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1. Kinsey, V. E. and Merriam, F. C., *Arch Ophthalmol.* **44**, 370 (1950).
2. McMillan, S. F., Ryerson, S. J., and Mortenson, R. A., *Arch. Biochem. Biophys.* **81**, 119 (1959).
3. Reddy, D. V. N., Klethi, J. and Kinsey, V. E., *Invest. Ophthalmol.* **5**, 594 (1966).
4. Sippel, T. O., *Invest. Ophthalmol.* **5**, 568 (1966).

5. Salmony, D., *Brit. J. Ophthalmol.* **44**, 29 (1960).
6. Kinoshita, J. H., Kern, H., Merola, L. O., *Biochim. Biophys. Acta* **47**, 458 (1961).
7. Beutler, E., Duron, O., and Kelly, B. M., *J. Lab. Clin. Med.* **61**, 882 (1963).
8. Srivastava, S. K. and Beutler, E., *Biochem. Biophys. Res. Commun.* **28**, 659 (1967).
9. Kinoshita, J. H., *Arch. Ophthalmol.* **72**, 554, (1964).

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Western Equine Encephalitis and Eastern Equine Encephalitis Virus Antigens Derived from Sucrose-Acetone Treated Chicken Embryos (32728)

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Subsequent to the initial report by Mohler (1) describing the use of chicken embryos for the production of Western equine encephalitis (WEE) and Eastern equine encephalitis (EEE) virus complement-fixation (CF) antigens, several modifications have been suggested for rendering the antigens more useful in serological tests (2-4). DeBoer and Cox (3) determined that extraction of these antigens with certain organic solvents minimized the frequency of false positive reactions with human sera. Although useful CF antigens have been made from unextracted tissues infected with WEE and EEE viruses (4), extraction processes have been preferentially employed for obtaining high titered hemagglutinating (HA) antigens (5,6).

Murine tissue has been regarded as the material of choice for HA antigens made with EEE and WEE viruses even though the use of mice presents certain problems of maintenance, yield of antigen per animal, and attendant economic factors. In an effort to minimize some of the disadvantages encountered in production of mouse brain antigens, the use of sucrose-acetone extracted chicken embryo homogenate was investigated as a source of WEE and EEE antigens.

Materials and Methods. Viruses. The strain of EEE virus used for production of antigens

was isolated from mosquitos collected in New Jersey in 1960 and passaged three times in mice (EEE/NJ, M₁, SM₂).¹ It was supplied by Dr. Roy Chamberlain of the National Communicable Disease Center, Atlanta, Georgia. The Fleming strain of WEE was used in its eighth egg passage and third mouse passage subsequent to its receipt in this laboratory (WEE/Fleming, XE₈, SM₃).

Antigen preparation. Ten batches of antigen for each of the two types of virus were prepared with virus from common seed pools. Eleven-day-old embryonated chicken eggs were inoculated intra-allantoically with 0.2 ml of virus diluted to 10⁻² in phosphate buffered saline (PBS), pH 7.2, containing 200 μ of penicillin and 200 μg of streptomycin per ml. At least 120 eggs were inoculated for each batch. Embryos dying after 18 hours of incubation at 37°C were removed along with the allantoic fluid for processing. A portion of the embryos was mixed with an equal volume of allantoic fluid and homogenized. After remaining at 4°C overnight, the homogenate

¹ The letters M, SM, and E, and the numerals designated in subscripts indicate passage history in the adult mouse, suckling mouse, and egg, respectively. The letter X indicates that the history of WEE virus prior to its acquisition at the National Communicable Disease Center is unknown.

TABLE I. Hemagglutinin and Complement-Fixing Titers of WEE Chicken Embryo Antigens.

Batch no.	Crude antigens				Sucrose-acetone treated-antigens			
	Untreated		Frozen-thawed ^b		Frozen		Lyophilized	
	HA ^a	CF ^a	HA	CF	HA	CF	HA	CF
1.	640 (6.4)	64	ND ^c	ND	1280 (6.4)	4	1280 (6.2-6.6)	8
2.	40 (6.2)	8	ND	ND	640 (6.4)	8	1280 (6.2)	8
3.	40 (6.2)	8	ND	ND	1280 (6.6)	4	1280 (6.4-6.6)	4
4.	<10 (6.0-6.6)	4	<10 (6.0-6.6)	4	5120 (6.2-6.4)	2	1280 (6.2-6.4)	4
5.	20 (6.0)	8	10 (6.0)	4	320 (6.2)	4	320 (6.0-6.2)	4
6.	<10 (5.8-6.4)	4	<10 (5.8-6.4)	8	400 (6.2)	4	400 (6.2-6.4)	4
7.	<10 (5.8-6.4)	2	<10 (5.8-6.4)	2	200 (6.2)	2	200 (6.4)	2
8.	10 (6.0)	4	<10 (5.8-6.4)	4	400 (6.2)	4	400 (6.2)	8
9.	<10 (5.8-6.6)	8	<10 (5.8-6.6)	16	200 (6.2-6.4)	16	200 (6.2)	4
10.	<10 (5.8-6.6)	16	<10 (5.8-6.6)	16	200 (6.4)	8	100 (6.4)	4

^a HA = Reciprocal of hemagglutinin titer followed in parenthesis by the pH of the adjusted goose erythrocyte suspension yielding maximum HA titers. CF = Reciprocal of the optimal antigen dilution fixing at least 70% of the complement in the complement-fixation test.

^b The crude antigen was alternately frozen and thawed for five cycles.

^c ND = Test not done.

was centrifuged at 600g for 20 min. The supernate was removed and centrifuged at 10,000g for 60 min. Thimerosal (merthiolate) was added to the supernate in a final concentration of 1:10,000. This was considered the crude 50% antigen.

The remaining portion of embryos was processed according to the sucrose-acetone (S-A) method of Clarke and Casals (6). This consisted of preparing a 20% homogenate of embryos in 8.5% sucrose and extracting two times with 20 vol. of acetone each time. After the second extraction, the acetone was aspirated and the sediment dried under vacuum. This was then reconstituted with PBS, pH 7.2, to a volume equal to twice that of the original embryo pool. The suspension was then centrifuged at 10,000g for 60 min and the resulting supernatant fluid was considered the final S-A antigen.

A number of vials from each batch of S-A antigen were lyophilized in 2 ml volumes for a period of 20-24 hours under a vacuum of 5 μ of mercury and sealed in an atmosphere of dry nitrogen. The rest of the antigens were stored at -60°C until tested.

Serologic testing. A block CF (7) titration was performed on each batch of antigen in which serial twofold dilutions of antigen were

mixed with serial twofold dilutions of pooled, hyperimmune guinea pig antiserum. The optimal dilution, recorded as the antigen titer, was taken as the highest dilution of antigen which gave the greatest fixation with the specific guinea pig antiserum. Control S-A and 50% crude antigens were prepared from uninoculated eggs and tested in an identical manner.

Hemagglutinin titers of the antigens were determined by the method of Clarke and Casals (6) in 12 \times 75-mm tubes and were assigned as the last dilution of antigen in a series of twofold dilutions which gave a complete hemagglutination pattern of the goose erythrocytes adjusted at various pH capacities using pH-adjusting diluents.

Results. The CF and HA titers of crude 50% and S-A antigens made from WEE infected chicken embryos are shown in Table I. Complement-fixing titers generally varied between 1:2 and 1:16 among the extracted and unextracted antigens, regardless of the method of treatment. However, the extracted antigens yielded demonstrable hemagglutinins much in excess of those found in the crude 50% antigens. Hemagglutinin titers of the S-A antigens ranged from 1:100 to 1:5120. By contrast it is noted that half of the crude

TABLE II. Hemagglutinin and Complement-Fixing Titers of EEE Chicken Embryo Antigens.

Batch no.	Crude antigens				Sucrose-acetone treated-antigens			
	Untreated		Frozen-thawed ^b		Frozen		Lyophilized	
	HA ^a	CF ^a	HA	CF	HA	CF	HA	CF
1.	1280 (6.2-6.4)	64	ND ^c	ND	5120 (6.4)	32	5120 (6.4)	32
2.	1280 (6.2)	64	ND	ND	5120 (6.4)	64	5120 (6.4)	64
3.	1280 (6.4)	64	ND	ND	5120 (6.4)	32	10240 (6.4)	32
4.	320 (6.0)	64	640 (6.0-6.4)	32	5120 (6.4-6.6)	32	5120 (6.4-6.6)	16
5.	5120 (6.0-6.4)	32	10240 (6.4)	128	10240 (6.4)	32	10240 (6.4)	32
6.	800 (6.4)	64	800 (6.4)	32	6400 (6.4-6.6)	32	6400 (6.4)	64
7.	200 (5.8)	64	40 (6.0)	16	2000 (6.2-6.4)	32	2000 (6.2-6.4)	32
8.	800 (6.4)	32	400 (6.4)	32	2000 (6.2-6.4)	32	2000 (6.2-6.4)	32
9.	800 (6.4)	32	1000 (6.4)	32	4000 (6.4)	128	4000 (6.4)	32
10.	100 (6.0-6.2)	64	100 (6.2)	64	2000 (6.4)	64	2000 (6.4)	32

^a HA = Reciprocal of hemagglutinin titer followed in parenthesis by the pH of the adjusted goose erythrocyte suspension yielding maximum HA titers. CF = Reciprocal of the optimal antigen dilution fixing at least 70% of the complement in the complement-fixation test.

^b The crude antigen was alternately frozen and thawed for five cycles.

^c ND = Test not done.

50% WEE antigens prepared had titers of less than 1:10 by HA test.

Complement-fixing and HA titers for EEE antigens exceeded those of the WEE antigens. The results from ten batches of EEE antigens are listed in Table II. Again, little variation was detected in CF reactivity regardless of the method of preparation employed. Hemagglutinin titers in the EEE antigens were comparatively high and ranged between 1:2,000 and 1:10,240 in the S-A antigens. On the average, HA titers in the S-A antigens were approximately eightfold greater than in the crude 50% preparations.

Lyophilization of both WEE and EEE S-A antigens resulted in little, if any, significant variation in CF and HA titers and resulted in a product that was more economical to store and ship.

An attempt was made to increase the titers of the crude 50% antigens, as well as obviate nonspecific CF reactivity (8) by repeatedly freezing and thawing the preparations for five cycles and centrifuging at 10,000 rpm for a period of 1 hour. Seven out of the 10 batches for each virus were treated in this manner. In general, this treatment neither reduced nor enhanced the CF and HA titers.

Discussion. Many laboratories are better

equipped to work with embryonated eggs than to maintain the number of suckling mouse litters needed for making large batches of EEE and WEE antigens. In addition, chicken embryos are a more economic source of these viruses on the basis of yield of antigen per host unit. In our experience, the average volume of S-A antigen produced per 12-day-old chicken embryo was approximately 7.5 ml, while that of a suckling mouse was about 0.3 ml (data not shown). Thus chicken embryo has yielded about 25 times the volume of S-A antigen produced from suckling mouse brain.

Generally, EEE antigens prepared in suckling mouse brains and processed by the S-A method in this laboratory have had HA titers lower than those of chicken embryo derived antigens; whereas, the CF reactivity of these same mouse brain antigens has been equal to or slightly greater than that of the chicken embryo antigens.

In contrast to the results of EEE antigen comparisons, we have found that antigens made from suckling mouse brains infected with WEE virus have consistently yielded demonstrable CF and HA titers higher than those of chicken embryo antigens treated by the same method. The titers of extracted

chicken embryo antigens are shown in Table I to be between 1:100 and 1:5120 by HA test and between 1:2 and 1:16 by CF test; extracted mouse brain antigens have generally yielded titers between five and ten times higher than these values. Although the CF and HA titers of suckling mouse brain S-A antigens are higher than those of the chicken embryo antigens, the mass of infected tissue in the chicken embryo frequently compensates for the lower titer per gram of tissue.

In order to ascertain the usefulness of the sucrose-acetone extracted avian antigens for the serodiagnosis of human disease, three WEE and three EEE chicken embryo antigens were compared with corresponding sucrose-acetone extracted mouse brain antigens by hemagglutination-inhibition (HI) and CF tests using a small number of patients' sera. Preliminary HI studies indicated that the chicken embryo antigens were at least as sensitive to human antihemagglutinin as the mouse brain antigens. Most of the sera reacted in the HI tests at higher dilutions using the avian antigens. In CF tests, however, the type of antigen (mouse brain or avian) giving the highest titer varied from serum to serum.

Summary. A procedure is outlined for the preparation of WEE and EEE chicken em-

bryo CF and HA antigens. High-titered, economical antigens were prepared using the sucrose-acetone method. Differences in titer and volume between extracted chicken embryo and mouse brain antigens are discussed, and preliminary comparisons are made of the relative reactivity of human patients' sera when tested by CF and HA methods using antigens from these two sources.

1. Mohler, M. M., J. Am. Vet. Med. Assoc. **94**, 39 (1939).
2. Brown, G. C., Science **106**, 43 (1947).
3. DeBoer, C. J. and Cox, H. R., J. Immunol. **55**, 193 (1947).
4. Lennette, E. H., Wiener, A., Neff, B. J., and Hoffman, M. N., Proc. Soc. Exptl. Biol. Med., **92**, 575 (1956).
5. Casals, J. and Brown, L. V., J. Exptl. Med. **99**, 429 (1954).
6. Clarke, D. H. and Casals, J., Am. J. Trop. Med. Hyg. **7**, 561 (1958).
7. Public Health Service, "Standardized Diagnostic Complement Fixation Method and Adaptation to Micro Test," Public Health Monograph no. 74, PHS Publication no. 1228, U. S. Govt. Printing Office, Washington, D. C., 1965.
8. Casals, J. and Olitsky, P. K., in "Diagnosis of Viral and Rickettsial Infections," Horsfall, F. L., Jr., ed., p. 76. Columbia Univ. Press, New York, 1949.

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Cell Walls from *Mycobacterium tuberculosis* (BCG) as Vaccine Against *Mycobacterium leprae* Infections in Mice (32729)

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BCG vaccine, a living antituberculosis vaccine prepared from an attenuated strain of bovine tubercle bacilli, provides protection against foot pad infection of mice with *Mycobacterium leprae* (1). Although ordinary preparations of BCG cell walls are not effective as vaccines against tubercle bacilli, oil-treated cell walls provide good protection against pulmonary (aerogenic) infections of mice with *Mycobacterium tuberculosis* (2). To be effec-

tive the cell walls must be combined in a dry state with a suitable oil (3). We report here a study of the enhancement of resistance against *M. leprae* infections of mice stimulated by oil-treated BCG cell walls. Of necessity the study concerned the effect of route of administration and challenge.

Materials and Methods. One cell wall vaccine was prepared in the Rocky Mountain Laboratory by methods described elsewhere