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An Intracerebral Assay Procedure in Mice for Chemical Inactivation of Rabies Virus*

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An intracerebral assay technique in mice is described for testing chemical disinfectants acting on the rabies virus. The assay determines whether more than 99% of 10 000 mouse intracerebral LD_{50} of virus are inactivated within 1-2 1/2 minutes. By this test, several substances usually available for the treatment of animal bite wounds were found to be effective. They are: 1%-20% soap solutions; 43%-70% alcohol; 1 : 1000 (0.1%) or lower dilutions of two quaternary ammonium compounds, benzalkonium chloride and cetrimonium bromide; 1 : 10 000 or lower dilutions of iodine; acetone; and ether.

Several other substances tested for possible use in environmental disinfection were also found to be virucidal. They were: 3% caustic soda and commercial preparations of organic phenols, iodine, and a mixture of trisodium phosphate and sodium hypochlorite. No virucidal effect was exerted by a 2% aqueous solution of mercurochrome, a 1 : 1000 aqueous solution of thiomersal, or 3% formalin (1% formaldehyde).

A number of substances that inactivate rabies virus have thus become available for local treatment of bite wounds and for environmental disinfection. The assay procedure described may be useful in testing other disinfectants and chemical substances.

The speed with which rabies virus is inactivated at the site of infection may ultimately determine the fate of the exposed individual. Therefore, it was essential to develop a simple assay procedure for substances that rapidly inactivate the rabies virus. Until the present time, the tests used have consisted principally of local wound treatment in guinea-pigs (Ahuja & Suri, 1954, 1955; Dean et al., 1963; Kaplan et al., 1962; Perez Gallardo et al., 1957; Shaughnessy & Zichis, 1954; and Soloviev & Kobrinski, 1962), the blocking effect in mice (Wiktor & Koprowski, 1963) and exposure of untitrated rabies virus for varying lengths of time to chemical substances, followed by intracerebral inoculation of mice (Harms, 1963). The procedure described here increases the accuracy of the last-named test, and in addition reduces the time and expense of methods presently in use.

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MATERIALS AND METHODS

Virus

The lot of CVS fixed virus used in these experiments was kept frozen at −60°C to −70°C as a 20% mouse-brain suspension in 50% serum-water. Repeated titrations revealed a titre of $10^{6.3}$ LD_{50} virus per 0.03 ml original brain tissue. For the test, the suspension of the virus, after thawing, was centrifuged at 2000 rev/min for 10 minutes to remove the gross nervous tissue particles and the supernatant liquid was diluted twofold to contain an estimated 10^5 LD_{50} per 0.03 ml. A further 10-fold dilution was made by adding 0.2 ml of the virus to 1.8 ml of solutions of the chemical under test. This suspension, containing an estimated 10 000 LD_{50} of virus, was shaken vigorously by hand for exactly one minute. Then the virus was diluted 100-fold through two serial 10-fold dilutions in buffered-saline solution, and 0.03 ml of this material, containing approximately 100 LD_{50} of virus, was inoculated into groups of 5-10 mice (see the table). Each experiment included a group of controls inoculated at the end of the experiment to determine the accuracy of the estimated residual 100 LD_{50} of virus used as the test inoculum. The time taken for

making the 100-fold dilution and inoculation of mice with the test mixture was 1-1½ minutes. Mice were observed daily and the number of sick and dead recorded. Infected mice usually died in 6-10 days after inoculation and the experiment was terminated in 14-21 days.

Substances tested

Benzalkonium chloride (aqueous) (Zephiran) [1] is a mixture of alkylbenzyldimethylammonium chlorides. A 1% dilution was prepared from the 17% commercial preparation.

Cetrimonium bromide (aqueous) (Cetavlon) [2] is hexadecyltrimethylammonium bromide. Dilutions were prepared from powder and from a 40% commercial preparation.

Amphyl [3] is a general-purpose disinfectant used in hospitals, which contains, as active ingredients: 70% potassium ricinoleate, *o*-phenylphenol, *p*-t-amylphenol, and ethyl alcohol (6.2%).

Wescodyne [4] contains, as active ingredients: polyethoxypolypropoxypolyethoxyethanol iodine complex 9.10% and nonylphenoxypoly(ethyleneoxy)-ethanol iodine complex 8.74%. This provides at least 1.5% available iodine.

Tincture of iodine (2% iodine in 47% alcohol).

Aqueous solutions of iodine, as listed in the table.

Merbromin (Mercurochrome), dibromohydroxymercurifluorescein disodium salt, 2% aqueous solution.

Tincture of thiomersal (Merthiolate),[5] sodium ethylmercurithiosalicylate 1/1000 (0.1%) in 50% alcohol.

Aqueous thiomersal 1/1000 (0.1%), as preceding, but in aqueous solution.

Tincture of green soap, 65% soft soap, 33% alcohol, 2% lavender oil. The active ingredients of soft soap are vegetable oil 38%, oleic acid 2%, potassium hydroxide 9%, glycerol 5%.

Tincture of green soap diluted 1 : 3, containing one-third of the constituents listed in the preceding item.

Toilet soap solution, 1% and 10% aqueous solutions and 20% water-ethanol (95 : 5) solution of a commercial toilet soap, Ivory.[6]

Diversol C,[7] a chlorinated trisodium phosphate containing, as active ingredients, 3.25% sodium hypochlorite, 91.71% trisodium phosphate ($Na_3PO_4 . 12H_2O$), 0.01% potassium permanganate and 0.04% sodium lauryl sulfate.

Other substances tested included *ethanol, methanol, sodium hydroxide, acetone, ether, chloroform* and *formalin*, in the concentrations given in the table.

RESULTS

The table shows the results of the inactivation of rabies virus obtained in six separate experiments.

Quaternary ammonium compounds. Both benzalkonium chloride and cetrimonium bromide were virucidal up to dilutions of 1/1000.

Ethanol and methanol. Concentrations of 43% and greater were shown to be virucidal.

Iodine solutions. Inorganic iodine was effective at 5% aqueous concentration. The action of the tincture can be ascribed to the alcohol concentration (47%). The organic iodine complex Wescodyne was effective at 0.5% concentration, even though the available iodine content was very low (less than 1/10 000). An aqueous solution of iodine at 1/10 000 was also virucidal, but the 1/25 000 concentration did not inactivate the virus.

Thiomersal. The virucidal action of the tincture was due to the 50% concentration of alcohol, an aqueous solution of the compound at the same concentration (1/1000) being ineffective.

Soap solutions. All soap solutions at 1% or higher concentrations were effective.

Acetone, 3% sodium hydroxide, Diversol and *Amphyl* were virucidal; *ether* and *chloroform* were partially virucidal, but 3% *formalin* (1% formaldehyde) and a 2% aqueous solution of *merbromin* failed to inactivate rabies virus.

DISCUSSION

Deep extensive wounds inflicted by animals suspected of having rabies require thorough irrigation with non-irritating but effective virucidal solutions. Cleansing with a soap solution, and the

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[7] Diversey Corp., Chicago 6, Ill., USA.

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Substance	Dilution	Deaths/number of mice inoculated in experiment					
		1	2	3	4	5	6
Aqueous benzalkonium chloride (Zephiran)	1/500	0/10	1/5	0/10			0/10
	1/1 000	0/10		0/10			0/10
	1/2 000			6/10			9/10
	1/2 500		4/5				
	1/3 000			10/10			
Aqueous cetrimonium bromide (Cetavlon)	1/500 [a]	0/10	0/5	0/10		1/10	0/9
	1/1 000	0/9		0/10		5/10	4/10
	1/2 000			2/10		10/10	10/10
	1/2 500		2/5				
	1/3 000			10/10		10/10	
Ethanol	70 %	0/10	0/5				
	50 %			2/8			
	45 % (gin)		0/7				
	43 % (whisky)		1/7	0/10			
	43 % (whisky)		0/7				
	40 % (vodka)			4/10			
	40 %			5/10			
	35 %		5/5	10/10			
Methanol	70 %					0/10	
Acetone	Undiluted				0/10		
Ether	Undiluted				4/10		
Chloroform	Undiluted					0/3 [b]	
Tincture of green soap (65 % soft soap, 2 % lavender oil, 33 % alcohol)	Undiluted				0/4 [b]		
	1/3				0/10		
Ivory toilet soap	20 %					0/10	
	10 %					0/10	
	1 %					0/10	0/10
Iodine	Tincture (2 % I in 47 % alcohol)				0/6 [b]		
	Aqueous 5 %				0/2 [b]	0/10 [c]	
	Aqueous 1/10 000						0/10
	Aqueous 1/25 000						10/10
	Wescodyne (available iodine approx. 1/10 000)				0/10		0/10
Sodium hydroxide	3 %					0/10	
Formalin	3 % (1 % formaldehyde)					0/10	
Thiomersal (Merthiolate)	Tincture (thiomersal 1/1 000 in 50 % alcohol)				0/10		
	Aqueous 1/1 000				10/10		
Mercurochrome (Merbromin)	2 % aqueous solution				10/10		
Amphyl	1/200				0/10		
Diversol	1/125						0/10
None (controls)	Estimated LD_{50}: 100	5/5	7/7	5/5	7/7	6/6	5/5
	10	5/5	7/7	5/5	6/7	5/6	5/5
	1	5/5	4/7	1/5	6/7	3/6	1/5
	0.1	0/5	1/5	0/5	1/7	1/6	1/5

[a] In experiments 5 and 6 a commercial preparation (40 %) of cetrimonium bromide was used to make the dilutions; in experiments 1, 2, and 3 dilutions were made from the powder form.

[b] The remainder of 10 mice died of toxic reaction of the chemical.

[c] Further 10-fold dilution was made (=10 LD_{50} virus) to avoid toxic reaction of the chemical.

application of 1%-2% benzalkonium chloride have proved to be effective in the treatment of rabies-infected wounds in guinea-pigs (Dean et al., 1963; Kaplan et al., 1962; Perez Gallardo et al., 1957; Shaughnessy & Zichis, 1954).

However, some quaternary ammonium compounds at 1% concentration may have a deleterious effect on tissues. Benzalkonium chloride and cetrimonium bromide are two quaternary ammonium compounds that show a marked lethal effect on the rabies virus at dilutions of 1 : 500 and 1 : 1000. At this concentration the two compounds can be used for irrigating deep wounds without harmful effect. A 1% soap solution or a highly dilute solution of iodine (1/10 000), which have been shown to be virucidal, should also be acceptable for this purpose.

Ether, acetone, soap, and quaternary ammonium compounds were among the lipid solvents tested and their effectiveness may be attributed to their direct action on the lipid-containing coat of the rabies virus. Highly dilute iodine (1/10 000) quickly inactivates the influenza virus, a myxovirus, presumably by acting on the lipid in its coat (Hoyle, 1964). This is perhaps the mechanism of action on the rabies virus, which is considered to possess several characteristics of the myxovirus group (Johnson, 1965). It is probable that ethanol acts by denaturing the lipoprotein in the virus coat.

The assay procedure is sufficiently sensitive to detect the inactivation of more than 99% of approximately 10 000 mouse intracerebral LD_{50} of rabies virus. Substances can therefore be easily tested with this method for use in wound treatment and environmental disinfection. Among the factors to be considered in the practical application of these laboratory results to wound treatment are: the removal of blood and organic debris, because of their neutralizing action on many disinfectants; thorough cleansing of wounds with soap and water before the application of chemical disinfectants; and, finally, the removal of all traces of soaps or anionic detergents before the application of quaternary ammonium compounds, because of their mutually neutralizing effect.

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RÉSUMÉ

La séro- et la vaccinothérapie, après morsures suspectes, appliquées selon les recommandations d'un Comité OMS d'experts de la Rage, n'excluent pas un traitement local par des produits possédant une action virulicide. Les auteurs ont une longue expérience des recherches dans ce domaine et ont déjà publié de nombreux résultats; ils exposent dans le présent travail une méthode nouvelle pour déterminer si un produit inactive rapidement le virus rabique. Au cours de leurs essais, ils ont inoculé des souris par voie intracérébrale, en utilisant le virus rabique CVS conservé sous forme de suspension de cerveau de souris à 20% dans un mélange eau-sérum à 50%, congelé entre −60°C et −70°C. La suspension de virus, décongelée, a été centrifugée à 2000 tours/minute pendant 10 minutes; la dilution au ½ du liquide surnageant a donné une suspension dont le titre a été estimé à 10^5 DL_{50} de virus pour 0,03 ml. Le mélange de 0,2 ml de cette suspension et de 1,8 ml de solution du produit à essayer contient 10^4 DL_{50} de virus. Après agitation pendant exactement une minute, la suspension a été diluée au 1/100 en solution tamponnée. Des groupes de 5 à 10 souris ont reçu, par animal, 0,03 ml de ce mélange contenant théoriquement 100 DL_{50} de virus. La dilution et l'inoculation aux souris ont été effectuées en 1-1,5 minute. Les souris ont été observées chaque jour et le nombre des animaux malades et morts a été enregistré. Les souris infectées sont mortes habituellement 6-10 jours après l'inoculation et l'expérimentation a été terminée en 14-21 jours.

Ces essais ont montré l'activité virulicide satisfaisante de deux dérivés de l'ammonium quaternaire, le chlorure de benzalkonium et le bromure de cétrimonium, aux concentrations de 0,1-0,2% — concentrations sans danger pour les tissus traités —, des solutions savonneuses à 1-20%, de l'alcool éthylique à 43-70%, de la solution aqueuse d'iode à 1/10 000, de l'acétone et de l'éther. Plusieurs autres substances ont montré une activité virulicide: la soude caustique à 3%, des préparations commerciales de phénols organiques et un mélange de phosphate trisodique et d'hypochlorite de sodium. En revanche, la solution aqueuse de mercurochrome à 2%, la solution aqueuse de thiomersal à 0,1% et la formaldéhyde à 1% n'ont eu aucune action virulicide.

On dispose donc, pour le traitement local et la désinfection des morsures, de nombreux produits inactivant le

virus de la rage. Le procédé décrit permet d'essayer d'autres produits chimiques désinfectants. Comparée aux méthodes antérieures qui utilisaient le traitement local des blessures chez le cobaye, l'effet de blocage chez la souris, ou l'inoculation intracérébrale de virus rabique non titré exposé pendant des temps variables à des produits chimiques, cette nouvelle méthode a l'avantage d'être plus précise, plus rapide et plus économique.

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