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THE USE OF FLUORESCENT LABELLED DIPHTHERIA ANTITOXIN FOR THE DIAGNOSIS OF DIPHTHERIA CASES

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Introduction

In Turkey diphtheria is still a very significant infectious disease among children. In the early diagnosis of suspected cases, the conventional method of direct smearing of throat swabbings is acknowledged to lead to frequent confusion, even in the best of hands.[1] Furthermore, the 12 to 24 hour wait for the results of such cultures often causes a critical delay in treatment. In recent years, the Fluorescent Antibody Technique (FAT) has been successfully applied to the detection of many bacterial, viral, mycotic and protozoal agents in human disease.[2,3,4] One of the advantages of the FAT lies in its rapid and simple identification of a specific organism by binding it to its specific antibody which has been previously linked to a fluorescent substance. Reports of earlier studies using FAT in diphtheria are in some respects conflicting. Whittaker, Nelson and Fink in 1961 first reported the successful use of this technique on indirect smears from diphtheria cases, using labelled antitoxin.[5] Several other reports since then have described the use of the technique both with antitoxin and antibody conjugates staining both fresh material and cultures.[6–9] The present study was undertaken in an attempt to use this fluorescent antibody technique for a more rapid identification of diphtherial organisms, as well as to compare the results with conventional methods of direct smear and culture.

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Materials and Methods

Specimens: Cotton swabbings were taken from suspected sites (throat, nose, or tracheal cannula) of patients seen in the outpatient department and in the Infectious Disease Service of the Hacettepe Medial Center over a period of three months. The swabs were taken in duplicate by rubbing the suspected area vigorously, and were usually made by one or the other of the authors. One swab was used to innoculate the culture media described below. The second was used in making the smears for conventional and FAT staining.

Conventional Culture Methods: Plates of blood agar, Loeffler and Modified Tinsdale medium of tellurite [10] were all inoculated with one of the swabs and incubated for 24 hours, the tellurite plates for 48 hours. The cultures were then examined for growth under a dissecting microscope and suspicious colonies were smeared and stained by both the Neisser and the Gram methods. Diagnosis was based on morphologic characteristics.

Examination of Direct Smears: These were prepared from the second of the two swabs. Two smears were used for direct staining by the Gram and Neisser methods. At least two slides were made for examination by FAT. The slides for FAT were first air-dried, then fixed in acetone for ten minutes at room temperature. One drop of the labelled antitoxin was placed on the smeared area, spread evenly and incubated in a moist chamber for thirty minutes at room temperature. Following this the smears were washed by gentle agitation in a slide container using buffered saline (pH 7.2) which was changed five times during a ten minute period. Slides thus prepared were examined promptly thereafter under a fluorescent microscope. (Occasionally the slides were stored at 4 C and examined the following day.) A Spencer AO microscope fitted with a bright-light condensor was used in combination with an Osram HB-200 mercury vapor arc light fitted with an exciter filter (Corning 209, 4 mm.) and a preventing filter (Wratten gelatin filter 2B).

Preparation of FITC Labelled Antitoxin: Antitoxin* containing 4000 units of purified antitoxin per milliliter was salted out with ammonium sulfate and an electrophoretic pattern was made of the resulting serum, indicating a pure solution of gamma globulin. The protein content of this solution was estimated by the Biuret method. Carbonate-bicarbonate buffer was added to make a final dilution of ten per cent. FITC was then mixed in a proportion of 0.05 milligrams per milligram of protein. The resulting solution was mixed with a mechanical agitator for 24 hours at 4 C. Dialysis was carried out in a

* Kindly supplied by Dr. A. Tasman of the Rijks Institute of Utrecht, Holland.

dialyzing tube against buffered saline (pH 7.2) for four to five days, until the dialyzing fluid was almost free of fluorescence. The conjugated antiserum was then put through a millipor filter and stored at 4 C until used.

Results

Before studying diphtheria suspects, the specificity of the conjugate was checked against a number of organisms which may be encountered in the throat: Hemolytic Streptococcus, Hemolytic Staphlococcus aureus, E. coli, Ps. aeruginosa, and Proteus. These were obtained from cultures taken from the clinical microbiology laboratory. Staphlococcus aureus and Hemolytic Streptococcus gave a 1 to 2 plus degree of fluorescence; the remaining organisms produced no discernible fluorescence. Park-Williams 8 strain (obtained from the Central Hygiene Institute of Ankara) was also studied and demonstrated a uniformly stronger (3 plus) fluorescence. In none of these preliminary stainings was a typical "halo" noted around the organisms.

In 28 clinically suspected cases of diphtheria, conventional and fluorescent techniques were used (Table 1). Of these, there were three in which definite bacillary forms could be appreciated under fluorescent light. Reasonably strong fluorescence was designated 3 plus, leaving the designation 4 plus for those in which a halo could be seen. This halo phenomenon was not observed. Of the three cases in which definite bacillary forms could be appreciated, two had a positive culture. On the other hand, in five of the positive cultures, the corresponding fluorescent study was felt to be negative. Twenty cases had negative cultures and fluorescent smears. The same was true of the hospital personnel and contacts examined, with the exception of one child who had nasal diphtheria. As has so often been reported [1] our study confirmed the lack of correlation between direct smear and culture (Table 2).

Fluorescent tests were also performed on cultures from five positive cases, taking the smears from Loeffler slants. In these, the FAT showed bacillary forms of varying intensity of fluorescence and one showed a faint halo phenomenon.

Discussion

With the FITC conjugated diphtheria antitoxin used in this study the most intense fluorescence was observed in the P-W 8 strain of C. diphtheriae. This however did not exhibit an intense halo such as has been observed in this laboratory with FITC conjugated E.E. coli antibody. [3] The fluorescence with P-W 8 strain and several smears from positive cultures was, however, stronger than that seen in control studies carried out on cultures of other organisms (H. Streptococcus, H. Staphlococcus aureus, Ps. aeruginosa, and

TABLE 1

A COMPARISON OF RESULTS OF CONVENTIONAL SMEAR AND CULTURE METHODS WITH FLUORESCENT ANTIBODY TECHNIQUE IN PATIENTS, FAMILY CONTACTS AND HOSPITAL PERSONNEL

Subjects	Conventional		Fluorescent	
Suspect Cases	Smear	Culture	Result	Interpretation
T.B.	ø	ø	-/-/-	ø
T.Z.	ø	ø	ø	ø
E.D.	+	+	ø	ø
C.V.	ø	ø	-/-/-	ø
D.K.	+	ø	ø	ø
A.B.	ø	ø	ø	ø
E.E. (Nose)	ø	ø	ø	ø
E.E. (Throat)	ø	+	-/-/-	ø
S.Y.	+	ø	+	ø
M.B.	ø	ø	ø	ø
L.P.	ø	+	ø	ø
E.A.	ø	ø	ø	ø
V.Y.	+	+	ø	ø
I.P.	+	+	+	ø
C.C.	ø	ø	ø	ø
G.K.	ø	ø	ø	ø
A.K.	ø	+	-/-/-/-	+
M.D.	ø	ø	ø	ø
H.K.	+	ø	+	ø
A.D.	ø	ø	-/-/-/-	+
S.S.	+	ø	ø	ø
M.K. (Trachea)	ø	ø	ø	ø
A.A.	ø	ø	ø	ø
S.Y.	ø	ø	ø	ø
C.E.	ø	ø	ø	ø
F.N.	+	+	-/-/-/-	+
F.N.	+	ø	-/-/-	ø
N.D.	+	ø	-/-/-	ø
Family Contacts of Positive Cases				
S.P. (Nose)	+	+	ø	ø
S.P. (Throat)	ø	ø	ø	ø
M.A. (Nose)	ø	ø	ø	ø
M.A. (Throat)	ø	ø	ø	ø
Doctors on C.D. Ward and in Clinic				
R.K.	ø	ø	ø	ø
E.G.	ø	ø	ø	ø
S.E.	ø	ø	ø	ø
B.K.	ø	ø	ø	ø
S.I.	ø	ø	ø	ø
G.O.	ø	ø	ø	ø
S.Y.	ø	ø	ø	ø

TABLE 2

SUMMARY OF COMPARATIVE RESULTS OF FLUORESCENT ANTIBODY TECHNIQUE AND CONVENTIONAL CULTURES

	Culture + FAT +	Culture + FAT (—)	Culture (—) FAT +	Culture (—) FAT (—)
Suspect Patients	2	5	1	20
Family Contacts of Positive Cases	–	1	–	3
Doctors on C.D. Service and in Clinic	–	–	–	7

Proteus). Moody and Jones, Allen and Cluff, and Whittaker, in their studies on this subject, noted some cross-staining with various other pathogenic and non-pathogenic organisms.[5,6,7] Moody suggests that the use of conjugated antiserum previously adsorbed with the cross-reacting bacteria would cause a lessening of this cross-staining.[7] Whittaker et al state that the difference can be readily appreciated by morphological characteristics.[5] Moody and Jones, using antitoxin, noted a weak to moderate staining in six out of six strains of atoxigenic C. diphtheriae.[7] Similarly, Allen and Cluff demonstrated 1 to 3 plus staining among five atoxigenic diphtheria strains as well as in C. xerosis and diphtheroids.[6] It is interesting to note that Allen obtained varying degrees of staining among the same toxigenic C. diphtheriae using two different commercially prepared antitoxins. While our limited studies with cultures of C. diphtheriae and other bacteria show results comparable to these studies in that many examples of cross-staining may occur, it is surprising that we have not encountered this same cross-staining on the smears examined directly from throat swabs of suspected cases.

It is considered to be of some value to determine why the diphtheria bacilli which later grew out in culture and which were often seen quite easily on conventional smear did not bind to the conjugated antitoxin. As suggested by some investigators, it is possible that many bacilli observed by direct smear may not be producing toxin at the time and therefore not be stainable with the FAT.[5,6] A second consideration is suggested by the studies of Tasman who found a very labile substance in the saliva of some diphtheria patients capable of inhibiting the formation of diphtheria toxin.[11,12] Should this occur, the toxin might be rendered unstainable. Lastly, it is possible that the amount of antitoxin in the serum was not sufficient to combine with the toxin of the bacilli. We are considering the use of a more highly concentrated serum in future studies.

It is interesting to note that some brightly fluorescent epithelial cells were observed in several direct smears conjugated with the labelled antitoxin. This suggested the possibility that these cells had adsorbed diphtheria toxin. Should this actually be the case, it might be possible to stain the adsorbed toxin with an antitoxin conjugate by incubating the saliva or throat swabbings of diphtheria suspects with some suitable medium or tissue such as human erythrocytes. Butler's studies demonstrating hemagglutination of red cells incubated with diphtheria toxoid upon addition of antitoxin suggest this may be feasible.[13]

Another, perhaps more remote, possibility is suggested by the work of Dowdle and Hansen, who successfully used an antiphage conjugate to label B. anthracis.[14] Since toxigenic C. diphtheriae is known to possess a lysogenic phage, a similar antiphage B conjugate might be effective in identifying toxigenic C. diphtheriae.

Conclusion

With the fluorescent technique used in this study it was possible to demonstrate the presence of fluorescence in diphtheria bacilli in the throat of two out of eight cases which had positive cultures by examination of direct smears. Fluorescence was also demonstrated on smears taken from cultures in five cases. Halo phenomenon was seen in one of the latter. However, in this study the degree of fluorescence was not sufficient or consistent enough to allow the positive identification of diphtheria (toxigenic or atoxigenic) organisms from suspected cases.

Summary

The authors present a study to determine the usefulness of the Fluorescent Antibody Technique in diagnosing cases of diphtheria. The results of this study indicate that the degree of fluorescence is not sufficient or consistent enough to allow positive identification of diphtheria organisms in suspected cases. Possible causes of the lack of fluorescence are discussed and alternative techniques suggested.

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