Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 107 MAY, 1961 No. 1

SECTION MEETINGS

CLEVELAND, O.	
University Hospitals	February 20, 1961
University Hospitals	March 20, 1961
DISTRICT OF COLUMBIA	
Georgetown University	March 30, 1961
ILLINOIS	
University of Illinois Med. School	March 14, 1961
IOWA	
State University of Iowa	March 29, 1961
MICHIGAN	
Lafayette Clinic, Detroit	March 16, 1961
MINNESOTA	
University of Minnesota	March 23, 1961
SOUTHERN	
Touro Infirmary, New Orleans	February 22, 1961
SOUTHERN CALIFORNIA	
University of Southern California	March 16, 1961
SOUTHWESTERN	
Southwestern Medical School	March 17-18, 1961

Identification of Poliovirus Isolates with Fluorescent Antibody. (26516)

M. H. HATCH, S. S. KALTER* AND GLORIA W. AJELLO
Communicable Disease Center, Public Health Service, U. S. Department of HEW, Atlanta, Ga.

Isolation and identification of enteroviruses from clinical specimens by routine methods generally require 2 or more weeks. Fluorescent antibody procedures, which have been applied to rapid diagnosis of influenza by Liu (1) and to demonstration of several viruses in tissue culture(2,3), also appear to offer the possibility of more rapid identification of enteroviruses isolated from clinical material. This report presents data concerning use of fluorescent antibody for identification of poliovirus isolates. A preliminary report of some of these data has already been made(4).

Materials and methods. Hyperimmune monkey poliovirus typing sera prepared by Wenner *et al.*(5) were used for preparation of fluorescent antibodies. Trypsinized rhesus monkey kidney cells were employed for isolation of viruses. Buffered saline consisted of 0.14 M NaCl, 0.01 M $NaH_2PO_4 \cdot H_2O$, with

* Present address: Head, Virology Section, School of Aerospace Medicine, Brooks Air Force Base, Texas.

the final pH adjusted to 7.0-7.2 by addition of 40% NaOH solution.

Globulins were precipitated from monkey antisera by 50% saturation with $(NH_4)_2SO_4$ and labeled with either fluorescein isocyanate (6) or fluorescein isothiocyanate(7). The latter compound was added in powdered form to globulin solutions at pH 9 with stirring in an ice bath (personal communication, Riggs, 1958). Before use, conjugates were absorbed twice with monkey liver powder(8) and once with a suspension of monkey kidney cells.

For fluorescent antibody study, 85 stools previously tested by routine methods were selected from storage and given to 2 of the authors (MHH and GWA) who had no knowledge of the earlier results. The specimens were then processed entirely as unknowns. Ten to 20% stool suspensions were prepared, penicillin and streptomycin added to final concentrations of 4000 units/ml and 2000 μg/ml, respectively, and 0.1 or 0.2 ml portions inoculated into tubes of monkey kidney cells. As soon as a 1 to 2+ cytopathic effect (CPE) was observed, maintenance fluid was poured off and cells rinsed with 1 ml of 0.02% versene in buffered saline. One ml of fresh versene was added and tubes incubated about 15 minutes at 37°C. Cells were then removed from the glass by gentle shaking and sedimented by centrifuging at 2000 RPM for 5 minutes. Smears of sedimented cells were made on slides with a loop as in preparing bacterial smears. Slides were air dried and fixed in acetone at room temperature for 10 minutes. They were either stained immediately or stored at −20°C until stained. Control slides of uninoculated monkey kidney cells and known poliovirus infected cells were prepared at the same time slides were made on test specimens. All stools which produced a toxic reaction in monkey kidney cells were passed a second time. Staining in all these cases was done on second passage material. The majority of stools (21/27) producing no CPE in first passage were also passed a second time. In these instances, negative tubes were incubated 7 days, frozen, thawed and 0.1 ml passed to fresh tubes. Second passage tubes remaining negative were harvested at the end of 7 days for staining. Tubes on 6 stools that did not produce CPE in first passage were harvested and stained at the end of this passage. In many experiments, Leighton tube preparations of monkey kidney cells were also inoculated with stool suspensions. Coverslips from these tubes were removed at the 1 to 2+ CPE stage, air dried, and fixed in acetone as above. The direct method of fluorescent antibody staining was employed throughout. Conjugates were used undiluted or diluted 1:2 with buffered saline. All preparations were examined under ultraviolet illumination using a Corning 5970 or 5840 exciter filter. A Wratten 2B gelatin filter was used in the microscope ocular. Results of fluorescent antibody method were compared to those obtained previously with the routine neutralization method used for identification of polioviruses in this laboratory.

Results. Conjugates were checked in various ways for specificity of staining. In several cross staining experiments, smears or Leighton tube coverslip preparations of cells infected with poliovirus 1, 2 or 3 were stained with antipoliovirus 1, 2 and 3 conjugates. In each case, only the homologous system showed significant staining. Occasionally, slight cross staining was noted between poliovirus Types 1 and 3, but staining with homologous conjugate was always definitely brighter. Smears were also made from poliovirus infected tubes showing varying degrees of CPE. The brilliance of staining as well as the number of stained cells increased as CPE increased to about the 2+ stage. However, smears prepared from tubes showing 3+ or 4+ CPE did not stain well, possibly because of release and dispersion of viral antigen from cells. Similar observations were made with Leighton tube material. Lastly, inhibition tests were performed by the one-step method(9). When a mixture of equal parts of undiluted, unlabeled homologous monkey poliovirus antiserum and undiluted homologous conjugate was used to stain smears, staining was markedly inhibited. Significant inhibition of staining did not occur when unlabeled normal monkey serum was mixed with homologous conjugate. Results

TABLE I. Comparison of Fluorescent Antibody and Routine Procedure in Identification of Poliovirus Isolates.

Enterovirus type in stool	No. by routine procedure	No. by fluorescent antibody procedure
Polio 1	22	19
" 2	2	2
" 3	14	13
Non-polio*	47	47†
Total	85	

* Eight Coxsackie B2, 3 Coxsackie B5, 1 Coxsackie A9, 1 ECHO 4, 5 ECHO 9, 29 no enteroviruses.

† Fluorescent antibody results negative with poliovirus conjugates indicating no poliovirus present.

of the comparison of fluorescent antibody and routine procedures are shown in Table I. Fluorescent antibody method correctly typed 34 of the 38 polioviruses present in the stools. In the remaining 4 cases, although the presence of virus was clearly recognized by CPE in tissue culture, there was no staining by the poliovirus conjugates. Where stools contained a Coxsackie or ECHO virus or no enterovirus, poliovirus fluorescent antibodies gave negative results. Thus, there were no false positive staining reactions in the 47 cases from which poliovirus was not isolated by routine methods.

Discussion. The reasons for the failure of fluorescent antibody staining to detect poliovirus in the 4 instances cited are not known. Two of these stools were reprocessed later, stained with fluoresecnt antibody and the viruses successfully typed as poliovirus, Type 1. However, on the basis of the data presented, fluorescent antibody is not a complete substitute for routine methods in poliovirus identification. Further studies are necessary to determine whether (a) better correlation can be obtained between fluorescent antibody and routine methods and (b) fluorescent antibody can be used as a screening procedure with routine methods being applied to those agents which fail to type with poliovirus conjugates. The absence of false positive reactions in the 47 stools from which poliovirus was not isolated and the correctness of typing in all 34 instances in which polioviruses were identified would suggest the possible application of fluorescent antibody as a screening tool.

Certain advantages of fluorescent antibody method should be pointed out. First, laboratory identification of polioviruses in the stool could be made more rapidly with fluorescent antibody. One-half (17/34) the polioviruses were identified in first passage in tissue culture, generally within 72 hours after inoculation. In the remainder of the positive cases, second passage in tissue culture was necessary. In all but one instance this was due to toxicity of the stool suspension for monkey kidney cells. Even where more than one passage was necessary, fluorescent antibody was more rapid than routine procedure because of the titrations and neutralizations involved in the latter method.

Second, fluorescent antibody method was comparatively simple to carry out. Less material, particularly monkey kidney tissue cultures ,and fewer manipulations (i.e., pipetting, reading of tissue culture tubes) were involved in fluorescent antibody method. Such advantages might be offset to a certain extent by the rather expensive equipment necessary for fluorscence microscopy and by the time and experience required to initiate fluorescent antibody procedures. Over a long period of time, however, fluorescent antibody methods should prove simpler and more economical.

Where both Leighton tube preparations and smears were stained, both were satisfactory for identification of the isolate. However, recognition of infected cells after staining appeared somewhat easier with Leighton tube material. Characteristic fluorescent granules were frequently seen in the cytoplasm of infected cells stained *in situ* on the coverslips. In smear preparations, fluorescence tended to be seen as larger cytoplasmic masses, and all cells appeared rounded as a result of versene treatment.

Summary. A specific and relatively rapid direct fluorescent antibody staining method for laboratory identification of polioviruses isolated from stool samples is reported. Eighty-five stool specimens were processed as unknowns in a comparison between the routine method employing neutralization tests

and the fluorescent antibody method. Thirty-eight were found to contain poliovirus Types 1, 2 or 3 by the regular procedure, and 34 of these were correctly identified by the fluorescent antibody method. The other 4 of the 38 were negative by fluorescent antibody. The 47 specimens found not to contain poliovirus by the routine method were negative by fluorescent antibody. Eighteen of these contained other enteroviruses. Thus, although the fluorescent antibody procedure was found slightly less sensitive than the routine procedure, it was entirely specific in this study.

1. Liu, C., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 883.

2. Coons, A. H., *Internat. Rev. Cytol.*, 1956, v5, 1.

3. Buckley, S. M., *Arch. f. Virusforsch.*, 1956, v5, 388.

4. Kalter, S. S., Hatch, M. H., Ajello, G. W., *Bact. Proc.*, 1959, 89.

5. Wenner, H. A., Miller, C. A., Kamitsuka, P. A., Wilson, J. C., *Am. J. Hyg.*, 1954, v59, 221.

6. Coons, A. H., Kaplan, M. H., *J. Exp. Med.*, 1950, v91, 1.

7. Riggs, J. L., Seiwald, R. J., Burckhalter, J. H., Downs, C. M., Metcalf, T. G., *Am. J. Path.*, 1958, v34, 1081.

8. Coons, A. H., Leduc, E. H., Connolly, J. M., *J. Exp. Med.*, 1955, v102, 49.

9. Goldman, M., *ibid.*, 1957, v105, 557.

Received December 16, 1960. P.S.E.B.M., 1961. -107.

Evidence for an Hypothalamic Focus of Inhibition of Gonadotropin by Androgen in the Male.* (26517)

JULIAN M. DAVIDSON† AND CHARLES H. SAWYER
Department of Anatomy, University of California Medical Center, Los Angeles

Control of pituitary gonadotropic secretion is generally acknowledged to be partially a function of the level of circulating gonadal hormones. The view has recently become widespread that in the female this "feedback" influence of sex hormones is an indirect one, operating through the hypothalamus, wherein lie receptor structures which respond to the sex steroids by modifying adenohypophysial activity(1,2,3). This report presents evidence that also in the male an area within the hypothalamus responds to the gonadotropin-inhibiting influence of the sex-specific gonadal hormone, testosterone.

Methods. Testosterone propionate was implanted into the brains or hypophyses of 11 mongrel male dogs. The crystalline steroid was heated in a glass tube, and immediately on reaching the melting point some of the hormone was drawn by capillary action into the end of 22 gauge stainless steel tubing. After cooling, the steel tube was cleaned on the outside with solvents, and a minute amount of solid testosterone (of the order of 0.5 mg) retained at the tip. With the aid of the dog stereotaxic instrument(4) tubes so prepared were implanted through the calvaria, each tip being located in the brain or hypophysis as described in the Table.

Semen ejaculates were collected and evaluated as described previously(5). Sperm count and motility were measured on 2 to 4 occasions (6-20 days) before implantation and at intervals after implantation of testosterone. The dogs were autopsied 24-33 days postoperatively. Testes and prostates were removed, weighed, fixed in SUSA solution, sectioned, and stained with hematoxylin and eosin. Brains were perfused and fixed in 10% formalin and sectioned by the freezing method for location of testosterone implantation sites. After removal of the tubes, testosterone was generally still clearly visible at their tips.

Results. Only dogs with pre-implantation ejaculates containing over 100 million highly motile spermatozoa were used in this study. In one group (Table I, Group I) implantation of testosterone into the ventral hypothalamus was followed by failure of ejaculation. In the case of dogs A and G, the first postoperative

* Supported by USPHS grant.

† USPHS Postdoctoral Research Fellow.