VARICELLA IN MONKEYS.

NUCLEAR INCLUSIONS PRODUCED BY VARICELLA VIRUS IN THE TESTICLES OF MONKEYS.

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The lesions observed in testicles of monkeys inoculated with emulsified human varicella papules and vesicles have been described in a previous paper (1). In the experiments reported at that time several species of monkeys were employed and also rats, rabbits, guinea pigs, and chickens. Significant lesions, nuclear inclusions, were found only in the testicles of African vervets 5 and 6 days after inoculation, and not in other inoculated tissues of the same animals, nor in the other experimental animals. Furthermore, similar inclusions were not found in the testicles of a vervet inoculated with normal skin. The inclusions were the eosin-staining nuclear bodies which are consistently associated with certain virus diseases and which, regardless of their nature, indicate to many workers the presence of a virus. Therefore, in view of these facts, it was deemed not unlikely that the acidophilic nuclear inclusions in the vervets' testicles were manifestations of the presence of a virus. The nature of the virus had not been studied at the time of the previous report. Recently, however, experiments were performed to obtain, if possible, additional information concerning the suspected virus and it is with the results of this work that the present paper deals.

Methods and Materials.

Monkeys Employed.—It was impossible to obtain vervets (*Cercopithecus lalandi*). A search for susceptible animals was made among other *Cercopithecus* monkeys. A few experiments showed that green monkeys (*Cercopithecus sabæus*) very closely allied to vervets were satisfactory for the work. It is essential that the monkeys be young, and all animals in which spermatogenesis had been established were discarded.

Inoculations.—Emulsified papules and vesicles collected from varicella patients, usually within the first 72 hours of the disease, were used as virus. The papules and vesicles were excised under aseptic conditions and emulsified by grinding in a mortar moistened with Locke's solution. Sand was not used. The emulsified material was taken up in 0.5–1.0 cc. of Locke's solution and portions of it were mixed as desired with equal amounts of Locke's solution, non-immune serum, or immune serum. The mixtures were then injected into monkeys. More than 45–60 minutes never elapsed between the collection of the virus from the patients and its inoculation into animals. All monkeys were inoculated intratesticularly (0.2–2.5 cc.). The non-immune serum was obtained from varicella patients usually within the first 72 hours of the disease. The immune serum was obtained from convalescent varicella patients 14–22 days after the onset of the disease.

Removal and Examination of Testicles.—In previous experiments (1) it was determined that nuclear inclusions were present in the testicles on the 5th and 6th days after inoculation. Therefore in the experiments reported at the present time the monkeys were castrated* on the 5th day. Testicles removed for histological studies were fixed in Zenker's fluid with 5 per cent acetic acid, sectioned, and stained with eosin and methylene blue. A careful search for eosin-staining nuclear inclusions was made in numerous sections of each testicle. Details concerning the tinctorial reactions of the inclusions are given by Tyzzer (2), Lipschütz (3), Goodpasture (4), and others.

EXPERIMENTAL.

The experiments to be reported were conducted to determine whether the eosin-staining nuclear inclusions in monkeys' testicles inoculated with emulsified varicella papules and vesicles are specifically associated with the virus of varicella. In connection with this phase of the work six experiments, consisting of neutralization and reinoculation tests, were performed, and a detailed account of each is given below.

Experiment 1.—Monkeys L and M; green. December 23, 1925. 2 lesions were removed from each of 3 varicella patients, Cases 18, 19, and 20, 3 days after the onset of the disease. The papules and vesicles were emulsified together and taken up in 0.5 cc. of Locke's solution. The emulsion was divided into equal portions. Case 18 was bled on the 3rd day of the disease for non-immune serum; Case 17 was bled for immune serum 17 days after onset of the disease. Equal amounts of the sera, not inactivated, were mixed respectively with the two portions of emulsified papules and vesicles. The mixtures were injected immediately

* All operative procedures were conducted under ether anesthesia.

into 2 green monkeys as follows: Monkey L, 0.25 cc. of virus and immune serum in each testicle; Monkey M, 0.25 cc. of virus and non-immune serum in each testicle. The 4 testicles, removed 5 days later, were fixed and examined in the usual way for the presence of eosin-staining nuclear inclusions.

Although nuclear inclusions were not found in the testicles of Monkey L inoculated with virus and immune serum, typical ones were observed in the testicles of Monkey M inoculated with virus and non-immune serum.

Experiment 2.—Monkeys N and O; green. January 4, 1926. 5 lesions, vesicles and papules, removed from a varicella patient, Case 22, 48 hours after the appearance of the rash were emulsified and taken up in 0.5 cc. of Locke's solution. The emulsion was divided into equal portions. Non-immune serum was also obtained from Case 22 and was inactivated at 56°C. for 30 minutes. Convalescent serum from Case 17, collected and inactivated, December 23, 1925, was used as the immune serum. Equal amounts of the sera were mixed respectively with the two portions of virus emulsion. 2 green monkeys were inoculated immediately; Monkey N, 0.25 cc. of virus and immune serum in each testicle; Monkey O, 0.25 cc. of virus and non-immune serum in each testicle. Monkey N also received 3 cc. of the convalescent serum intraperitoneally; Monkey O, 3 cc. of the non-immune serum in a similar manner. 5 days later the monkeys were castrated. The testicles were fixed, sectioned, and examined in the usual way for the presence of nuclear inclusions.

Nuclear inclusions were not observed in the testicles of Monkeys N and O. No explanation of their absence from the testicles of Monkey O has been found.

Experiment 3.—Monkeys P and Q; green. January 8, 1926. 5 lesions, vesicles and papules, were removed from varicella patients,—2 from Case 24 on the 4th day of the disease, 3 from Case 25 within 36 hours after the appearance of the rash,—and ground up together. The emulsified material was taken up in 0.5 cc. of Locke's solution and divided into equal portions. The inactivated non-immune serum was a mixture of sera collected from Cases 18, 22, 24, and 25 on the 3rd, 2nd, 4th, and 2nd days of the disease respectively. The inactivated immune serum was a mixture of sera collected from Cases 17, 18, 21, and 23 on the 17th, 16th, 14th, and 14th days respectively after the appearance of the rash. Equal amounts of the non-immune and immune sera were mixed respectively with the two portions of virus emulsion. The mixtures were injected into 2 green monkeys as follows: Monkey P, 0.25 cc. of virus and immune serum in each testicle; Monkey Q, 0.25 cc. of virus and non-immune serum in each testicle. Monkey P also received 7 cc. of the immune serum intraperitoneally; Monkey Q,

7 cc. of the non-immune serum in a similar manner. 5 days later the testicles were removed, fixed, and examined in the usual way for the presence of nuclear inclusions.

Eosin-staining nuclear inclusions were found in the sections from Monkey Q inoculated with virus and non-immune serum. None were seen, however, in the sections from Monkey P inoculated with virus and immune serum.

Experiment 4.—Monkey A (1); vervet. November 12, 1924. 10 cc. of blood collected from a varicella patient, Case 1, 36 hours after the appearance of the eruption, was injected intravenously into Monkey A. Fluid from 30 vesicles was also collected at the same time and injected intradermally in left eyelid, in left and right thighs, and in right side of abdominal wall. While under observation the animal showed no manifestations suggestive of chicken-pox. No tissue was removed at this time for histological study.

Monkey A (1) was inoculated again, April 7, 1925. Emulsified varicella vesicles and papules from Cases 13 and 14 were injected into the right testicle. 6 days later the testicle was removed and fixed. Nuclear inclusions were not found in the sections.

Monkey A; Monkey R, green. January 9, 1926. 3 lesions were removed from varicella patients,—2 from Case 26 on the 2nd day of the disease, 1 from Case 27 on the 4th day of the disease,—emulsified, taken up in 0.5 cc. of Locke's solution. 0.25 cc. of the emulsion was injected into the left testicle of each monkey. Monkey R also received 7 cc. of fresh unclotted blood from Case 26. 5 days later the inoculated testicles were removed and fixed. A search was made in the usual manner for the presence of nuclear inclusions.

Monkey A was considered to be immune because of the two previous inoculations. Monkey R was normal and approximately as old as Monkey A. Nuclear inclusions were found in the normal testicle inoculated with varicella virus. None were seen, however, in the immune testicle inoculated with the same virus.

Experiment 5.—Monkey R, green. January 9, 1926. Monkey R was employed in Experiment 4. At that time the left testicle was inoculated with varicella virus. It was removed 5 days after the inoculation and sections of it showed nuclear inclusions.

Monkeys R, S, and T; green. February 3, 1926. 2 papules and 2 vesicles were removed from a varicella patient, Case 28, within 48 hours after the appearance of the rash, emulsified, and taken up in Locke's solution. There was 0.75 cc. of emulsion. The non-immune serum was collected from Case 25 on the 2nd day of the disease and inactivated. The immune serum was collected from

the same patient 12 days later and inactivated. 0.25 cc. of the virus emulsion was inoculated in the right testicle of Monkey R. 0.25 cc. of the emulsion was mixed with 0.25 cc. of the non-immune serum and then half of the mixture was injected into each testicle of Monkey S. 0.25 cc. of the virus emulsion was mixed with an equal amount of immune serum and then half of the mixture was injected into each testicle of Monkey T. In addition to the above injections Monkeys S and T received intraperitoneally 3.5 cc. of the non-immune and immune sera respectively. The testicles were removed and fixed 5 days later. A careful search through numerous sections revealed the presence of nuclear inclusions in both testicles of Monkey S. None were seen, however, in the testicles of Monkeys R and T.

Experiment 5, in which reinoculation and neutralization tests were conducted simultaneously, is especially interesting. Monkey R's left testicle inoculated with varicella virus, January 9, 1926, showed nuclear inclusions 5 days later. In the right testicle, however, inoculated 25 days later with varicella virus no inclusions were found. The non-immune and immune sera used in the neutralization tests were obtained from the same patient; the former on the 2nd day of the disease, the latter on the 14th. The non-immune serum mixed with virus did not prevent the formation of nuclear inclusions in the testicles of Monkey S. On the other hand, the immune serum inhibited the production of inclusion bodies in the testicles of Monkey T.

Experiment 6.—Monkeys U and V; green. April 6, 1926. 5 lesions, vesicles and papules, were removed from a varicella patient, Case 30, within 48 hours after the appearance of the eruption, and emulsified. The volume of the emulsion was made up to 0.5 cc. with Locke's solution. Patient 30 also supplied the non-immune serum. The immune serum was obtained from Case 29 on the 22nd day after the onset of the disease. Both sera were fresh and not inactivated. 0.25 cc. of each serum was mixed with equal amounts of the virus emulsion. The mixtures were injected immediately into 2 green monkeys as follows: Monkey U, 0.25 cc. of virus and immune serum in each testicle; Monkey V, 0.25 cc. of virus and non-immune serum in each testicle. In addition to the above injections, the monkeys received intraperitoneally 3 cc. of the immune and non-immune sera respectively. The monkeys were castrated 5 days later and a search for the presence of nuclear inclusions in the testicles was made in the usual way.

Eosin-staining nuclear inclusions were found in both of Monkey V's testicles inoculated with a mixture of virus and non-immune serum. On the other hand, inclusion bodies were not seen in Monkey U's testicles inoculated with a mixture of virus and immune serum.

The results of the six experiments described above are summarized in Table I.

TABLE I.

Summary of the Results of Reinoculation and Neutralization Tests.

Experiment	Monkey	State of monkey	Inoculum	Nuclear inclusions.
1	L	Normal	Virus + convalescent serum	−
	M	Normal	Virus + non-immune serum	+
2	N	Normal	Virus + convalescent serum	−
	O	Normal	Virus + non-immune serum	−
3	P	Normal	Virus + convalescent serum	−
	Q	Normal	Virus + non-immune serum	+
4	A	Actively immunized	Virus	−
	R	Normal	Virus	+
5	R	Actively immunized	Virus	−
	T	Normal	Virus + convalescent serum	−
	S	Normal	Virus + non-immune serum	+
6	U	Normal	Virus + convalescent serum	−
	V	Normal	Virus + non-immune serum	+

+ indicates presence of nuclear inclusions in testicles.
− " absence " " " " " "

DISCUSSION.

Neutralization and reinoculation tests are the accepted means employed to identify viruses and have been extensively used in investigations concerning vaccine virus (5), variola virus (5), poliomyelitis virus (6), Virus III (7–9), herpes virus (10, 11), and others. Technical details of the tests may vary somewhat with each virus, yet in every instance the usefulness of the tests is dependent either upon the specific virucidal properties of an immune serum or upon a specific refractory state of an immune animal.

Many viruses produce characteristic macroscopic lesions in animals, or cause marked changes in their condition. Such alterations and lesions serve as indications of virus activity. Under proper con-

ditions the occurrence of these changes is specifically prevented either by mixing the virus with a homologous immune serum prior to the inoculation of the animal or by injecting the virus alone into an animal previously immunized by means of the same virus. In addition to the characteristic macroscopic lesions already mentioned, many viruses also produce equally characteristic microscopic changes as indicated by the presence of inclusion bodies in the nuclei and cytoplasm of injured cells. Sometimes, however, the microscopic changes are the only manifestation of the presence of a virus (1, 12). When such a condition arises, there is no obvious reason why the microscopic changes, inclusion bodies, should not be used as guides or indicators with the same degree of readiness as that with which the macroscopic lesions are employed.

Many workers believe that the eosin-staining nuclear inclusions are the manifestations of the presence of certain filterable viruses, including the virus of varicella. Consequently at the time of the previous report (1) there were grounds for the belief that the nuclear inclusions found in the monkeys' testicles inoculated with emulsified varicella papules and vesicles were produced by the action of varicella virus. Proof of this, however, was obtained only recently by means of the experiments reported above, and consists, in brief, of the following considerations. Nuclear inclusions were not found in monkeys' testicles inoculated with a mixture of varicella virus and convalescent varicella serum. On the other hand, they were found in testicles inoculated with a mixture of virus and non-immune serum collected from varicella patients early in the disease. Furthermore, the inoculation of one testicle with varicella virus prevented the formation of nuclear inclusions in the other one when it was inoculated at a later date with the same virus.

So far as is known, the evidence presented in this paper is the only definite proof on record that experimental animals are susceptible in any way to the action of varicella virus.

CONCLUSION.

The eosin-staining nuclear inclusions found in the monkeys' testicles inoculated with emulsified tissue of human chicken-pox lesions are specifically associated with the virus of varicella.

BIBLIOGRAPHY.

1. Rivers, T. M., *J. Exp. Med.*, 1926, xliii, 275.
2. Tyzzer, E. E., *J. Med. Research*, 1905–06, xiv, 361.
3. Lipschütz, B., *Arch. Dermat. u. Syph., Orig.*, 1921, cxxxvi, 428.
4. Goodpasture, E. W., *Am. J. Path.*, 1925, i, 1.
5. Gordon, M. H., *Med. Research Council, Special Rep. Series, No. 98*, London, 1925, 12.
6. Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1910, liv, 1780.
7. Rivers, T. M., and Tillett, W. S., *J. Exp. Med.*, 1924, xxxix, 777.
8. Miller, C. P., Jr., Andrewes, C. H., and Swift, H. F., *J. Exp. Med.*, 1924, xl, 773.
9. Andrewes, C. H., and Miller, C. P., Jr., *J. Exp. Med.*, 1924, xl, 789.
10. Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 357.
11. Zinsser, H., and Tang, F.-F., *J. Exp. Med.*, 1926, xliv, 21.
12. Kuttner, A. G., and Cole, R., *Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 537.