Several reports(6-10,12-14) suggest various reasons for the disseminated nature of tuberculosis in silicotic individuals. Although somewhat limited, these results may suggest that the phenomenon of "blockade" of phagocytic cells could be of importance in helping explain the spread of tuberculosis in individuals exposed to extensive dust inhalation.

Summary. Mononuclear phagocytes which have been altered by ingestion of various particles, take up tubercle bacilli less readily than the same type cell in an unaltered state. This decreased phagocytic activity is observed with both attenuated and virulent tubercle bacilli.

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Tissue Culture Studies on Arbor Viruses of the Japanese B-West Nile Complex.* (23435)

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In October, 1955, a tissue culture section was established at the Virus Research Centre (VRC), Poona, India, with a view to developing tissue culture methods for the study of arthropod-borne (arbor) viruses in India. This communication presents some of the initial results obtained with viruses of the Japanese B-West Nile complex of the B Group of arbor viruses in monkey kidney epithelial cell and chick embryo cell tissue culture. Similar attempts with the Egypt 101 strain of West Nile virus in HeLa, KB, HELL, Detroit 6 (1,2,3,4) and human amnion and chicken kidney cells were unsuccessful in demonstrating significant cytopathogenic effects.

Materials and methods. Viruses: The viruses used in this study were the Egypt 101 strain of West Nile, the P4230 strain of West Nile isolated from the blood of an investigator at the VRC who became ill, presumably from a laboratory infection with West Nile virus(5), the Nakayama strain of Japanese B and the Tamil Nad virus recently isolated from *Culex vishnui* mosquitoes in South India and found to be serologically related to both West Nile and Japanese B(6). Details of the viruses inoculated are given in Table I.

Tissue culture technics: The two types of cell cultures used in this work were *Macacus rhesus* monkey kidney epithelial cells and chick embryo cells. The procedure for preparation of monkey kidney cells was that de-

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TABLE I. Data about Viruses Tested in Tissue Culture.

Virus	Source	Passage	Titer, mouse LD_{50}/.03 ml	Types of cells inoculated	Dilution of inoculum
E 101	M 9254	CE_5M_1*	ND	CEC	$10^{-1.3}$
E 101	M 9254	CE_5M_1	$10^{-7.9}$	MKEC	$10^{-1.3}$
P 4230	M 11353	M_6	$10^{-8.5}$	CEC	10^{-1}
P 4230	M 11353	M_6	$10^{-8.5}$	MKEC	10^{-1}
G 2266	M 12001	M_9	ND	CEC	10^{-1}
JBE	M 10838	M_{48}	$10^{-8.6}$	MKEC	10^{-3}
JBE	M 10838	M_{48}	$10^{-8.6}$	CEC	10^{-2}

* CE = chick embryo; M = mouse brain.
ND = not done.

scribed by Rappaport(7) with minor modifications. The chick embryo cell cultures were prepared by a procedure considerably modified from that of Dulbecco(8), the details of which are to appear elsewhere(9). Both preparations consisted of cells trypsinized in 0.2% trypsin in phosphate buffered saline containing penicillin and streptomycin(7) and resuspended after centrifugation in the lactalbumin hydrolysate-Hank's solution-calf serum medium described by Melnick *et al.*(10, 11). The cell suspensions were diluted to a concentration of 0.6-1.0 million cells per ml, as determined by preliminary cell count, and 0.5 ml volumes seeded into tubes. The tubes were incubated in the stationary state at 37°C. In the case of the monkey kidney cells, a good monocell layer was obtained in 6-9 days. Prior to virus inoculation the medium was replaced with lactalbumin hydrolysate-Earle's solution-calf serum(12). The chick embryo cultures attained good growth and were ready for use in 48-72 hours. Prior to virus inoculation the medium was replaced with lactalbumin hydrolysate-Earle's solution-calf serum plus 1 per cent chick embryo extract(9).

Bottle cultures of monkey kidney cells for plaque studies were prepared according to the technic of Hsiung and Melnick(13) with modifications to be described separately(9). The media used were the same as those used for tube cultures. A period of 6-9 days was required to produce a continuous monolayer of cells.

The tube cultures were inoculated with 0.1 ml of various virus dilutions or serum-virus mixtures. The bottle cultures were inoculated with 0.3 ml of virus or serum-virus mixtures. After incubation for 1 hour at 37°C to permit virus invasion, 10 ml of an agar nutrient medium overlay(9) were introduced into each bottle. The optimal conditions for exposure of cells to virus prior to the addition of the agar overlay have yet to be determined.

Following inoculation with virus the tube cultures were incubated at 37°C and examined daily for evidence of cytopathic effect. When such effects were observed the fluid was harvested and inoculated into fresh tubes either undiluted or in a series of 10-fold dilutions. The fluid in the original tubes was replaced with fresh medium. It has been observed that 10^{-2} to 10^{-3} dilutions of harvest fluid produce more rapid and decisive effects than undiluted fluid, a point of some technical importance. Tube cultures of monkey kidney cells were observed daily over a period of 10-15 days. Tube cultures of chick embryo cells were observed over a period of 6-7 days, following which nonspecific degeneration became a complicating factor.

Monkey kidney cells in bottle cultures were incubated at 37°C and observed for plaques from the sixth to the fifteenth postinoculation day. The plaques, visible as color-depleted areas in the neutral red-stained monocell sheet, were counted and recorded every other day.

Tissue culture neutralization tests. These tests were carried out in tube cultures of monkey kidney and chick embryo cells and in bottle cultures of monkey kidney cells, the latter by differential plaque counts. Tenfold dilutions of a titrated frozen or lyophilized tissue culture virus suspension were made extending 2 dilutions beyond the determined tissue culture

ID_{50}. Under exceptional circumstances fresh tissue culture fluid harvests were used. The virus dilutions were made in lactalbumin hydrolysate-Earle's solution-calf serum medium. Specific monkey immune sera and nonimmune control sera from the same animals were mixed without inactivation in quantities of 0.3 ml with 0.3 ml of diluted virus, shaken well and incubated at 37°C for 2 hours, and inoculated in quantities of 0.1 ml in tube cultures and in quantities of 0.3 ml in bottle cultures. Monkey kidney cell culture tubes were examined on the third, fifth, seventh and tenth days after inoculation and chick embryo cell culture tubes daily from the third to the seventh day.

Results. Adaptation of viruses to tissue culture: the general pattern of cytopathogenic effect produced by the Japanese B encephalitis-West Nile group of viruses in tissue culture.

1. *Tubes*: In the early passages in chick embryo cells a few cells showed rounding and ultimate degeneration. This reaction was noticed 3-5 days after inoculation of the virus. In the early passages such cells were scattered among other healthy-looking cells, but in higher passages more and more cells were affected until all were involved. The time between early rounding to complete degeneration was about 48 hours after the onset of the process.

In early passages in tubes of monkey kidney cells, only a few groups of cells appeared granular and distorted 3-5 days after inoculation. These cell groups were more like zigzag lines but in later passages appeared as clumps of 10-20 cells. The distorted cells became granular and rounded 2-4 days after onset of the process and separated from the glass wall. The cells which did not degenerate looked normal in outline but granular. The number of such less affected cells decreased with further passages.

It should be noted that the above-mentioned changes were never observed earlier than 3 days after inoculation, regardless of the virus dose.

2. *Bottles*: Most of the plaque work was done with the P4230 strain of West Nile virus. Early passage plaques were 1-3 mm in diameter with an uneven diffuse outline. The plaques were noticed on the sixth to eighth postinoculation day and there was not much increase in size up to the fifteenth day. The higher passage virus produced round plaques of diffuse outline and larger size—2-5 mm.

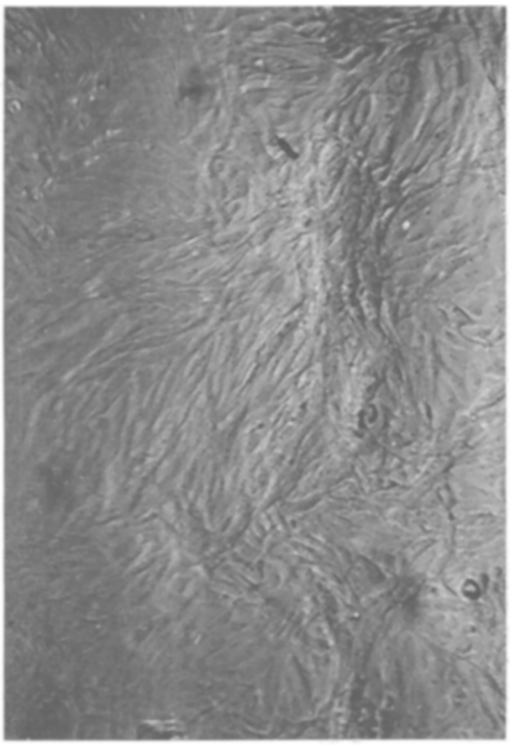

FIG. 1. Normal monkey kidney cells.

P4230 strain of West Nile: This virus gave detectable cytopathogenic effects in tube cultures on the first passage in both chick embryo and monkey kidney cells. Adaptation to chick embryo cells was somewhat more rapid than to monkey kidney cells, so that after 5-6 passages marked pathogenic effect was observed. However, with continued passage the rate and extent of pathogenic effect were enhanced. Cytopathogenicity for monkey kidney cells was well-marked by the 20th passage. Fig. 1 shows normal control monkey kidney epithelial cell growth in a tube for comparison with Fig. 2, which shows the cytopathogenic effect observed in such cultures 3 days after inoculation with 27th tissue culture passage of the P4230 strain. By this passage level plaque formation in bottle cultures was also well-established. The appear-

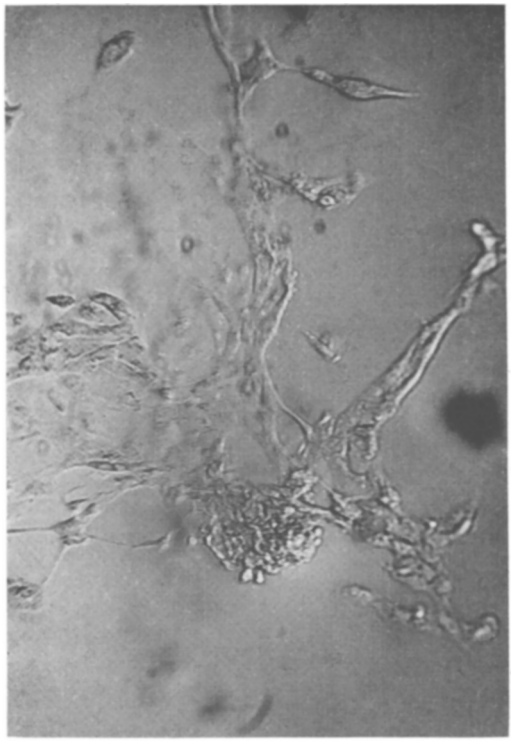

FIG. 2. Monkey kidney cells inoculated with 27th TC passage of P4230 3 days after inoculation with 0.1 ml of $10^{-2.3}$ dilution of the virus.

ance of the plaques 7 days after inoculation with 10^{-3} to 10^{-5} dilutions of 45th passage virus is shown in Fig. 3.

In Table II are presented the results obtained with P4230 virus harvested from 27th passage in monkey kidney cells when titrated in subadult mice (intracerebral inoculation) and tubes of monkey kidney cells. In the same table are shown titrations obtained with P4230 virus at its 23rd passage level in chick embryo tube cultures. These titrations were carried out only in mice and chick embryo tube cultures.

The results of the different methods of assay for infective units can be seen to correlate fairly well. The lower titer achieved by the plaque method can perhaps be attributed to the present lack of knowledge of the optimum conditions for plaque formation.

Egypt 101 strain of West Nile: This strain was readily adapted to chick embryo cells. The 5th chick embryo passage material was tested in monkey kidney cell tube cultures with results similar to those described for the P4230 strain.

Tamil Nad virus: This virus has been studied mainly in chick embryo culture to which it was easily adapted. By the 35th passage it had attained a tube tissue culture titer of $10^{-7.8}$ ($TCID_{50}$/ml) and an adult mouse titer (intracerebral) of $10^{-6.7}$ (LD_{50}/ml).

Japanese B encephalitis virus: Successful adaptation of 3 viruses of the Japanese B-West Nile complex to tissue culture with the development of definitive cytopathogenic effects led to attempts to establish the Japanese B virus in a similar manner. Both chick embryo and monkey kidney cell cultures were used. Early passages of virus into both types of culture resulted in only slight cell destruction in a few scattered areas. However, repeated passage in chick embryo cells led to an increase in degree of cytopathogenicity to the point where tube cultures could be used for virus titrations and neutralization tests.

Cytopathogenicity of this virus for monkey

TABLE II. Comparative Titrations of P4230 Strain of West Nile Virus and Japanese B Virus Adapted to 2-Cell Types in Tissue Culture.

Virus	Cell type	Tissue culture passage level	Titration in — Mice, LD_{50}/ml	Titration in — Tissue culture tubes, $TCID_{50}$/ml
P4230 strain of West Nile	Monkey kidney	27th*	10^{-7}	$10^{-7.5}$
	Chick embryo	23rd	$10^{-6.8}$	$10^{-7.3}$
Japanese B	Monkey kidney	6th	$10^{-8.3}$	$10^{-6.6}$
		25th	$10^{-2.5}$	$10^{-6.3}$
	Chick embryo	5th	—	$10^{-6.5}$
		22nd	$10^{-7.2}$	$10^{-6.0}$

* Endpoint of plaque titration of P4230 virus in bottle cultures of monkey kidney cells was 66×10^5.

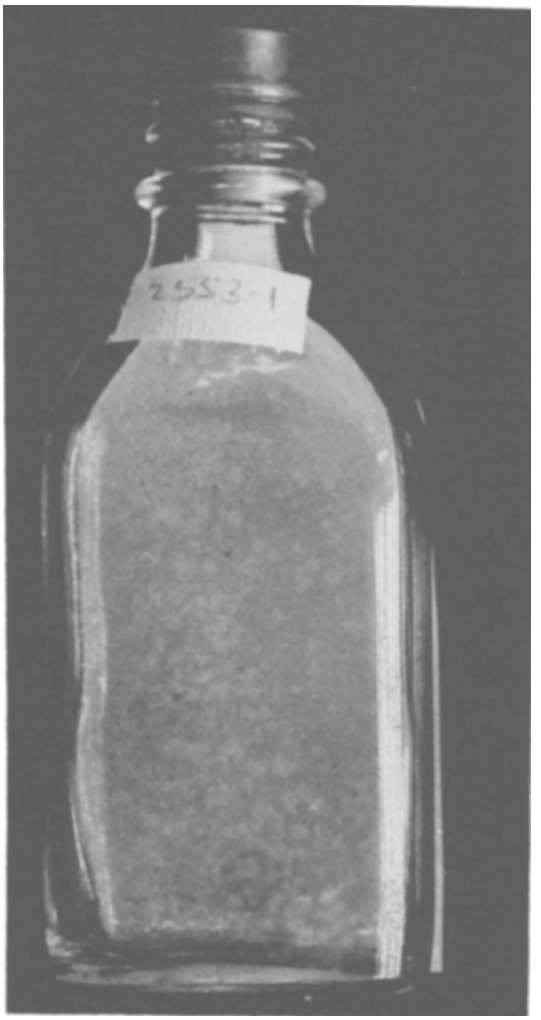

FIG. 3. Plaque formation by P4230 on monolayer of monkey kidney cells 7 days after inoculation with 10^{-8} dilution of virus.

kidney cells was likewise somewhat slow in developing but a marked increase in effect occurred by the 6th passage level and made the performance of tube culture titrations and neutralization tests possible. However, even by the 30th passage satisfactory plaque development had not been achieved, although a few poorly defined plaques were apparent.

An interesting aspect of the process of adaptation of Japanese B virus to monkey kidney cells has been the gradual decrease in the pathogenicity of this virus for mice. This does not seem to be true so far for the virus adapted to chick embryo cells. These results are shown in Table II, which gives comparative assays of infective units obtained by intracerebral titration in subadult mice and by tube culture titration at different tissue culture passage levels. Japanese B immune serum prepared in rhesus monkeys neutralized >2.6 logs of 22nd TC passage virus, demonstrating that it was modification specifically of JBE virus which resulted in the change of mouse pathogenicity after continuous MKEC passage. This neutralization test was carried out in monkey kidney tissue culture tubes.

Neutralization tests: Neutralization tests were done in both types of tube cultures and in monkey cell bottle cultures. The sera used were specific homologous immune sera prepared by inoculation of monkeys with mouse-brain virus suspensions.

Identity of the viruses after passage and adaptation was confirmed by testing in chick embryo and monkey kidney cell cultures, using preimmunization and specific immune sera prepared in rhesus monkeys for the homologous viruses. The monkeys were immunized with mouse-adapted strains which had not previously been cultivated in tissue cultures.

In Table III are shown the results of plaque neutralization tests carried out with the P4230 strain of West Nile virus in monkey kidney cells. The sera used were pre- and postimmunization bleedings from monkeys inoculated with mouse-brain suspensions of the Egypt 101 strain of West Nile and of Japanese B. Also included in the study is the serum obtained from the convalescent laboratory investigator who yielded the P4230 strain of West Nile.

In plaque neutralization Test 2 it will be noted that the preinoculation serum from R 56 produced more than a 10-fold decrease in the plaque count. Presumably this can be related to a nonspecific viricidal effect and indicates one of the complicating factors which must be kept in mind in interpreting the results. Such effects have been observed by various investigators in performing mouse neutralization tests with some of the arbor viruses. More experience will be necessary to determine the frequency of this phenomenon and whether it will prove necessary to eliminate such effects by heating or otherwise

TABLE III. Plaque Neutralization Tests with P4230 Strain of West Nile.

Monkey	Immunizing virus	Serum	Plaque counts, PFU/0.3 ml* Test 1†	Test 2†
R 102	Japanese B	Preinoculation	‡	70 × 10^3
		Postinoculation	> 45 × 10^3	2.1 × 10^3
			<270 × 10^3	
R 24	West Nile (Egypt 101)	Preinoculation	‡	180 × 10^3
		Postinoculation	1.3 × 10^3	.5 × 10^3
R 56	*Idem*	Preinoculation	‡	15 × 10^3
		Postinoculation	1.05 × 10^3	0 × 10^3
	West Nile (P4230)	Human convalescent	.15 × 10^3	0 × 10^3
		Virus-control	270 × 10^3	350 × 10^3

* PFU = plaque forming units.
† Test 1 carried out with 19th passage and Test 2 with 23rd passage tissue culture virus.
‡ Confluent plaques at highest dilution tested.

Note: Results of first test are an avg of plaque counts for bottles inoc. with 10^{-2} and 10^{-3} dilutions of virus. Control titer is based on plaque count of 10^{-4} dilution of virus.

Results of second test are an avg of plaque counts in bottles inoc. with 10^{-4} and 10^{-5} dilutions of virus with pre-sera and 10^{-3} and 10^{-4} dilutions of virus with post-sera. Control titer is based on an avg of plaque counts with 10^{-4} and 10^{-5} dilutions of virus.

treating the test sera.

These results served to confirm the identity of the agents producing the cytopathogenic effects and to demonstrate the potential usefulness of the 2 types of technics. The partial neutralization of a strain of West Nile virus by Japanese B immune serum (Table III) is in accord with the known immunological relationship between the 2 viruses first demonstrated by Smithburn *et al.* with the standard intracerebral mouse neutralization test(14).

Summary. The adaptation of 2 strains of West Nile virus, of Tamil Nad virus and of Japanese B encephalitis virus to chick embryo cell and monkey kidney cell cultures is described. After a variable number of passages, cytopathogenicity was sufficiently enhanced that tube cultures could be used for virus titrations with all 3 viruses. Tissue-culture-adapted strains of West Nile virus could be studied by the plaque titration technic in bottle cultures of monkey kidney epithelial cells. This has not been possible so far with Japanese B virus. Neutralization tests with all 3 viruses were successfully performed in tube cultures and the plaque titration method was used for neutralization tests with 1 strain of West Nile. It is believed that the technics used in these studies have potentialities as tools in epidemiological research on West Nile, Tamil Nad, and Japanese B encephalitis viruses and presumably other members of the arbor virus group.

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