Propagation of Bunyamwera, West Nile, Ilheus, and Br I Viruses in Human Cells in Tissue Culture.* (21044)

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The data to be presented demonstrate the sustained propagation of West Nile, Egypt 101, Ilheus, Bunyamwera, and Br I viruses in tissue cultures of human normal lymph nodes, and suggest that prolonged propagation of Bunyamwera virus in these human cells has altered its pathogenicity for mice. These studies also indicate that these viruses have little cytopathogenic effect under these experimental conditions.

Materials and methods. Viruses. Bunyamwera, West Nile, Egypt 101. and Ilheus viruses are mosquito-borne viruses capable of causing encephalitis in man and of inhibiting growth of certain mouse tumors. Egypt 101 is a recently isolated virus(3) which appears to be serologically identical with West Nile virus, but differs from the original isolate in its pathogenicity for man(11,12). Detailed literature surveys are available elsewhere(4,13). Br I virus was isolated from mouse brains by Dr. Hilary Koprowski during studies of intracerebral heterologous tumor growth (personal communication). Prior to growth in tissue cultures, all viruses were maintained by intracerebral passage in mice. Identity of Ilheus, West Nile, and Egypt 101 viruses was established before and during the tissue culture studies by neutralization tests with known specific antisera (neutralization indices of 100 or greater). Br I virus was recently obtained from Dr. Koprowski and serologic identification was not attempted. Neutralization studies with Bunyamwera virus failed to show neutralization of more than one log using 2 sera supplied by other laboratories or sera from rabbits repeatedly inoculated with this virus in our own laboratory. The virus is, nevertheless, considered to be Bunyamwera because the original stock from which these studies were initiated was so designated, and because its infectious characteristics for the mouse (described in text) are in agreement with the original descriptions(8) and have remained constant throughout studies of this virus in mice at this institute. *Tissue cultures.* The cells were grown from histologically normal lymph nodes obtained by therapeutic node dissection from patients with various types of cancer. The roller slide technic was used. This entailed the embedding of tissue fragments in chicken plasma clot on a free cover slip which was then placed in a roller tube with 2.0 ml of a nutrient medium consisting of 45% Z 16 balanced salt solution(5), 50% cell-free ascitic fluid from humans with cancer, and 5% chick embryo extract. Cultures were incubated in roller drums at 37°C for 6 to 14 days prior to the introduction of virus (a single exception was incubated for 28 days prior to inoculation) and were not used for virus inoculation unless cellular outgrowth occurred. The cells were chiefly fibroblastic. Lymphocytes were common in young cultures, but rare after 10 days. It has therefore been assumed that the cells which supported the virus growth were fibroblasts. Cultures were fixed and stained at the end of each study. Cytologic study of living cultures was not attempted. *Virus technics.* For primary inoculations 0.2 ml of 1.0% virus-infected mouse brain was added to 3 or more tubes of a tissue culture, and an equal number of control tubes of the same culture received normal mouse brain suspension. The initial virus dilutions thus became 10^{-3}. For subsequent passages, supernatant fluids of the 3 replicate tubes were pooled and 0.2 ml was transferred undiluted to new cultures containing 1.8 ml of nutrient solution. Cultures were re-fed at intervals of 4 to 10 days, thus causing a further 10-fold (or greater) dilution of the original mouse-brain inoculum. Viruses were titered in 18-20 g female white

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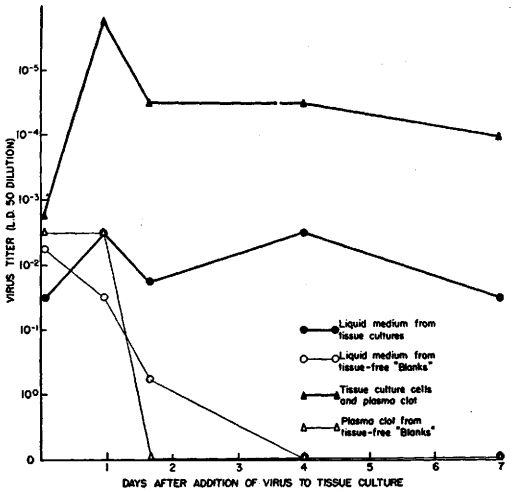

FIG. 1. Demonstrating rapid disappearance of Bunyamwera virus when incubated in the absence of cells, in contrast to persistence of virus in cells and in the supernatant fluid from tissue cultures.

Swiss (Banks) mice by intracerebral inoculation of 0.03 ml of serial 10-fold dilutions of tissue culture fluid (or a triturated suspension of cells and clot in one experiment). Three mice were used per dilution. LD_{50} titers were calculated by the Reed-Muench method(6). If undiluted fluid (10° dilution) contained some virus, but less than an LD_{50}, it is plotted in the figures midway between 0 (no virus demonstrable) and 10°. Control and infected cultures were treated identically through all re-feedings, passages, and titrations.

Results. Bunyamwera virus. Proofs of propagation. When Bunyamwera virus was added to a tissue culture "blank" consisting of glass, plasma clot, and nutrients, but without any cells, detectable virus decreased rapidly in the first 24 hours, and no virus was detectable when tested at 4 days or thereafter (Fig. 1). In marked contrast, in one experiment, Bunyamwera virus was consistently maintained within a single tissue culture (re-fed at weekly intervals) for 94 days. Another similar study was maintained for 42 days. The virus was still present at the end of the studies when the cultures were fixed for cytologic examination. The similarity of form of the virus propagation curves in the parallel studies of tissue and of culture medium (Fig. 1) suggests that the titrations of virus in the medium reflect the amount of virus within the cells. The virus titers in the cells (plus plasma clot) average about 2.5 logs higher than in the liquid medium. That this difference represents the concentration of virus in cells, rather than in the clot, is indicated by the fact that there was no more virus in the clot than in liquid medium in the tissue-free "blanks".

By consecutive transfer from culture to culture, Bunyamwera virus was maintained through a total of 20 separate tissue cultures for a total lapsed time in culture of 159 days. The virus (in tissue culture medium) was stored in a dry-ice box on 7 occasions between tissue culture passages for a total period of 213 days. At no time during this passage study was the virus propagated in any tissue except the human lymph-node cells in tissue culture. On one occasion the tissue culture medium containing virus was Seitz filtered prior to transfer to new tissue cultures. Inoculation of mice with medium from the normal-mouse-brain-control tissue cultures of each passage caused death of only 3 out of a total of 315 mice.

Computation of the dilution of the original mouse-brain inoculum during these studies indicates that at the end of the 20th consecutive tissue culture passage the *original* inoculum had reached a dilution of $10^{-26.0}$. In contrast, the LD_{50} intracerebral titer of the original virus inoculum was $10^{-7.5}$. This quadrillion-fold difference must be due to virus propagation. In the study in which virus was maintained in a single continuous culture for 94 days, the dilution of the original inoculum by the time this tissue culture was infected (18th tissue culture passage) was 10^{-23}, and by the time the culture was fixed (after 12 re-feedings) the original inoculum had been diluted to 10^{-35}. In a single culture in which virus was maintained for a period of 42 days, titrations were performed on 18 occasions. These titers fluctuated between 10^{0} and $10^{-3.5}$ without apparent relationship to total lapsed time in culture or to time of re-feeding of the cultures. Further evidence of the propagation of Bunyamwera virus was provided by 3 studies in which the virus titer in culture medium was determined

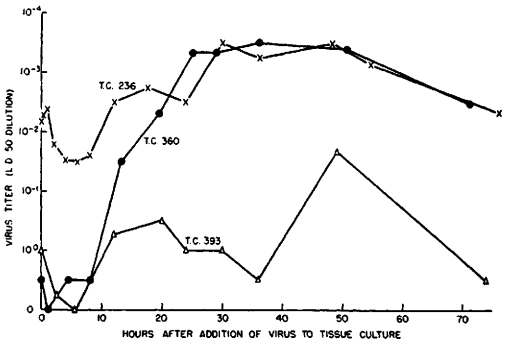

FIG. 2. Demonstrating increasing virus titers in culture medium during 3 days following addition of Bunyamwera virus to tissue cultures.

at frequent intervals following inoculation (Fig. 2). During the first few hours after virus inoculation, there was a drop in titerable virus. By 12 hours there was an increase, and peak titers were reached between 18 and 24 hours. Examination of these curves (especially tissue cultures 236 and 393) suggests that the infective cycle for this virus is approximately 18 to 24 hours. In culture 360, there was a greater than one-thousand-fold increase in virus titer during the 24 hours following inoculation.

In addition to the 20 serial passages already discussed, Bunyamwera virus was transferred in several sublines in human lymph node tissue cultures for various types of special studies. In all, this virus was inoculated into 36 different tissue cultures (*i.e.*, lymph nodes from 36 patients). Virus propagation could not be demonstrated in 6 (17%) of these attempts. These failures were not due to poor cellular growth or to inadequate virus inoculum, and are still unexplained. It seems most probable that virus could not be demonstrated in these 6 cultures because it had not propagated, but it is conceivable that it had mutated to a form which was avirulent for mice.

Adaptation of Bunyamwera virus in tissue cultures. There is some evidence that during these passages in human cells there was adaptation of Bunyamwera virus to this new host. This is suggested by a progressive change in the picture of disease produced in mice. The original mouse-brain-passage Bunyamwera virus killed mice in 3 to 5 days even when inoculated at the minimum lethal dose. Paralysis occurred rarely and lasted only a few hours before death. Usually death occurred suddenly with few signs of sickness other than a brief terminal convulsion. After the virus had been through 10 passages (58 days) in human cells, it caused a more chronic illness of mice. Mice injected with this passaged virus frequently died 10 to 14 days after inoculation, and it became common for paralysis to persist for 2 or more days before death; and a few mice recovered after several days of paralysis. The prolonged period of illness and prolonged incubation time with the tissue-culture-passaged virus was observed even when mice were inoculated with as much as 1000 times the LD_{50}, indicating that the change in character of the disease in mice was a result of change in pathogenic character of the virus rather than a dose phenomenon. Fig. 3 summarizes the data which suggest virus adaptation. In this figure are included results from all cultures tested between days 2 and 7 after virus inoculation. From one to 4 samples were studied during this period in each passage. Studies earlier than day 2 were excluded because they might measure non-propagating virus or sub-maximal proliferation. Studies later than day 7 were excluded because they were available for only a few cultures. The lowermost curve presents the virus titers, as measured by lethality for mice. These titration data are incomplete because several cultures were tested undiluted only; however, it is apparent that there was no consistent tendency toward increased or decreased virus titer (as measured in mice) during serial passage in culture. The remaining 2 parts of Fig. 3 illustrate the progressive change in pattern of the illness produced in mice by the virus during serial tissue-culture-passage. For these curves only those mice inoculated with undiluted medium were considered. The middle section of the figure illustrates the progressive prolongation of average survival time of mice inoculated with virus grown in successive tissue culture passages. In order to permit a computation of average survival times for each passage, those mice which were alive at the end of the 2-week observation

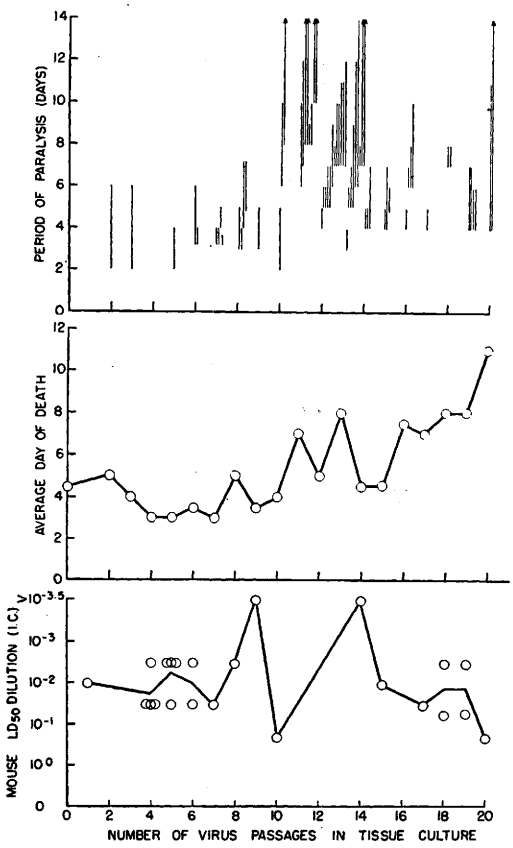

FIG. 3. Demonstrating change in type of illness produced in mice by Bunyamwera virus during course of prolonged passage in tissue cultures of human cells. Lower portion of figure shows virus titers as measured in mice. Middle portion shows progressive increase in survival time of mice inoculated with undiluted tissue culture medium from the serial passages. Upper portion shows duration of paralysis in individual mice (arrows indicate recovery from paralysis).

period were included in the calculation as if they had died on day 15. The uppermost part of the figure indicates the prolonged paralysis frequently seen in mice inoculated with high tissue-culture-passage virus. Each line indicates the duration of paralysis in one mouse. The paucity of lines on the left half of this chart reflects the rarity of prolonged paralysis from early passage virus. It can be seen, by comparison of these curves that the changes in the pattern of illness in the mice are not a function of virus titer. For example, in passages 4 through 7 titers are almost identical with the titers in passages 17 through 19, but average survival time of mice is 3 days and 8 days respectively for these same groups.

That these changes were due to adaptation is also suggested by the fact that when "adapted" virus (13th tissue culture passage), was carried through 3 mouse passages, it regained the original pathogenic pattern of rapid death preceded by convulsions or very brief paralysis (even at the minimum lethal dose).

Cortisone and virus growth in culture. Because of evidence that cortisone treatment increases susceptibility of mice to Bunyamwera virus(9), and has similar effects on other viruses in various hosts(1,2,7,9), the effect of cortisone acetate and dehydro-cortisone acetate (compound F) on the growth of this virus in these tissue cultures was studied. Cortisone was used in a final concentration in the culture medium of 5, 50, and 500 γ/ml. Compound F was used at 50 γ per ml. Control cultures contained the same solvents and suspending agents as were used for the steroids. There was no significant effect upon virus propagation as judged by serial titration of virus in the culture medium (Fig. 4). These results suggest that cortisone affects host susceptibility to virus infection indirectly, rather than by an effect upon the parasitized cell. Interpretation must be tentative, however, because it is possible that the acetate form of these steroids is not active at the cellular level, and could not be broken down by the fibroblastic cells.

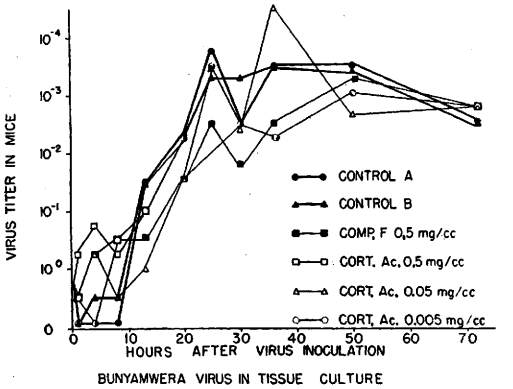

FIG. 4. Demonstrating failure of cortisone acetate or dehydrocorticosterone acetate to influence rate or degree of virus propagation in cells in tissue culture.

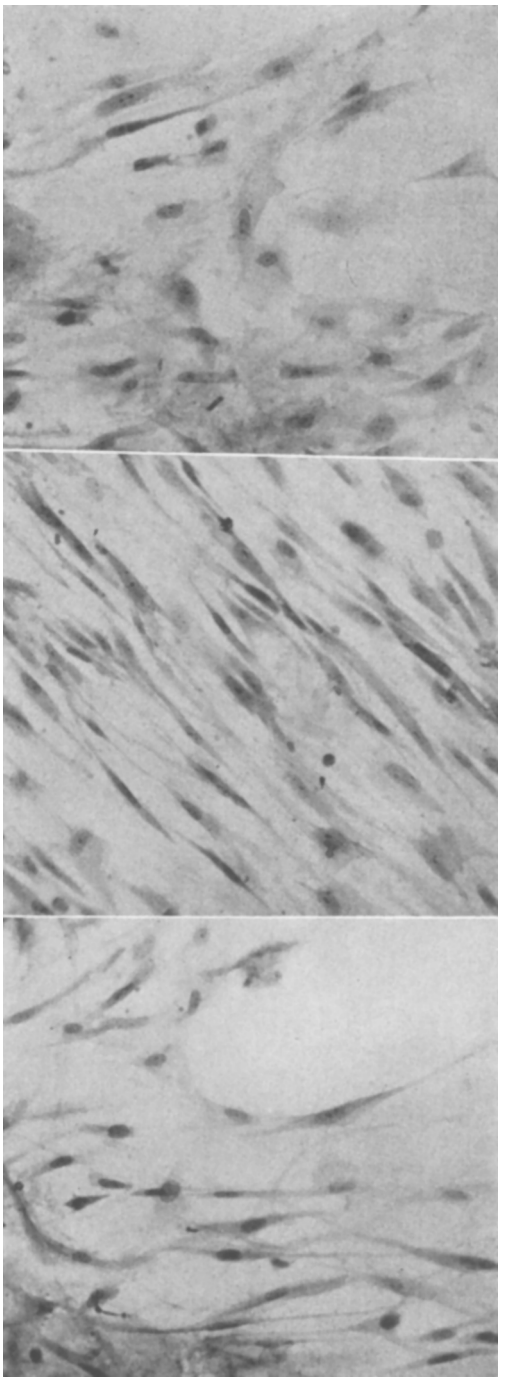

FIG. 5 (top). Normal appearing fibroblastic type cells from human lymph node in tissue culture. Culture was 54 days old and had been inoculated with Bunyamwera virus 42 days before fixation. Virus was still present at end of experiment. Stained by Jacobsen's technic with May-Gruenwald and Giemsa stains. T.C. No. 278.

FIG. 6 (middle). Normal-mouse-brain control for culture in Fig. 5.

FIG. 7 (bottom). Same as Fig. 5 except that this culture had supported growth of Egypt 101 virus for 39 days.

Cytopathology. There was a surprising lack of cell damage in virus-infected tissue cultures, indicating that Bunyamwera virus is able to grow in human fibroblasts without killing the cells, or that the rate of cell proliferation is equal to the rate of cell destruction. Areas of degenerating cells were occasionally seen, but did not appear more common than in the normal-mouse-brain control series of tissue cultures. There was poorer cell growth in many virus-infected cultures than in parallel controls, suggesting inhibition of cell propagation, but this difference was not sufficiently consistent to be convincing, and quantitation of cell growth was not attempted. Fig. 5 shows normal fibroblastic cells after 42 days of virus proliferation. Fig. 6 is the normal-mouse-brain control of the same culture.

West Nile viruses. The Egypt 101 isolate of West Nile virus was inoculated into 12 lymph node cultures. Virus propagated, or at least persisted, in 7 (58%). The reason for the failures was not determined, but it was not absence of cell growth nor absence of virus in the inoculum. The longest serial passage of Egypt 101 virus was through 4 consecutive tissue cultures for a total of 79 days. The titer of the original inoculum in this study was 10^{-6} (*i.e.* mouse LD_{50}). The dilution of the original mouse-brain inoculum produced by 4 culture passages and 12 re-feedings was 10^{-17}, indicating that at least a 10-billion-fold increase of virus had occurred. The tissue-culture virus was Seitz filtered prior to the fourth passage. In another culture, not included in the above series, Egypt 101 virus was demonstrable for 39 days. There were 9 re-feedings during this period, thus resulting in 10^{-11} dilution of the original mouse-brain inoculum. This indicates a 10000-fold increase of virus in this single tissue culture, since the original inoculum for this culture titered 10^{-7}.

Smithburn's original isolate of West Nile virus was inoculated into 4 cultures and prop-

agated in 2. There is no apparent explanation for the 2 failures, and it is of interest that both of the cultures which failed to support growth of Nest Nile virus did support growth of Bunyamwera virus in parallel studies. The longest persistence of West Nile virus was 37 days. During this period the culture was re-fed 5 times, resulting in dilution of the original inoculum to 10^{-7}. There was no consistent cytopathology attributable to either the Egypt 101 isolate (Fig. 7) or the original isolate of West Nile virus.

Ilheus virus. Ilheus virus was inoculated 10 times into lymph node cultures and propagated, or at least persisted for several days, in 7. The longest successful serial passage was through 4 cultures for a total of 163 days in culture, and a final dilution of the original mouse brain inoculum to 10^{-27}. The original inoculum had a titer of 10^{-6}. Each of these 4 serial passages was maintained (with persistence of viable virus) for over 30 days. No convincing evidence of cytopathology was seen.

Br I virus. Br I virus was inoculated into 6 cultures of human nodes and propagated in all. Four of these were in serial passage for a total duration of 135 days. The original inoculum, which titered $10^{-7.2}$, was diluted to 10^{-23} by these passages and re-feedings. This implies at least a 10^{15}-fold increase in virus in tissue culture. The virus was Seitz filtered before one of these passages. As with the other viruses used in this study, there was no definite cytopathology.

Viruses in tissues other than lymph nodes. In addition to normal lymph nodes, tissue cultures of normal human skin and a variety of human neoplasms were used in these studies. Most cultures from these tissues contained fibroblastic as well as epidermal or neoplastic cells. In only a few of the cultures of cancer was it possible to be certain that neoplastic cells were growing. It was not possible to ascertain within what cell types the virus propagated. Bunyamwera virus was inoculated into a skin culture once and did not propagate. It probably did propagate in a culture of epidermoid carcinoma, as evidenced by a titer of $10^{-1.5}$ at 3 days after inoculation, at which time cultures were fixed. This culture was proved by back-implantation to contain cancer cells (see Fig. 31 to 33 in ref. 10), and appeared to be a pure culture of cancer cells. Hence it is highly probable that the virus was propagating in epidermoid cancer cells. Microscopically, necrosis of cells was more common in the virus-infected cultures than in the normal-mouse-brain controls, but many areas of healthy appearing carcinoma cells remained. Bunyamwera virus also propagated in cultures from reticulum sarcoma for 17 days. These cultures contained principally lymphoid (? lymphosarcoma) cells, but fibroblastic cells were also frequent. Egypt 101 virus persisted for 9 days in cultures of the same epidermoid carcinoma mentioned above. As with Bunyamwera virus there was suggestive but inconclusive evidence of cytopathology attributable to the virus. This virus also grew for 22 days in another epidermoid carcinoma culture and a renal adenocarcinoma culture, and for 24 days in a culture of rectal adenocarcinoma (see below). These cultures contained carcinoma cells, but also many fibroblasts. Egypt 101 virus also persisted for 34 days after inoculation into tissue cultures of melanoma. This virus was also demonstrated, without addition of stock virus, in cultures of malignant melanoma from 2 patients who had Egypt 101 virus infection with viremia. In each of these 3 cultures of melanoma the cells appeared fibroblastic, and there was no proof that melanoma cells were present. Egypt 101 virus was also inoculated into 3 cultures of human leukemic blood buffy coat. There was no evidence of virus propagation, but neither was there any evidence of cellular proliferation. Ilheus virus was inoculated into the same epidermoid carcinoma culture mentioned above and apparently propagated as evidenced by repeated recoveries over a period of 9 days. No definite cytopathology occurred.

Virus growth in cells from immune patient. Egypt 101 virus grew well for 24 days in tissue cultures of rectal adenocarcinoma (see Fig. 35 in reference 15) from an Egypt 101-immune patient, but disappeared promptly after addition of 0.2 ml of the same patient's immune serum. Addition of the

same serum to parallel cultures infected with Ilheus virus did not prevent continued propagation of the Ilheus virus. It has recently been reported that infectious canine hepatitis virus grows well in tissue cultures of kidneys from ICH-immune dogs(14).

Summary. 1. Bunyamwera virus was grown in roller-slide tissue cultures of human lymph nodes (fibroblastic type cells). Propagation of virus was demonstrated by increases in virus titers in short-term virus growth studies, by long persistence of virus in tissue cultures as contrasted with rapid disappearance in cell-free preparations, and by persistence of virus after serial passages had diluted the original virus inoculum far beyond its minimal infective dilution. 2. Similar but less extensive studies demonstrated the propagation in human fibroblast tissue cultures of West Nile, Egypt 101, Ilheus, and Br I viruses. 3. Bunyamwera virus showed changes interpreted as an adaptation phenomenon during serial passages in tissue cultures. These changes were reversed by serial intracerebral passage in mice. 4. None of these viruses caused appreciable cytopathology under these experimental conditions, even after prolonged growth in tissue culture. 5. Bunyamwera, Egypt 101, and Ilheus viruses were also grown in tissue cultures from various human cancers. It was not possible to determine whether virus was propagating in the cancer cells, or in non-neoplastic stromal cells. 6. Egypt 101 virus propagated in cells from an immune patient, but not in the presence of immune serum.

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1. Kass, E. H., Ingbar, S. H., Lundgren, M. M., and Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1951, v37, 780.

2. Kilbourne, E. D., and Horsfall, F. L., Jr., *ibid.*, 1951, v76, 116.

3. Melnick, J. L., Paul, J. R., Riordan, J. T., Barnett, V. H., Goldblum, N., and Zabin, E., *ibid.*, 1951, v77, 661.

4. Newman, W., and Southam, C. M., *Cancer,* 1954, v7, 106.

5. Parshley, M. S., and Simms, H. S., *Am. J. Anat.,* 1950, v86, 163.

6. Reed, L. J., and Muench, H., *Am. J. Hyg.,* 1938, v27, 493.

7. Schwartzman, G., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 835.

8. Smithburn, K. C., Haddow, A. J., and Mahaffy, A. F., *Am. J. Trop. Med.,* 1946, v26, 189.

9. Southam, C. M., and Babcock, V. I., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 105.

10. Southam, C. M., and Goettler, P. J., *Cancer,* 1953, v6, 809.

11. Southam, C. M., and Moore, A. E., *Am. J. Trop. Med.,* 1951, v31, 724.

12. ———, *Am. J. Trop. Med. and Hygiene,* 1954, v3, 19.

13. ———, *J. Immunol.,* 1954, in press.

14. Cabasso, V. J., Stebbins, M. R., Norton, T. W., and Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 239.

15. Southam, C. M., *Cancer,* 1954, v7, 394.

Received February 25, 1954. P.S.E.B.M., 1954, v86.

Specificity of Antineoplastic Phenomenon Induced in Mice by Carcinogenic Agents. (21045)

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Following the injection of methylcholanthrene into mice an antineoplastic (AN) factor appears in their spleens(1). This response appeared to be specific in that the AN factor was not engendered in homozygous mice through inoculations with phenanthrene and with cooking oil (the diluent), and it was not present in spleens from uninoculated mice. The AN phenomenon endured through a period that appeared to coincide with the latent period of carcinogenesis. This supposition was further supported by the absence