LOCALIZATION OF ANTIGEN IN TISSUE CELLS

IV. Antigens of Rickettsiae and Mumps Virus*

By ALBERT H. COONS, M.D., JOHN C. SNYDER, M.D., F. SARGENT CHEEVER, M.D., AND EDWARD S. MURRAY, M.D.

(From the Department of Bacteriology and Immunology, Harvard Medical School, and the Department of Public Health Bacteriology, Harvard School of Public Health, Boston)

PLATE 5

(Received for publication, August 6, 1949)

The availability of a method for the microscopic localization of antigenic material in tissue cells (1–3), employing fluorescence microscopy and antibody conjugated with fluorescein, has prompted its application to two dissimilar infectious agents, rickettsiae and mumps virus. It was thought that prompt, specific, microscopic evidence of the presence of these agents might speed investigations concerning them, and perhaps open the way to studies not heretofore possible. The present paper will describe a few simple experiments which demonstrate immunologically specific staining of the rickettsiae of epidemic typhus and Rocky Mountain spotted fever, and of mumps virus.

EXPERIMENTAL

1. Rickettsiae

Materials and Methods.—Conjugates of rabbit and horse serum were prepared as previously described (2). The sera used as sources of antibody were: (*a*) Concentrated therapeutic antityphus (epidemic) rabbit serum (Lederle[1]), prepared by the injection of rickettsial vaccine (4) from yolk sac suspensions.[2] (*b*) Antityphus (murine) horse serum (5) prepared in 1937 for Dr. Hans Zinsser by injection of formalinized peritoneal washings from x-rayed rats infected with murine typhus (6).[3] (The basic characteristics of these conjugates are summarized in

* This work was supported by grants-in-aid from the Life Insurance Medical Research Fund, the Helen Hay Whitney Foundation, the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service, the National Foundation for Infantile Paralysis, and was conducted with the aid of the Commission on Virus and Rickettsial Diseases, Army Epidemiological Board, Office of The Surgeon General, United States Army, Washington, D. C.

[1] Supplied through the courtesty of Dr. Herald R. Cox, Lederle Laboratories.

[2] Conjugates prepared from this serum stained normal yolk sac suspensions presumably because they contained anti-egg antibodies, and could not be used in the study of yolk sac material.

[3] The conjugate from this serum did not stain normal yolk sac suspensions. The serum was supplied through the courtesy of Dr. Edsall and Dr. McComb of the Massachusetts Department of Health Biologic Laboratories.

Table I.) (*c*). Concentrated therapeutic anti-Rocky Mountain spotted fever rabbit serum (Lederle[1]).

Strains of epidemic typhus (*Rickettsia prowazeki*) employed were the Breinl strain, and a strain recently isolated from a case of Brill's disease. The Long Island strain[4] of Rocky Mountain spotted fever (*Dermacentroxenus rickettsi*) was used.

Smears were prepared from the intestines of human body lice infected with typhus by the method of Snyder and Wheeler (8). Eastern cotton rats were injected intracardially as described by Snyder and Anderson (9). Relatively large numbers of *R. prowazeki* and *D. rickettsi* respectively were obtained in smears from the peritoneal and pericardial exudates of Eastern cotton rats appropriately infected after exposure to filtered x-radiation (600 to 700 roentgens) by a technique similar to that used for white mice by Liu, Snyder, and Enders (10).

TABLE I

Comparison of Titer and Staining Efficiency of Typhus Conjugates

	Protein	Complement fixation titer*	Staining‡
	per cent		
Rabbit serum (antityphus-epidemic)	7.41	1/160	
Preparation 5	0.44	1/80	+++
Preparation 46	0.50	1/160	+++
Preparation 51	1.68	1/80	++
Horse serum (antityphus-epidemic)	7.15	1/40	
Preparation 59	0.99	1/40	+++

* All sera were diluted to isoprotein concentration (0.44 per cent); titers are expressed in terms of these solutions.

‡ Tested on frozen sections of infected cotton rat spleen after dilution to isoprotein concentration (0.44 per cent) and absorption with mouse liver powder (2).

Frozen sections were prepared by the method of Linderstrøm-Lang and Mogensen (11). This method is potentially hazardous to laboratory personnel when employed with highly infectious material. Smears and frozen sections were unfixed except for drying.

Uninfected control material was examined in each case. Staining and specific inhibition of staining were carried out as described in the accompanying paper (2).

Results.—Staining of rickettsiae and rickettsial antigen was specific. The murine typhus antiserum conjugate stained epidemic typhus rickettsiae well, as was anticipated from its known ability to agglutinate these forms (5).

In the smears of infected yolk sac suspensions, it was possible to distinguish between heavily infected and normal suspensions with ease. Many tiny fluorescent cocco-bacillary bodies could be seen in the infected material. The minimum number of typhus rickettsiae which are required for definite identification with fluorescent antibody has not been determined.

Smears from single infected lice also showed such forms, allowing a rapid decision provided there were several organisms per high power field. There was

[4] Obtained through the courtesy of Dr. Harry M. Rose (7).

a tendency for the exoskeleton of insects to stain non-specifically. Specific inhibition performed on parallel smears was successful. As a further control of specificity, smears were made from the intestines of normal bedbugs, which contain numerous rickettsial forms. These are believed to be non-pathogenic and have no relation to typhus or Rocky Mountain spotted fever in animal tests, but it has been impossible to distinguish them from pathogenic rickettsiae on the basis of morphology or tinctorial qualities. However, these rickettsiae in smears of bedbugs did not stain with either fluorescent antityphus or anti-Rocky Mountain spotted fever serum.

TABLE II

Specificity of Rickettsial Staining

X-rayed cotton rats—peritoneal smears—Rocky Mountain spotted fever				
	Normal	Infected RMSF*	Normal	Infected RMSF
	Treated with normal rabbit serum 15 min.		Treated with anti-RMSF rabbit serum 15 min.	
	Rinsed with saline, then treated with fluorescein-antibody solution 15 min.			
	Washed in buffered saline, 10 min.			
	Mounted in buffered glycerol, and examined under the fluorescence microscope			
	Results—staining			
	Normal	Infected	Normal	Infected
Preparation 51 (antityphus)	0	0	0	0
Preparation 52 (anti-RMSF)	0	++++	0	0

* Rocky Mountain spotted fever.

In smears from the peritoneum and pericardium of cotton rats infected with the Breinl strain, rickettsiae were found lying free in the exudate, adherent to the surface of cells, and packed in the cytoplasm of macrophages and serosal cells (Fig. 1).

Frozen sections of the unfixed liver and spleen of a cotton rat moribund with experimental typhus showed rickettsiae lying extracellularly in the liver sinusoids and the splenic red pulp. Many Kupffer cells were enlarged and their cytoplasm contained typical cocco-bacillary forms (Fig. 2); the cytoplasm of some of them showed diffuse staining. The liver cells themselves did not stain. In the spleen the macrophages and reticulum cells of the red pulp contained

rickettsiae, although in smaller numbers than in the liver. In addition to those observed lying extracellularly in the red pulp, an occasional form was visible between the cells in the lymphoid follicles. The sections from a normal cotton rat failed to retain any of the stain. Specific inhibition was successful.

Smears from the peritoneal exudate of x-rayed cotton rats infected with Rocky Mountain spotted fever showed organisms both intra- and extracellularly and in addition a few of the cells exhibited diffusely stained cytoplasm which we interpret as indicating the presence of dispersed antigenic material. Reciprocal experiments with typhus and Rocky Mountain spotted fever smears and conjugates homologous for one or the other indicated that there was no cross-staining between these forms. One such experiment is outlined in Table II.

As tested on smears and frozen sections of typhus-containing material, formalin, methanol, and Zenker's fluid all markedly diminished or completely destroyed antigenic activity. Acetone did not impair staining, and is a potential fixative for rickettsial antigens.

2. Mumps Virus

Success with rickettsiae led us to attempt to extend the method to mumps virus.

Materials and Methods.—Fluorescein-antimumps monkey serum conjugate was prepared as described (2). The antiserum was obtained from a monkey convalescent from experimentally induced mumps (Enders strain). It had a complement fixation titer (12) of 1/2048 against monkey gland antigen. Before use as a stain it was absorbed twice with human or mouse liver powder (2).

Parotid glands were removed surgically from one normal monkey and three monkeys infected 4 or 5 days previously by instilling 2 ml. of a 1:40 suspension of infected monkey gland into Stenson's duct on each side after the method of Johnson and Goodpasture (13). After removal, pieces of each gland were sealed in glass, quick-frozen, and stored in a dry ice cabinet until use. One gland from each animal was tested for the presence of mumps virus by the injection of embryonated eggs *via* the amniotic sac route (14); in all instances mumps virus was isolated.[5] In one case the piece of gland used for this procedure was the same piece from which frozen sections had been cut a few minutes before. Further evidence of successful infection of these monkeys was the fact that each subsequently developed antibodies in significant titer against the mumps virus.

Frozen sections, staining of sections, and controls of specificity were prepared and carried out as described in reference 2.

Results.—The infected glands showed irregularly distributed yellow-green staining of single acini or small groups of them. This staining could be specifically inhibited. Sections of the parotids of the normal monkey showed no staining.

The principal concentration of antigenic material, as determined by specific

[5] We are grateful to Dr. Tien-Hsiuo Chu and Joan B. Daniels for carrying out the infectivity tests.

staining, was in the cytoplasm of the acinar cells. The infected (*i.e.*, stained) acini were scattered irregularly throughout the lobules (Fig. 3), and their number in relation to the uninfected acini varied markedly from gland to gland, and from lobule to lobule. Most of the cells in an infected acinus contained antigenic material, although occasionally one or two cells only were involved. When this was the case, the amount of material in the infected cell was small, as though this represented the beginning of infection in that focus.

Within the cytoplasm of the antigen-containing cells, the staining had a granular appearance (Figs. 4 and 5). The visible granules were usually very fine, but larger particles were also found, and occasionally roughly spherical stained objects 1 to 2 micra in diameter were observed. This granular specific staining was limited to the cytoplasm; the nuclei were free. Often the cell outlines of the stained cells were distorted, and sometimes seemed disintegrating, liberating specific material into the lumen of the acinus (Fig. 5).

The ducts not infrequently contained specifically stained cellular debris and granular material. Occasionally impressive amounts of antigen could be seen in the lumen (Fig. 6), and a few spherical granules about 1 micron or smaller in diameter could be seen in the cytoplasm of the epithelial cells lining the ducts. It was quite definite that antigenic material, probably virus, invaded the duct epithelium, but there was no appearance suggesting that the epithelial cells were disintegrating at this stage and the concentration of antigen in the ductal epithelium was quite low where it was present.

The stroma of the glands was free of staining.

DISCUSSION

The data presented here suggest the potency of the labelled antibody as a histochemical tool, particularly if it can be applied, as seems likely, to a diversity of antigens. Some of the technical precautions necessary in using it in this way have been described in an accompanying paper (2); its limitations are as yet unexplored.

The procedures previously available for the identification of rickettsiae have required animal inoculations and serologic tests involving at least 2 weeks for completion. The fluorescein-antibody technique makes it possible to identify and to locate these two varieties of rickettsiae in less than 2 hours, although the minimum number of organisms necessary for these procedures has not yet been determined. Furthermore, antigenic material has been identified in infected human body lice when these were no longer infectious as a result of inadvertent thawing. Applications of the method to various problems concerning pathogenic rickettsiae are in prospect.

The specific localization of mumps virus antigen in the experimentally infected monkey parotid represents a definite advance in the precision of virus

detection. The focal nature of the distribution of antigen, probably for the most part active virus, is in complete agreement with the histological findings of Johnson and Goodpasture (15). Their description of the various cytological stages in the development of the lesion in the acinus, from a single swollen cell to the disintegration of all its cells, is exactly paralleled by the deposit of fluorescent antibody. The absence of necrosis in the ductal epithelium is consistent with the smaller amount of antigen found there.

What promise this use of labelled antibody holds for the study of other viruses it is not yet possible to say. Attempts with others, particularly perhaps with the neurotropic viruses, are clearly desirable. However, one such attempt,[6] a preliminary experiment with Columbia SK virus, was unsuccessful.

SUMMARY

Rickettsiae of epidemic typhus fever and Rocky Mountain spotted fever have been microscopically localized and identified in smears of exudates and tissue sections from infected cotton rats by means of homologous antibody labelled with fluorescein. Epidemic typhus has also been identified in smears from single infected human body lice.

Mumps virus antigen has been microscopically localized in the parotid of the experimentally infected monkey by the same method. The antigenic material, probably active virus, was found in the cytoplasm of the acinar cells. Such infected acini were scattered irregularly throughout the gland. Some antigen could be seen in the lumens of the parotid ducts and small amounts were present in the cytoplasm of the epithelial cells lining the ducts.

We are indebted to Miss Jeanne M. Connolly for technical assistance.

BIBLIOGRAPHY

1. Coons, A. H., Creech, H. J., Jones, R. N., and Berliner, E., *J. Immunol.*, 1942, **45**, 159.
2. Coons, A. H., and Kaplan, M. H., *J. Exp. Med.*, 1950, **91**, 1.
3. Kaplan, M. H., Coons, A. H., and Deane, H. W., *J. Exp. Med.*, 1950, **91**, 15.
4. Cox, H. R., *Science*, 1941, **94**, 399.
5. Zinsser, H., and Castaneda, M. R., *J. Exp. Med.*, 1933, **57**, 391.
6. Zinsser, H., and Castaneda, M. R., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 840.
7. Rose, H. M., Duane, R. B., and Fischel, E. E., *J. Am. Med. Assn.*, 1945, **129**, 1160.
8. Snyder, J. C., and Wheeler, C. M., *J. Exp. Med.*, 1945, **82**, 1.
9. Snyder, J. C., and Anderson, C. R., *Science*, 1942, **95**, 23.
10. Liu, P. I., Snyder, J. C., and Enders, J. F., *J. Exp. Med.*, 1941, **73**, 669.

[6] In collaboration with Dr. Robert Rustigian.

11. Linderstrøm-Lang, K., and Mogensen, K. R., *Compt.-rend. trav. Lab. Carlsberg*, 1938, **23**, série chimique, 27.
12. Enders, J. F., and Cohen, S., *Proc. Soc. Exp. Biol. and Med.*, 1942, **50**, 180.
13. Johnson, C. D., and Goodpasture, E. W., *J. Exp. Med.*, 1934, **59**, 1.
14. Habel, K., *Pub. Health Rep., U.S.P.H.S.*, 1945, **60**, 201.
15. Johnson, C. D., and Goodpasture, E. W., *Am. J. Path.*, 1936, **12**, 495.

EXPLANATION OF PLATE 5

All photomicrographs were taken through the fluorescence microscope after treating the object with homologous antibody conjugated with fluorescein. The lightest areas represent the yellow-green fluorescence of deposited fluorescein antibody. The blue-gray autofluorescence of the tissue makes visible the topography of the organ.

FIG. 1. Peritoneal smear from cotton rat infected with the Breinl strain of epidemic typhus. Large cell with cytoplasm choked by rickettsiae specifically stained by fluorescent antibody. Scattered extracellular rickettsiae are also present. × 600.

FIG. 2. Liver from cotton rat infected with typhus. Two Kupffer cells contain many rickettsiae in their cytoplasm. Free rickettsiae in the sinusoids. Frozen section. × 600.

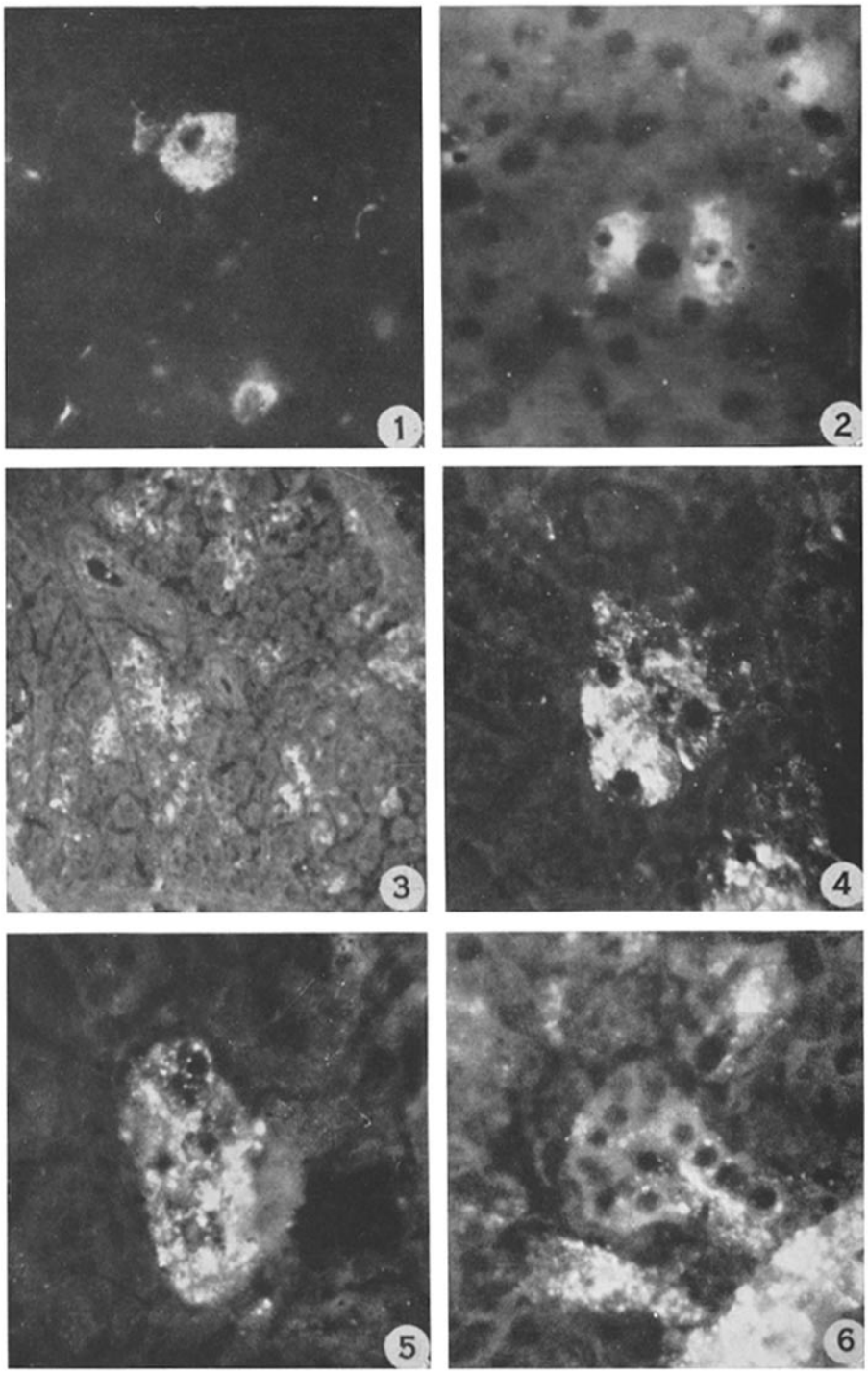
FIGS. 3 to 6. Monkey parotids in experimental mumps on the 4th or 5th day after infection. Frozen sections.

FIG. 3. Irregular focal distribution of mumps antigen in the acini. × 150.

FIG. 4. Single acinus showing granular deposit of antibody over the cytoplasm of the cells. ×600.

FIG. 5. Single acinus with distorted cell outlines and scattered antigenic material. × 600.

FIG. 6. Section through a duct, showing antigen in the lumen and granules in the cytoplasm of the epithelial lining cells. The diagonal white cloud in the lower right-hand corner is an artefact produced by a fold in the section. × 600.



(Coons *et al.*: Rickettsiae and mumps virus antigen)