera indicating an abundance of M protein, scarceness of M protein in type 1 is suggested by the weak precipitin reaction.

The specificity of the FA-staining reactions of types 1, 3, 5, 6, and 14 L forms with the homologous FA conjugates is demonstrated by data presented in Table II. The FA conjugates react in much higher titer with homologous L forms than with heterologous L forms except in the case of the type 1 which also exhibits a relatively weak homologous precipitin reaction. It is of interest

TABLE II. FA-Staining Reactions Between Type-Specific Conjugates and Streptococcal L Forms.

L forms	FA titer with type-specific conjugates* 1	3	5	6	14
1	*40*	<20	<20	20	20
3	<20	*160*	<20	<20	<20
5	<20	<20	*80*	<20	20
6	<20	20	<20	*640*	20
14	<20	20	<20	<20	*160*

* Reciprocal of highest dilution showing 3+ or 4+ staining reaction.

that type 6 L forms, rich in M protein as detected by the precipitin method, react with FA conjugate in high dilution, whereas the type 1 L forms lean in M protein react with FA conjugate in low titer.

Although the above data indicated that L form immunofluorescence in this instance is dependent upon antibody to M protein, it is conceivable that under streptococcal antibody such as group-specific antibody is in part responsible for the reaction. This possibility was ruled out because Group A antiserum, when employed in 2-step inhibition tests, failed to inhibit the subsequent immunofluorescence of L forms with homologous FA conjugates. In these experiments L forms prior to exposure to the FA conjugate were treated with a 1:50 dilution of Group A antiserum. The reactivity of the FA conjugates with the treated L forms was equivalent to that exhibited with the untreated L forms.

The reactions between the FA conjugates and homologous streptococci rich in M protein are presented in Table III. The FA

TABLE III. FA-Staining Reactions Between Type-Specific Conjugates and Intact Streptococci Rich in M Protein.

Streptococci Type	Strain	FA titer with type specific conjugates* 1	3	5	6	14
1	T1/114/7	*640*	<20	<20	<20	<20
3	B930/24/5	40	*320*	20	<20	40
5	B90/3	<20	<20	*640*	<20	20
6	S43/100	<20	<20	<20	*1280*	20
14	T14/46/4	20	<20	80	20	*1280*

* Reciprocal of highest dilution showing 3+ or 4+ staining reactions.

conjugates react in high dilution with homologous types of streptococci although minimal cross-reactivity is detectable at low dilution. It is of interest that the type 1 FA conjugate reacts in high dilution with the type 1 streptococci which contain an abundance of M protein. This finding corroborates the view expressed above that the type 1 FA conjugate reacted in low titer with the type 1 L forms because of the scarceness of M protein. Indeed, in this connection, L forms after successive subculture on hypertonic agar medium have become unreactive

with type-specific FA conjugate and with type-specific precipitin antisera. This finding indicates that L forms after repeated passage *in vitro* may lose the type-specific M protein.

Discussion. The experiments of Freimer *et al*(1), which employed a modified Ouchterlony technique, demonstrated the diffusion of M protein from L-form colonies into the agar medium, thus suggesting a continuous production of M protein, which, in the absence of a bacterial cell wall, passes freely into the surrounding medium. The accumulation of an appreciable quantity of extracellular M protein and its absence in isolated protoplast membranes suggested than an insignificant amount of synthesized antigen persisted within multiplying L forms. The present data confirm the diffusion of M protein out of L-form colonies and into the agar. It is of interest, however, that M protein was readily detected in L-form colonies when examined by the immunofluorescent technique. It is unlikely that this finding is dependent upon a nonspecific cross-reaction. There is, for instance, no doubt that the type-specific fluorescein-labeled antisera employed here contained anti-M antibodies(4). Furthermore, specific fluorescence in the absence of significant heterologous staining was noted with 5 different types of L forms and homologous conjugated antisera.

Although the FA method employed in the experiments reported above was used to identify subcultures of L forms, it has now been employed successfully to identify original isolates. The FA procedure provides a simple and rapid method for direct detection of M protein in L-form colonies, affording an expedient method for identification and classification.

Summary. M protein was detected in growing L-form colonies of Group A streptococci by an immunofluorescent technique which employs type-specific fluorescein-labeled antibody. The type-specificity of the FA-staining reactions exhibited by the L forms was confirmed by parallel precipitin studies. These data suggest the feasibility of identifying the L-form colonies of Group A streptococci by detecting the M protein with the FA method.

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Abnormal Limb Development in *Rana catesbeiana.** (29797)

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The occurrence of limb abnormalities has been encountered in nature as a result of injury and has been well documented from defined experiments involving such representatives from the animal kingdom as the amphibians, crustaceans, and insects(1). In contrast to these abnormalities which in most cases were produced physically by cutting or breaking, certain teratogenic chemicals have proven to be effective in mammals and birds (2). Other approaches have been reported with urodele Amphibia using localized ultraviolet irradiation(3) and still another experi-

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