Summary. The melanin isolated from feathers of turkey poults injected with DL-lysine-2-C^{14} was radioactive. Part of the radioactivity was lost when melanin was autoclaved with 6.N HCl. With the protein removed, "melanin" was only slightly soluble in 0.05.N NaOH. Radioactivity was also distributed in aspartic acid, glutamic acid and lysine of the feather keratin.

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Hamster Kidney Cell Cultures in Laboratory Aspects of Epidemiologic Studies on Japanese B Encephalitis Virus.* (24797)

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Propagation of Japanese B encephalitis (JBE) virus in cultures of hamster kidney cells (HKC) has been studied fairly extensively(1,2) but these cultures have not been used in testing field materials collected during epidemiologic investigations. Therefore, an attempt was made to put this new laboratory tool to test with the expectation that it might have certain advantages over the use of mice. This report records results of comparative application of the *in vitro* and *in vivo* method for serum survey studies on distribution of JBE antibodies and to record results of comparative virus isolation attempts carried out in suckling mice and in HKC for recovery of JBE virus from field collected materials.

Methods. Preparation and maintenance of HKC cultures for routine laboratory use has been described(2). For our studies, these tube cultures were inoculated and observed for specific cytopathogenic responses in a manner analogous to procedures routinely in use with mice. Details of the mouse method have been recently reviewed(3) and the currently used procedure varied but slightly. Briefly reviewed, the neutralization procedure employed a screen test using 2 dilutions of virus with undiluted serum which allows for estimation of a serum neutralization index. A neutralization index of >1.6 logs was interpreted as positive; indices ranging between 1.1 and 1.6 logs as equivocal and <1.1 logs as negative. All sera were inactivated at 56°C for ½ hour prior to testing.‡ The serum-virus mixtures were incubated 2 hours at 37°C. The unit volume for tissue culture inoculation was 0.1 ml; for white mice 0.03 ml by the intracerebral route. The HKC culture contained 1 ml of maintenance fluid prior to inoculation. HKC cultures were observed daily for 7 days and mice were held for 14 days. Standard diluent for tissue culture virus in-

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‡ A neutralization procedure using inactivated serum was chosen to minimize the influence of labile "accessory factor" known to be necessary for demonstration of maximum neutralizing activity in test sera. Since comparative tests were performed at an interval of several years elimination of the labile factor was essential.

TABLE I. Results of HKC and Mouse Neutralization Tests* with JBE Virus on Sera Collected in Sumatra in 1954.

Age group (yr)	Antibody incidence ratio			
	Mouse test		HKC test	
	+	±	+	±
4– 5	0/5	0/5	0/5	0/5
6–15	1/5	"	"	1/5
16–25	2/5	2/5	3/5	0/5
26–41	4/4	0/4	4/4	0/4
42+	4/5	0/5	3/5	1/5
Total	11/24	2/24	10/24	2/24

* HKC virus doses were 400 and 40 TCD_{50} respectively for higher and lower dilutions used in test. Mouse virus doses varied between 100-200 and 10-20 MLD_{50} respectively for higher and lower dilutions used in test.

ocula was 10% normal calf serum in Hanks' lactalbumin maintenance medium and for mouse brain virus, 10% normal rabbit serum saline. Virus isolation tests were carried out by similar methods of inoculation. Suckling mice were used, however, instead of adults and .01 ml was given intracerebrally. To compare sensitivity of the 2 methods, dried or frozen suspensions of field collected specimens were diluted in a 10-fold series and aliquots of each dilution tested in HKC and in mice.

Serum survey studies. In 1954, serum specimens were collected from more than 100 residents of southern Sumatra, Indonesia, as an activity of U.S. Army Medical Research Unit in Kuala Lumpur, Malaya.§ Bloods were collected aseptically in bleeding venules and the serum separated and stored in frozen state until thawed for testing for JBE antibody by the mouse method described above. Aliquots of these survey specimens were lyophilized in 1956, brought to United States and stored at 4°C until retested in HKC cultures in 1958. For our purpose, 25 of these dried sera were selected by stratified random selection procedure, equal numbers from each of the 5 age groups (0-5; 6-15; 16-25; 26-41; >41 years). The result on one serum was discarded because this serum had not been tested previously in the mouse test. Table I records responses elicited in the indicator systems. With only 4 sera were differences observed between results of the 2 methods. In each instance, the difference involved equivocal responses.

Comparative isolation studies. Lyophilized mouse brain suspensions representing aliquots from 3 early passages of JBE virus isolated from mosquitoes collected in Malaya were available for study§. Available for testing also were frozen aliquots of 4 original emulsions of mosquitoes which had been collected 12 years before in Japan and were known to have contained virus(4). Finally, the dry icebox files of frozen materials available in this laboratory contained aliquots of 3 original suspensions of human brain from which JBE virus had been isolated approximately 11 years previously(5). Virus recovery was accomplished equally well in tissue cultures and in mice (Table II). The 3 early passage mouse adapted viruses from Malaya induced cyto-

TABLE II. Results of Isolation Attempts from Suspect Materials Using Hamster Kidney Cell Cultures and Suckling Mice.

Identification		HKC*		Suckling mice*	
		Titer TCD_{50} (neg. log)	Result	Titer MLD_{50} (neg. log)	Result
MM	182	>2.0	+	>2.0	+
	192	"	"	"	"
	193	"	"	"	"
JM	317	"	"	"	"
	534	Tox.	" †	"	"
	538	4.3	+	3.7	"
	541	"	"	3.6	"
HB	A	Tox.	0 ‡	<und.	0
	P	"	"	" §	"
	D	"	"	" ‖	"

MM = Malayan mosquito virus, early mouse passage, dried material. JM = Japanese mosquito emulsion; original material stored at dry icebox temperatures. HB = Human brain emulsion; original material stored at dry icebox temperatures.

Tox. = No titer; suspect material toxic for HKC cultures.

* Titrations done in 10-fold dilution series; 0.1 ml dose for HKC and 0.01 ml dose for mice; 4 tubes/dilution and 6 mice/dilution.

† Additional passage of pooled tissue culture fluids revealed presence of virus to titer of approximately $10^{-3.0}$/0.1 ml.

‡ Additional passage of pooled tissue culture fluids failed to reveal presence of virus.

§ 2 mice found dead on 18th and 19th day respectively in titration series; further passage failed to reveal presence of virus.

‖ 2 mice found dead on 18th day in titration series; further passage failed to reveal presence of virus.

§ Unpublished data summarized in Progress Reports of U. S. Army Med. Res. Unit, Malaya, submitted to Communicable Disease Division, Walter Reed Army Institute of Research, 1953-1956.

pathogenic changes in tissue cultures and deaths in mice in all dilutions tested. Similarly, the lot 317 and lot 534 original Japanese mosquito preparations yielded virus in all dilutions tested. The 2 Japanese mosquito isolates (lots 538 and 541) tested in sufficient dilution to yield endpoints, indicated that the HKC cultures were possibly more sensitive to presence of virus than were suckling mice. Each of the tissue culture titers was $10^{-4.3}$ and the mouse titers were $10^{-3.7}$ and $10^{-3.6}$. The 3 human brain suspensions were toxic for tissue cultures and irregular deaths occurred in mice. Further passages both in mice and tissue cultures failed to reveal any residual active virus in these preparations. Each of the recovered viruses was identified as JBE virus by neutralization in HKC cultures with specific rabbit hyperimmune serum.

Discussion. Results of the serum survey studies leave little doubt that the *in vitro* HKC culture method can be substituted for the *in vivo* mouse tests now used in laboratory aspects of epidemiologic studies designed to gain information on distribution of JBE antibodies in different geographic localities. The results of the 2 types of tests differed only in 4 sera in which an equivocal response was obtained by one method. Since an equivocal result in this type of test simply means that the test cannot be interpreted as positive or negative, complete agreement in 20 of 24 tests and, in fact, in all tests not involving an equivocal result, is an indication of excellent association. This degree of reproducibility is as good or better than might be expected had mouse neutralization tests been performed again on these same sera instead of tissue culture tests. In view of the marked susceptibility of the HKC culture to other members of the arthropod-borne viral encephalitis group(1,2), it seems probable that this *in vitro* method can be applied to studies designed to gain similar information about distribution of antibodies for several of the arthropod-borne viral infections.

The use of HKC cultures for recovery and identification of JBE virus from field collected materials compared favorably with the use of suckling mouse in studies summarized here. However, the toxic effects of certain of the inocula, the occasional spontaneous degeneration of monolayers following inoculation and the difficulty of assessing the cytopathogenic response of the monolayer, all contributed to a general feeling of dissatisfaction with the results obtained following the primary inoculation. However, subsequent passage left no doubt as to the effectiveness of the method when combined with a second passage. The *in vitro* method appeared to be slightly more sensitive to minimal quantities of unadapted virus than did the suckling mouse.

Summary. Comparative neutralization tests on 24 sera from residents of Indonesia carried out both in adult mice and in HKC cultures gave essentially identical results. Comparative isolation attempts using field collected mosquitoes, early mouse passages from field isolates and human brain tested in HKC cultures and suckling mice showed the *in vitro* indicator system to be as sensitive and possibly more sensitive to the presence of minimal amounts of virus. The implications of these findings were discussed for immediate application in public health laboratories.

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