been ascertained, since, at least with New Guinea B, use of the regular Hsiung-Melnick formula suffices. Furthermore, the lack of neither arginine nor oxalacetic appreciably affects New Guinea B virus plaquing efficiency; their inclusion, however, does aid in plaque clarity.

Summary. Host range susceptibility studies with mouse adapted dengue types 1-6 have shown that cultures prepared from kidney tissues of primate origin are more sensitive to dengue virus plaque formation than culture systems originating from tissues of non-primates, *e.g.*, porcine kidney. Plaques developed in cultures of rhesus monkey kidney (LLC-Mk_2 passage line) and porcine kidney (PS-Y-15 passage line) in 4-7 days for dengue types 2, 4, 5, and 6, and in 9-13 days for types 1 and 3. About 99% of dengue 2 was adsorbed onto primate kidney cultures in 15 minutes, but adsorption of virus in PS-Y-15 cultures was poor even after 120 minutes. When comparing multiplication rates of the dengue viruses in infant mice and two passage cell cultures, synthesis of new virus proceeded at about the same rate in both hosts. However, higher titers were usually evident in mouse brain preparations.

1. Hotta, S., Evans, C. A., J. Inf. Dis., 1956, v98, 88.
2. ———, Proc. Soc. Exp. Biol. and Med., 1956, v93, 153.
3. Diercks, F. H., Hammon, W. McD., ibid., 1958, v97, 627.
4. Siegal, M. M., Beasley, A. R., Wellings, F. M., Lichter, W., Williams, J. A., Fed. Proc., 1961, v20, 443.
5. Schultze, I. T., Schlesinger, R. W., Virology, 1963, v19, 40.
6. Karabatsos, N., personal communication.
7. Buckley, S. M., Nature, 1961, v192, 778.
8. Wiebenga, N. H., Am. J. Hyg., 1961, v73, 365.
9. Halstead, S. B., Sukhavachana, P., Nisalak, A., Proc. Soc. Exp. Biol. and Med., 1964, v115, 1062.
10. Miles, J. A. R., Austr. J. Sci., 1963, v25, 466.
11. Georgiades, J., Stim, T. B., McCollum, R. W., Henderson, J. R., Proc. Soc. Exp. Biol. and Med., 1965, v118, 385.
12. Hsiung, G. D., Diagnostic Virology, Yale Univ. Press, New Haven-London, 1964.
13. Hull, R. N., Cherry, W. R., Johnson, I. S., Anat. Rec., 1956, v124, 490.
14. Inoue, Y. K., Yamada, M., J. Bact., 1964, v87, 1239.
15. Eagle, H., Science, 1959, v130, 432.
16. Mintz, B., ibid., 1962, v138, 594.
17. Wallis, C., Melnick, J. L., Texas Rep. Biol. and Med., 1961, v19, 701.

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Role of the Spleen in Interferon Production in Mice.* (31311)

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Heretofore, most experiments with interferon have utilized *in vitro* systems. Although *in vivo* interferon production has been previously reported(1,2), little is known about the site of interferon synthesis *in vivo*. Several investigators have demonstrated that leukocytes are capable of synthesizing this inhibitory substance(3,4), and it was therefore considered pertinent to determine to what extent leukopoietic organs are involved in the formation of interferon. The present report relates certain findings which indicate that the spleen plays a major role in interferon synthesis.

Materials and methods. Virus strains. The Cincinnati strain of Newcastle disease virus (NDV), obtained from Dr. Robert N. Chanock of the National Institutes of Health, and the Indiana strain of vesicular stomatitis virus (VSV), obtained from the American Type Culture Collection, were maintained by

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passage in the allantoic cavity of 10- to 11-day-old chick embryos. The NDV stocks used in these experiments titered $10^{10.2}$ EID_{50} per ml, and contained 5120 HA units per ml. The VSV pools titered $10^{6.3}$ PFU per ml as measured in L cells.

Cell cultures. L cells. Strain L-929 cells were obtained from Dr. Russell W. Brown of Tuskegee Institute. Stock cultures were maintained by weekly subculture in Eagle's minimum essential medium (MEM) with 10% calf serum (without antibiotics).

Mice. Two hundred 5- to 8-week-old male Swiss mice, ranging in weight from 27.7 to 43.2 g, with a mean weight of 37.7 g, were used as the source of test materials for these experiments.

Surgical procedure. The mice to be used in these experiments were prepared for surgery in the following manner. The hair over the splenic area was removed by the use of Nair.† Following depilation the mice were randomized and divided into 2 groups of 100 animals each. One group underwent splenectomy, and the other served as controls, experiencing sham operations (abdominal incision and closure). Anesthesia was accomplished by intraperitoneal injection of 2.3 mg of sodium pentobarbital. Following cleaning of the skin with 70% ethyl alcohol and Betadine,‡ a transverse incision (1 cm) was made through the skin, muscle, and peritoneum, from a point just above the spleen along its longitudinal axis. The spleen was excised following ligation of the pedicle by a single suture placed as close to the spleen as possible. The abdominal muscles and peritoneum were sutured as a unit. Sutures were then applied to the skin. (Black cotton sutures were used throughout the surgical procedure.) The sutured skin was coated with a thin layer of collodion.

The animals were allowed a 3-week recovery period prior to stimulation of interferon production. During the recovery period, deaths occurred among 2 control mice and 11 splenectomized animals (7 of the latter were in the same cage). Further evidence of the lack of any serious detrimental effects of splenectomy was obtained when the average weights of the 2 groups were found to be the same at the end of the experiment.

Production of interferon test preparations. Control and splenectomized groups were again randomized after recovery from surgery. A quantity of diluted NDV sufficient for all inoculations was prepared prior to the beginning of the inoculation and stored in 3 ml aliquots at —20°C until ready for use. Two-tenths ml of NDV dilution ($10^{8.2}$ EID_{50}) was injected into the tail vein of each mouse. Since the groups were large and required considerable time for bleeding and organ removal, inoculations were staggered to provide uniform time intervals within each group. Each mouse was tagged with a number so that harvests could be made in order of inoculation. At the designated intervals after inoculation, the animals were anesthetized with sodium pentobarbital, and harvests of serum and organs were made. Spleen and liver tissues were weighed, minced with scalpels, and extruded through a syringe. The tissue fragments thus obtained were then placed in organ culture, in screw-capped soft glass prescription bottles, as a 20% suspension in Earle's balanced salt solution (BSS) with 15% fetal calf serum. After a 12-hour incubation at 36°C, the suspension fluids were harvested and centrifuged at 525 $\times$ g for 15 minutes. The supernates were then stored, along with the serum, at 4°C until the completion of all interferon production phases of the experiment. Serum, spleen, and liver test materials were also obtained from a group of 13 control mice which received no stimulator virus but had been subjected to the sham operation.

After all test preparations had been obtained, they were dialyzed at pH 2.0 for 5 days, followed by a 48-hour dialysis at pH 7.4 against Earle's BSS. The dialyzed fluids were subjected to low speed centrifugation to remove precipitates. All preparations were then filtered through a 0.22 μ Millipore® filter prior to storage at 4°C. All filters were pre-rinsed in 2 changes of approximately 400 volumes of distilled water to

† Carter Products, Inc., New York.

‡ Tailby-Nason Co., Inc., Dover, Del.

TABLE I. Comparison of VSV Plaque Counts in L Cells Pretreated with a 1:20 Dilution of Serum from Control and Splenectomized Mice.

Time	Avg plaque counts* Control	Splenectomized	P value‡
0	161.5	168.7	.60
2	151.0	142.7	.20
4	67.8	138.0	<.001
† 8	.3	77.3	<.001
12	.0	93.8	<.001
18	32.0	135.0	.002
25	98.3	107.0	.30
30	87.3	104.8	.40

* Averages computed from 6 cultures per sample based on 159.1 plaques in untreated cultures except where noted.

† Separate experiment based on 148 plaques in untreated cultures.

‡ Computed by standard t test.

remove any inhibitors which might be present on the filter membrane.

Assay of test materials. All test materials were assayed in monolayer cultures of L cells, using a plaque reduction method with VSV as the challenge virus. The cultures were initiated in MEM containing 10% calf serum plus 400 units penicillin and 200 μg streptomycin per ml. One day later, the growth medium was replaced with MEM containing 5% calf serum and 5% of test material (serum or tissue extract from control or splenectomized animals). The cultures were then incubated for 12 hours at 36°C, at which time the test materials were decanted, the cultures rinsed twice with Earle's BSS, and challenged with 0.5 ml of VSV diluted to give a plaque count of about 150. Virus adsorption was carried out at 36°C for 1 hour, after which the monolayers were again rinsed twice with Earle's prior to overlay with 4.5 ml of growth medium containing 1% methyl cellulose.§ The cultures were incubated for 56 hours at 36°C, at which time the methyl cellulose medium was diluted with Earle's and poured off. Cultures were then stained with crystal violet, and plaque counts were made under a photographic enlarger at a magnification of 7 diameters.

Results. Serum interferon test preparations from control and splenectomized mice were assayed at a dilution of 1:20. The results are presented in Table I and Fig. 1. They clearly show that the interferon from the serum of control animals reached a maximum between 8 and 12 hours. Inhibition of VSV plaque formation by interferon in serum from splenectomized animals was consistently much lower than that obtained with serum from sham-operated animals. Since relatively little interferon was detected in the serum from splenectomized mice, it may be concluded that the spleen is a very important producer of interferon in the intact host, and that the difference in interferon content of the serum from the two groups is attributed to removal of the spleen.

Although these results implicate the spleen as a major source of interferon in the intact host, direct proof of the spleen's importance in this type of resistance against infection requires actual demonstration that this organ is highly efficient in the synthesis of interferon.

Prior to the experiments herein reported, we inoculated C57/Bl mice intravenously with 10^6 EID_{50} of NDV. Spleens, lymph nodes, and bone marrow were removed at 0, 2, 4, 8, 12, 18 and 24 hours. These organs were placed in an *in vitro* maintenance system, and assays for interferon showed total absence of interferon from cultures of lymph node and bone marrow. Interferon was found in the cultures of spleen fragments. The spleen removed at time zero produced no interferon, and the first demonstrable production was noted in cultures of spleen fragments removed 2 hours after inoculation. The maximum level of interferon was obtained in cultures of spleens removed 8 hours after inoculation. These levels exceeded the amount of interferon demonstrable in the serum by as much as 40-fold.

Based on these findings, we have followed interferon levels in organ cultures derived from tissues of the Swiss mice employed in the present experiment (spleens and livers in the sham-operated and livers in the splenectomized mice). No interferon activity was demonstrable in liver fragments obtained from either group. In sharp contrast, supernates from the spleen fragments manifested significant protective activity (Fig. 2) and

§ Methyl cellulose, Fisher Scientific Co., Fair Lawn, N. J.

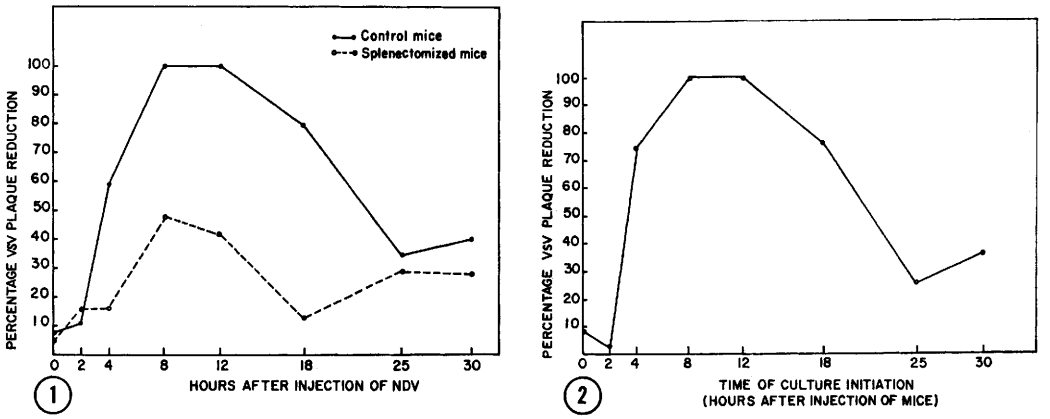

FIG. 1. Comparison of inhibition of VSV plaque formation in L cells treated with a 1:20 dilution of serum from control or splenectomized mice inoculated intravenously with NDV.

FIG. 2. Percentage VSV plaque reduction in L cells treated with fluids from organ cultures of mouse spleens excised at various times after intravenous injection of NDV.

the level of this activity corresponded closely with the inhibitory activity of the serum. Thus, the maximum protection was realized in organ cultures of spleens obtained at 8 and 12 hours, and minimal protective effects were noted with spleens removed at 0, 2, 25 and 30 hours.

Discussion. The present findings indicate that the spleen plays a major role in the production of interferon *in vivo*. Not only was interferon production impaired in splenectomized mice, but the spleens were shown to produce significant protective activity. In contrast, the liver was apparently much less involved in the process of interferon synthesis. These findings indicate that the spleen contributes to the defense of a host not only through a specific antibody synthesis but also by producing interferon. It has been suggested that macrophages are actively engaged in interferon synthesis(5). Our data indicate that the liver, which is rich in stationary macrophages, does not seem to play a major role in the mouse challenged with NDV.

De Somer and Billiau have also found the spleen to be critical in the production of interferon in response to *E. coli* endotoxin(6). Yet these workers have not associated the spleen with virus-induced interferon *in vivo*. It should be pointed out that De Somer and Billiau employed rats as the host and Sindbis virus as the inducer. Therefore, it is possible that the difference in our results may be due to the use of different hosts and viruses.

Summary. The effect of splenectomy on interferon formation in mice following intravenous injection of Newcastle disease virus is described. Serum from splenectomized mice had markedly lower protective activity than serum from control animals. Organ cultures of spleens and livers removed from these animals after injection of NDV showed interferon production in the spleen but not in the liver.

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1. Baron, S., Buckler, C. E., Science, 1963, v145, 1063.

2. Kono, Y., Ho, M., Virology, 1965, v25, 162.

3. Gresser, I., Proc. Soc. Exp. Biol. and Med., 1961, v108, 799.

4. Glasgow, L. A., Habel, K., J. Exp. Med., 1963, v117, 149.

5. Glasgow, L. A., ibid., 1965, v121, 1001.

6. De Somer, P., Billiau, A., in preparation.

Received March 18, 1966. P.S.E.B.M., 1966, v122.