Skin, Liver, and Corneal Lesions Produced by Injections of High Infectious Titer and UV-Irradiated Preparations of Herpes Simplex Virus in Rabbits and Guinea Pigs* (34047)

TADASU TOKUMARU
(Introduced by T. N. Harris)
The Children's Hospital of Philadelphia, Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

It previously was shown that the intravenous injection of high-titer suspensions of herpes simplex virus had a pyrogenic effect in rabbits and guinea pigs (1) and that such viral suspensions also had cytotoxic effects when inoculated into various tissue cultures (2).

As had been observed previously in toxicity studies with influenza virus (3, 4), the intracerebral inoculation of a suspension of the herpes simplex virus caused the inoculated animal to develop tonic convulsions when it was twirled by the tail. Attempts then were made to abolish the infectivity of the virus by UV irradiation. The irradiated preparation lost its infectivity for mice, but it was still capable of producing tissue lesions, such as erythema and induration of the skin, in guinea pigs and pyrogenic effects, hemorrhagic necrosis of the liver, and opacity of the cornea in rabbits when injected intradermally, intravenously, and intracorneally, respectively.

The above effects of the UV-resistant components of the herpes simplex virus were completely neutralizable by specific antiserum against this virus. Storage, subjection to a pH of 4, heating to 56°, or formalinization resulted in a partial or complete loss of these toxic effects.

Materials and Methods. The H strain of herpes simplex virus was propagated in primary rabbit kidney (RK) cell cultures, and the infected culture fluid, which hereafter will be referred to as the virus, was harvested as previously described (2). It contained 64 syncytium-producing units and $10^{9.3}$ plaque-forming units (pfu) per ml. After its cytotoxic titer had been determined by 4-hr readings as previously described (2), it was processed as soon as possible and used without storage.

Abolition of the virus infectivity. Ultraviolet (UV) irradiation. Five-milliliter amounts of the virus were placed in a petri dish (internal diameter: 9 cm) under an ultraviolet irradiator (The Drummond Scientific Company, Broomall, Pennsylvania; light source: Sylvania Germicide Type A.G15TB with reflector producing 829 $\mu W/cm^2$ at a distance of 17.8 cm), irradiated for 6 min with mechanical rocking (90 times/min; excursion of 2.5 cm), transferred to a clean petri dish and again irradiated for 4 min.

Heat inactivation. The virus was held at 56° for 45 min in a Wassermann tube in a serological bath.

Inactivation at pH 4. The virus was adjusted to pH 4 by the addition of 0.1 *N* HCl with stirring with a magentic stirrer, allowed to stand at 26° for 60 min and then neutralized with 0.1 *N* NaOH.

Ether inactivation. The virus was stirred (magnetic stirrer) with an equal volume of anesthetic ether (Squibb) for 60 min at 4°. The aqueous phase was then separated by centrifugation at 2000 rpm for 30 min and placed under a gentle stream of nitrogen.

Formalinization. This was carried out by a

* This investigation was supported by a research grant (AI-05612) from the National Institute of Allergy and Infectious Diseases, U. S. Public Health Service, Bethesda, Maryland.

previously described technique (5) using 1:400 and 1:1000 dilutions of formalin. The infectivity of the virus subjected to the above-described inactivation techniques was found to be negative by a plaque titration. In all of these procedures uninfected RK cell cultures were included as controls. The untreated virus was filtered twice through a Swinnex-25 Millipore membrane with a pore size of 0.22 mμ (Millipore Filter Corporation, Bedford, Massachusetts). The infectivity of the filtrate was $10^{2.2}$ pfu/ml.

Animals. Rabbits weighing about 4.5 kg, guinea pigs weighing about 500 g, and mice of both sexes, weighing 25 g, were supplied by Rockland Farms, Gilbertsville, Pennsylvania.

Inoculation procedure. Pyrogen-free disposable plastic syringes and pyrogen-free saline (Abbott) as the diluent were used as previously described (1). The following volumes were injected: intravenously in rabbits—2 ml/kg (in the ear vein); intradermally in guinea pigs—0.1 ml (in the clipped flank); intracerebrally in mice—0.03 ml; intraocularly in rabbits—0.1 ml [into anterior chamber, cornea, or conjunctival sac with a 1.0-ml syringe and a 30-gauge needle after the application of a 1% solution of Ophthaine (proparacaine hydrochloride, Squibb)].

Determination of rectal temperature. A YSI scanning telethermometer set was used as previously described (1).

Titration of infectious virus in organ homogenates. The animals were killed with ether. Pieces of the removed infected organs were fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin. The remaining portion of each organ was ground in a mortar with sand, and a 20% suspension in saline was prepared from the ground material. After centrifugation of the suspension at 5000 rpm for 20 min, the supernatant fluid was collected and its virus content titrated in RK cell cultures.

Preparation of immune serum and virus neutralization. After collecting samples of the preimmunization serum (hereafter referred to as normal serum) from six rabbits, they were infected by application of the virus to the scarified cornea. All of the rabbits developed a keratoconjunctivitis which healed by approximately Day 8 after the inoculation. The sera were collected 4 weeks after the inoculation and heated at 56° for 30 min. The sera from two of the rabbits had virus-neutralizing antibody (N-Ab) titers of 1:1024. Serum samples obtained from these two rabbits before the immunization had no demonstrable herpes-neutralizing antibodies. The immune sera from these two rabbits were pooled and used in this study. The pooled sera will hereafter be referred to as herpes antiserum. Neutralization of the virus was carried out in serological bath at 37° for 60 min. We also used a gamma-globulin (N-Ab titer = 1:4096) preparation (Gamulin, Pittman-Moore, Division of Dow Chemical Company, Indianapolis, Indiana) and obtained with it neutralization effects comparable to those obtained with the rabbit antiserum. The herpes antiserum was always used in a final dilution of 1:256.

Results. Tonic convulsions and death. A severe central nervous disturbance developed within 18 hours after the intracerebral injection of the untreated virus in mice (Table I), as evidenced by hyperirritability and particularly by the tonic convulsions and subsequent death which were produced when the animal was twirled by the tail. The latter phenomenon was rarely seen when virus concentrations of less than $10^{7.0}$ pfu/0.03 ml were injected intracerebrally. A significant amount of the above activity of the virus was destroyed by only five cycles of freezing and thawing, and it was completely destroyed by exposure of the virus to UV irradiation or a pH of 4.0. It also was absent from a Millipore filtrate of the virus.

Skin lesions. The virus exposed to UV irradiation or a pH of 4.0 still retained toxic effects for guinea pig skin, in which it produced erythema and induration within 18–24 hr after intradermal injection (Fig. 1). These toxic effects were completely neutralized by the herpes antiserum. The mean sizes of skin lesions produced by UV-irradiated virus and the virus exposed to a pH of 4.0 were about 63% and 22%, respectively, of those produced by the control virus, whereas the activ-

TABLE I. The Effects of Intracerebral Injections of Herpes Simplex Virus in Mice.

Virus preparations	Total no. of mice	Reactivity at 18 hr (no. of mice)			Virus infectivity (pfu/brain, 20% suspension)
		Tonic convulsion and death	Sick	No symptoms	
					$\log_{10}$
Untreated virus $10^{8.0}$ pfu/0.05 ml	24	16	8[a]	0	5.0, 4.9, 5.0, 5.3, 5.5 (mean 5.14 ± 0.25[b])
Frozen-thawed virus $10^{6.9}$ pfu/0.05 ml	24	6	9[a]	9[a]	4.0, 4.1, 4.2, 4.4 (mean 4.18 ± 0.17)
UV-irradiated virus $<10^{1.0}$ pfu/ml	24	0	0	24	<1.0, <1.0, <1.0
pH 4-treated virus $<10^{1.0}$ pfu/ml	10	0	0	10	<1.0, <1.0, <1.0, <1.0
Control tissue culture medium	10	0	0	10	

[a] Died within 48 hr.
[b] Standard deviation.

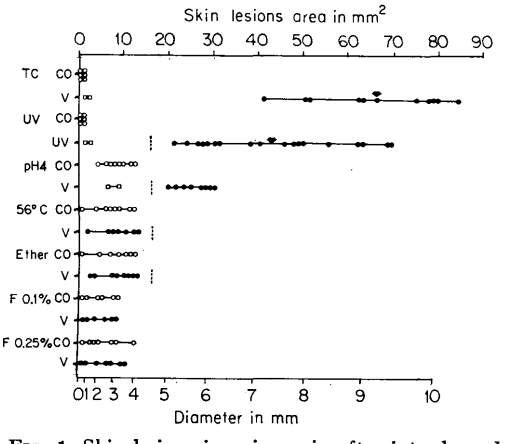

FIG. 1. Skin lesions in guinea pig after intradermal injection of the virus. CO = Control consisting of uninfected tissue culture fluid. V = Virus. F = Formalin. The lesions were measured by the size of the area of erythema surrounding the central induration. Diameter of the skin lesions in millimeters is shown at the bottom. The broken line indicates the lesions having a diameter of at least 4.5 mm (smaller lesions were not regarded as specific). Each point indicates the size of one injection site (there were two injection sites per guinea pig). The arrows indicate mean values. The squares (□—□) show the neutralization of the lesion-producing activity by antiserum. The controls (uninfected tissue culture fluid) are shown by the open circles (○—○), and the test samples by the closed circles (●—●).

ity was lost by the other treatment methods listed in Fig. 1 and by Millipore filtration.

The indurations produced by the virus preparation showed vesiculation without an increase in size after 3 days, at which time erythema began to disappear. Histologically the lesions produced by the preparations of virus exposed to UV irradiation or a pH of 4.0 showed relatively mild changes consisting of dilation of the capillaries, petechiae in the corium, hemorrhages in the subcutaneous tissues, a few necrotic cells, and some cellular infiltration.

Pyrogenicity. As shown in Fig. 2, the pyrogenicity of the virus was found to be largely resistant to UV irradiation but only partially resistant to exposure to a pH of 4.0. It was destroyed by heating at 56° for 30 min, formalinization, or Millipore filtration.

Neutralization tests indicated that all of the pyrogens present in the virus preparation were antigenically specific and that their stability was comparable to that of the pyrogens of influenza virus when treated by the same procedures (6).

Liver lesions. The rabbits used for the pyrogenicity studies were examined for liver damage 20 hr after the injections of the untreated virus material. In contrast to the

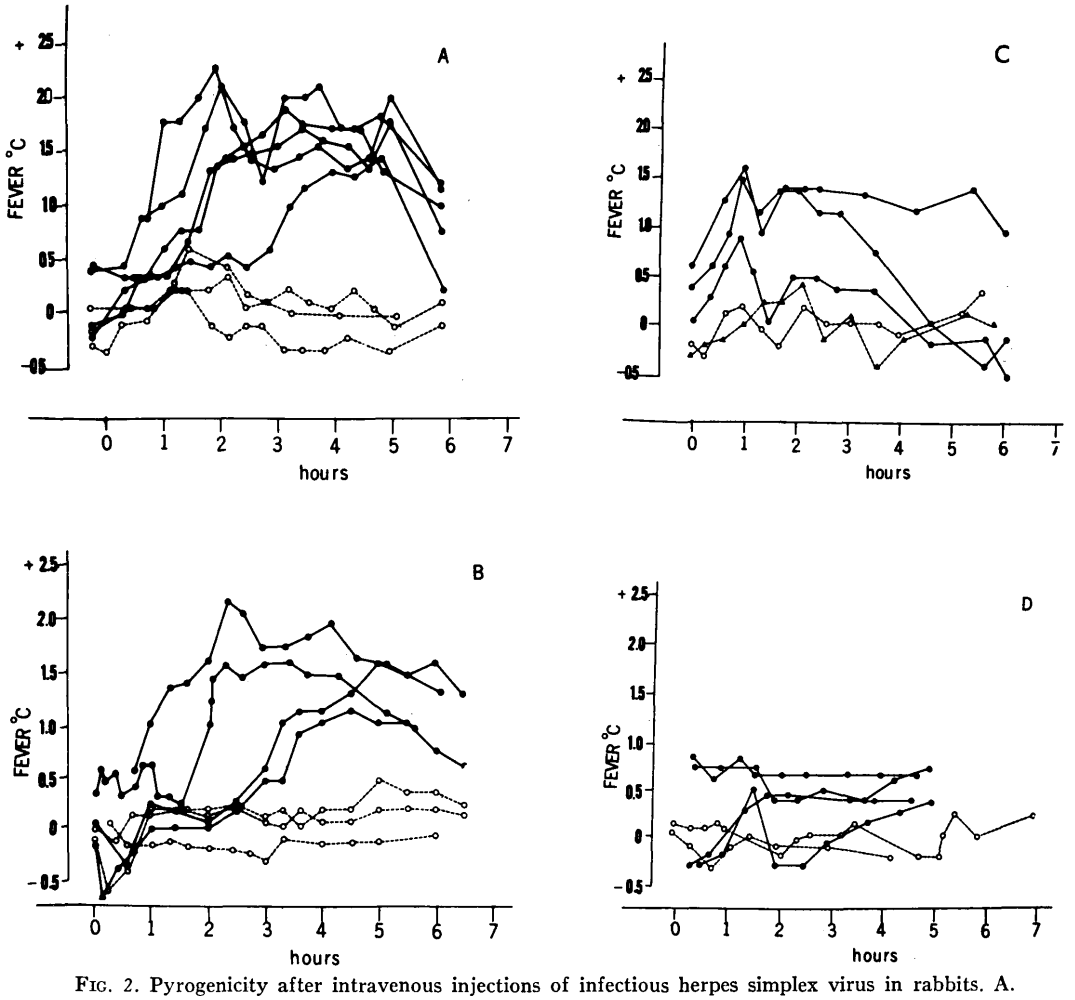

FIG. 2. Pyrogenicity after intravenous injections of infectious herpes simplex virus in rabbits. A. Virus + normal serum (●—●) and virus + antiserum (○—○). Each line represents one rabbit. B. UV-irradiated virus (●—●) and UV-irradiated uninfected tissue culture fluid (○—○). C. pH 4-treated virus (●—●) and formalinized virus (○ - - - ○). D. Heated virus (56° for 30 min) (●—●) and heated uninfected tissue culture fluid (○ - - - ○).

spleen and adrenals, which contained appreciable quantities of infectious virus (Table II), the livers showed a minimal amount of infectious virus. The livers of three of five rabbits showed hemorrhage, necrosis, and a formation of multinucleate giant cells (Fig. 3

TABLE II. Virus Infectivity of Suspensions of Organs of Rabbits 20 hr after Intravenous Injection of Herpes Simplex Virus.

Rabbit no.	Heart	Liver	Spleen	Adrenal	Kidney	Lung	Brain
			$\log_{10}$ (pfu/ml of 20% suspension)				
1	<1.0	<1.0	<1.0	4.5	<1.0	<1.0	<1.0
2	<1.0	<1.0	2.6	6.3	1.2	1.2	1.2
3	<1.0	<1.0	2.7	3.9	<1.0	<1.0	<1.0
4	<1.0	<1.0	2.5	4.5	<1.0	<1.0	<1.0
5	<1.0	1.3	4.9	4.7	1.2	1.3	1.8

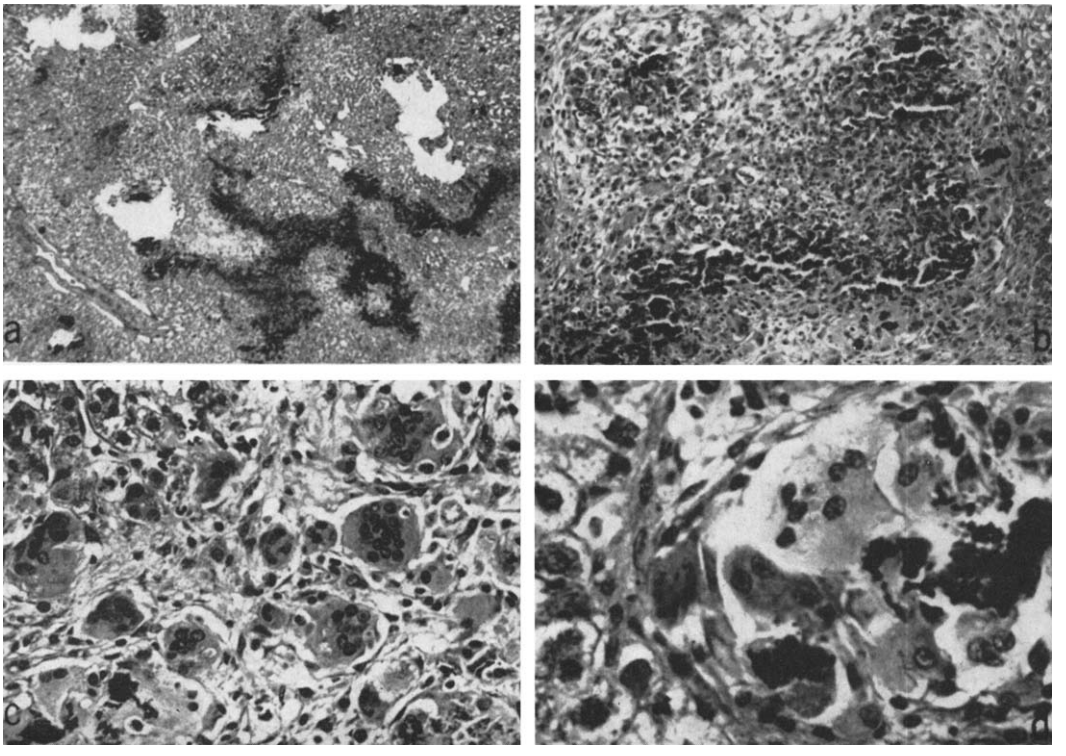

FIG. 3A. Rabbit liver 20 hr after intravenous injections of the virus + normal serum (a–d). a, Magnification 60×. Areas of necrosis, deeply stained with hematoxylin, and other areas of hemorrhage; b, 120×. An area of necrosis surrounded by multinucleate giant cells; c, 430×. A number of multinucleate giant cells. No intranuclear inclusion bodies. d, 650×. An area of necrosis and surrounding giant cells. No intranuclear inclusion bodies.

a–d), and the livers of the other two showed chiefly hemorrhages and fat-laden cells with shrunken nuclei with little or no cellular infiltration. Injections of the UV-irradiated virus produced hemorrhage, necrosis, and fat-laden cells without multinucleate cells in all of five rabbits (Figs. 3 e–f). All of these rabbits also showed an enlarged, flabby, yellowish liver. Less marked and more variable damage was found in the other organs listed in Table II. Rabbits injected with the untreated virus which had been previously treated with the herpes antiserum showed no or only minimal changes in the liver and other organs (Fig. 3 g–h).

Corneal lesions. When UV-irradiated virus was serially diluted (as shown in Fig. 4) and injected intracorneally in rabbits (four rabbits per dilution), there was a development of conjunctival hyperemia and marked opacity within 24 hr. This effect was specifically neutralized by the herpes antiserum in all of the 12 rabbits tested. The results of the experiments showing the neutralization of this effect by the herpes antiserum are shown in Fig. 4. The conjunctival sac was found to be quite sensitive to the UV-irradiated preparation of the virus, which produced an edema when injected directly into the sac. When the UV-irradiated virus was injected into the anterior chamber in four rabbits, all of these animals showed an acute formation of a mass of exudate which covered the pupil within 4 hr (Fig. 4e) and which then persisted for another 24 hr, with development of a hypopyon. This was accompanied by an intense iridocyclitis, with little change in the retrocorneal surface. Although the less severe corneal opacities disappeared within 48 hr, the more severe ones persisted for 3–4 days. These effects were neutralized by the herpes antiserum. Intracorneal and retrocorneal in-

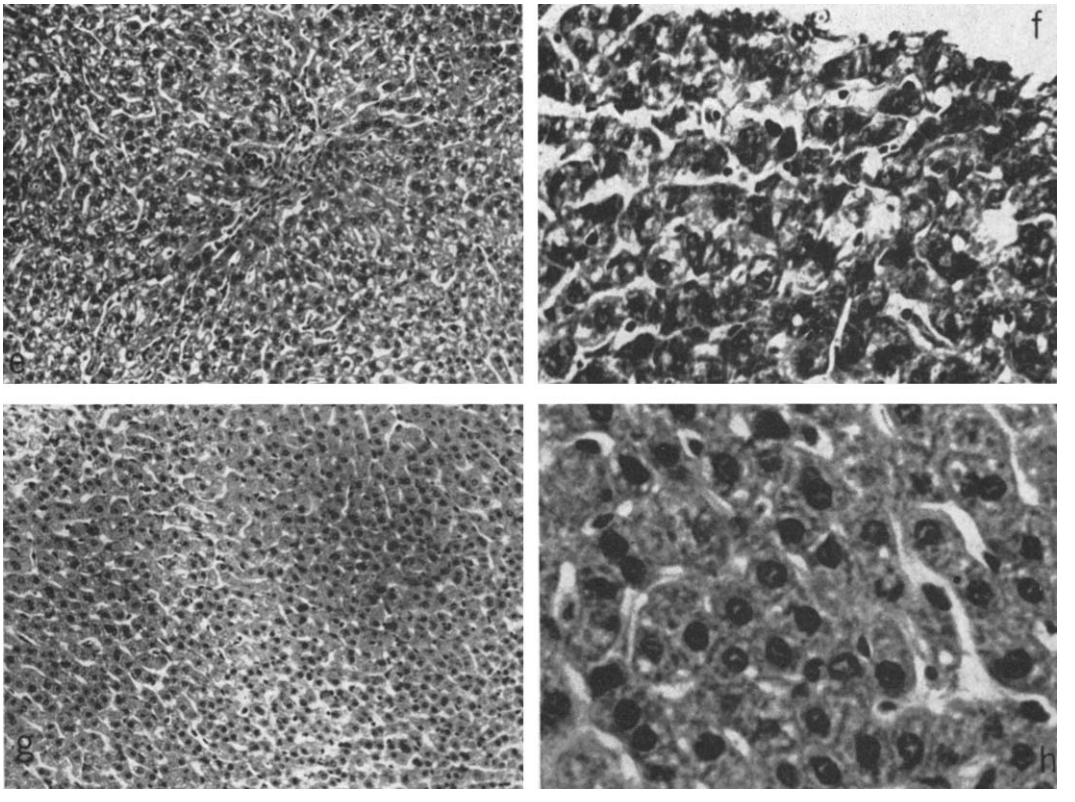

FIG. 3B. Rabbit liver 20 hr after intravenous injection of UV-irradiated virus + normal serum (e–f). e, 120×. Appearance of fat-laden cells; f, 650×. Rabbit liver 20 hr after intravenous injection of the virus + antiserum (g–h). g, 120×. Liver appears normal; h, 650×.

jections of the UV-irradiated virus produced similar reactions in five guinea pigs. It has been reported that the local injection of the surface exudate from the chorioallantoic membrane of the developing chick embryo infected with herpes virus can produce corneal opacities (7), and that more severe opacities (8) can be produced by repeated infections of the rabbit cornea (9).

Discussion. Among the various methods used to abolish the infectivity of herpes simplex virus, UV irradiation was found to be the least destructive for the tissue lesion–producing components of this virus. However, this procedure did destroy two important biologic activities of the virus; namely, a fatal tonic convulsion and a capacity to induce multinucleate giant cells. The latter-type reaction has recently been observed also in the livers of rabbits after intravenous injections of extracellular proteins (exotoxins) obtained from cultures of Type A hemolytic streptococci (10, 11).

It is interesting to note that the lesion-producing activities which were UV-resistant were limited to only a few tissues *in vivo* and that these were the tissues which are most sensitive to the effects of an active infection with the virus.

The experimental demonstration of *in vivo* tissue lesions produced by noninfective virus has rarely been reported for viruses outside of the myxovirus group (12–15). It is our intention to make further studies of various aspects of the modification of the host tissues produced by the above-described noninfectious preparations of herpes virus.

Summary. Tissue lesions consisting of erythema and induration of the skin, hemorrhagic necrosis of the liver, and corneal opacity were produced by UV-irradiated herpes simplex virus. These toxic effects were complete-

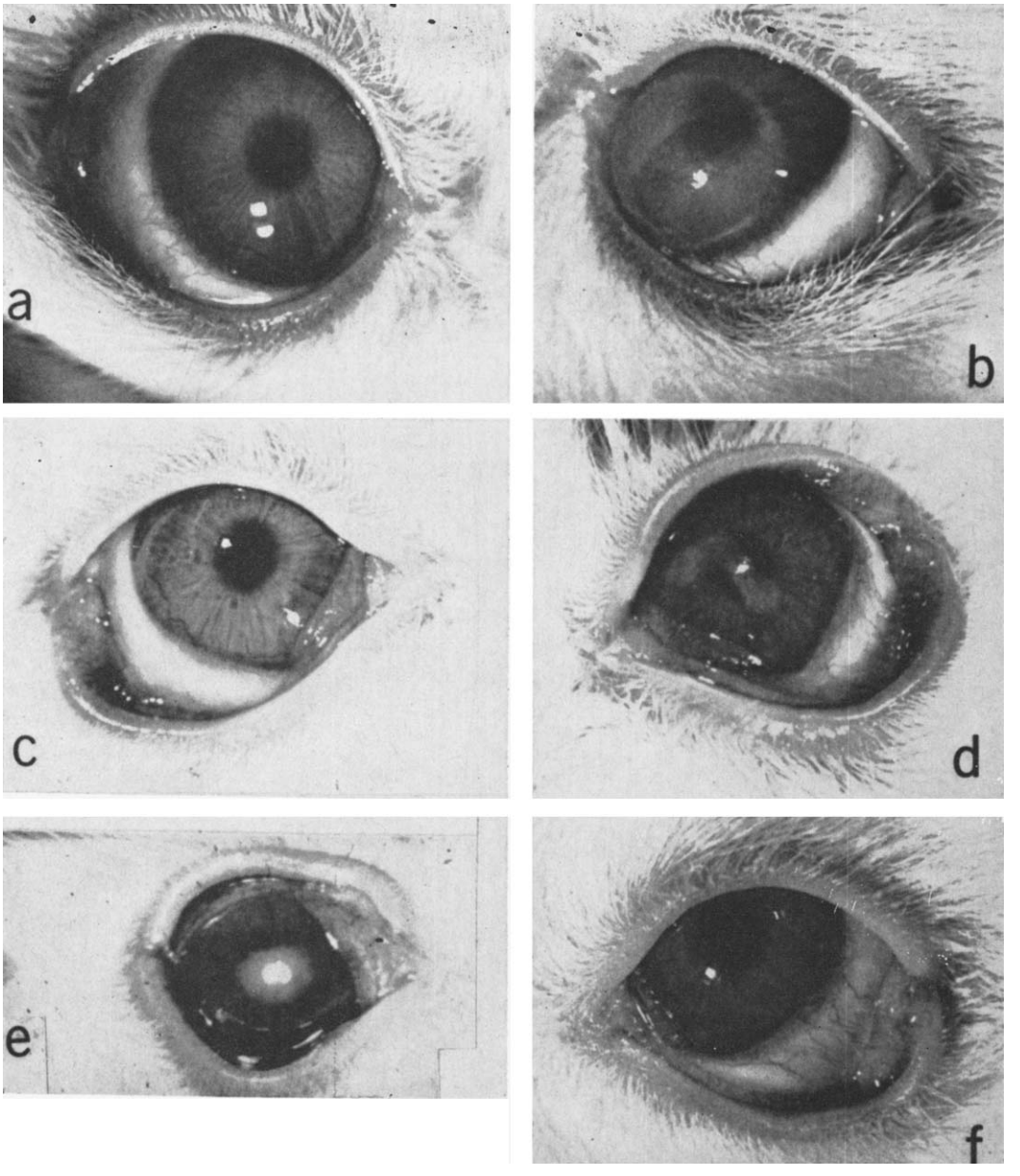

FIG. 4. Ocular reactions after intra-and retrocorneal injections of UV-irradiated virus (a–d, f; 24 hr after the intracorneal injection). a, dilution 1:2 + antiserum; b, same rabbit, opposite eye with dilution 1:2 + normal serum. Edema and opacification; c, dilution 1:4 + antiserum; d, same rabbit as in c, opposite eye with virus dilution 1:4 + normal serum. Corneal opacity at the center; e, retrocorneal injection of UV-irradiated virus (4 hr after the injection). Formation of an exudate within anterior chamber; f, dilution 1:8 + normal serum. No opacification, but conjunctival hyperemia and edema.

ly neutralized by herpes antiserum. Other biologic effects of the herpes virus, namely, fatal tonic convulsions and the formation of multinucleate giant cells in the liver, were destroyed by the UV irradiation of the virus.

1. Tokumaru, T., Arch. Ges. Virusforsch. **23**, 257 (1968).

2. Tokumaru, T., Arch. Ges. Virusforsch. **24**, 104 (1968).

3. Henle, G. and Henle, W., J. Exptl. Med. **84**, 623 (1946).

4. Mims, C. A., Brit. J. Exptl. Pathol. **41**, 586 (1960).

5. Hull, R. N. and Nash, J. C., Am. J. Hyg. **71**, 15 (1960).

6. Siegert, R. and Braune, P., Virology **24**, 218 (1964).

7. Sery, T. W., Richman, M. W., and Nagy, R. M., J. Allergy **38**, 338 (1966).

8. Sery, T. W., Am. J. Ophthalmol. **48**, (Part II), 57 (1959).

9. Williams, L. E., Nesburn, A. B. and Kaufman, H. E., Arch. Ophthalmol **73**, 112 (1965).

10. Zeiri, N., Bentwich, Z., Boss, J. H., Ginsburg, I., and Harris, T. N., Am. J. Pathol. **51**, 351 (1967).

11. Spira, G., Stilberstein, Z., Harris, T. N., and Ginsburg, I., Proc. Soc. Exptl. Biol. Med. **127**, 1196 (1968).

12. Kato, N., Okada, A., and Hara, H., Brit. J. Exptl. Pathol. **42**, 145 (1961).

13. Kato, N. and Okada, A., Brit. J. Exptl. Pathol. **42**, 253 (1961).

14. Wacker, W. B., Lipton, M. M. and Steigman, A. J., J. Immunol. **89**, 525 (1962).

15. Ogasawara, K. and Nakayama, M., Virology **6**, 288 , 1958.

Received Feb. 26, 1969. P.S.E.B.M., 1969, Vol. 131.