cytomegaloviruses has been developed employing human embryonic lung fibroblast cultures and an overlay containing 2% methyl cellulose. Neutralization tests employing the 80% plaque reduction endpoint were found to give reproducible results. Human adult sera exhibited titers of 1:8 to 1:64. Typing antisera were successfully prepared in monkeys, yielding titers of 1:32 to 1:256.

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1. Weller, T. H., Hanshaw, J. B., Scott, D. E., *Virology,* 1960, v12, 130.
2. Rapp, F., Rasmussen, L. E., Benyesh-Melnick, M., *J. Immunol.,* 1963, v91, 709.
3. Goodheart, C. R., Jaross, L. B., *Virology,* 1963, v19, 532.
4. Hotchin, J., *Nature,* 1955, v175, 352.
5. Rapp, F., Seligman, S. J., Jaross, L. B., Gordon, I., PROC. SOC. EXP. BIOL. AND MED., 1959, v101, 289.
6. Henson, D., Pinkerton, H., *ibid.,* 1963, v114, 130.
7. Smith, K. O., Rasmussen, L., *J. Bact.,* 1963, v85, 1319.

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Antibody Production in the Guinea Pig to Homologous Uvea.* (29521)

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Proposed as the cause of sympathetic ophthalmia by Elschnig(1,2,3) more than 50 years ago, auto-sensitization to uveal tissue has also been suggested recently as the cause of more common forms of uveitis. Experimentally these assumptions rest on fragmentary and seemingly contradictory evidence, which has been previously cited(4). A significant advance in the study of immunogenic uveitis was the recent confirmation by Aronson *et al*(5,6,7,8) of Collins'(9,10) original findings of uveitis in guinea pigs immunized with homologous uvea. Neither study, however, demonstrated circulating antibody nor hypersensitive responses with which the ocular lesions might be associated.

The present study, a continuation of earlier published work(4), was undertaken to develop an experimental model for the study of immunogenic uveitis. Because organ specificity has particular significance for immunologic disease, emphasis was placed on detection of immune responses to antigens peculiar to the uveal tissue. As the results will show, high titered complement fixing antibody reacting with an organ specific species antigen, was produced in guinea pigs by immunization with homologous uvea. Accompanying antibody production was the development of lesions in the uveal tract.

Materials and methods. Uveal tracts were dissected from normal pigmented guinea pig eyes removed immediately after exsanguination of the animals under chloroform or ether anaesthesia. For one experiment, uveal tracts from Hartley albino guinea pigs were used. The uveal tracts were washed in 3 changes of sterile physiologic saline, blotted lightly, and stored 5-10 uveas/vial at —50°C. Immediately before use the tissues were thawed, weighed, and ground in a mortar or Tenbroeck grinder with addition of sterile physiologic saline to make a 10% suspension. The suspensions were then emulsified with an equal volume of Freund's complete adjuvant, consisting of 1.5 volumes Arlacel A, 8.5 volumes light mineral oil, and 4 mg killed and dried tubercle bacilli/ml. The completed emulsion contained approximately 50 mg uveal tissue/ml, with the exception of one

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lot used for Group I below which contained 20 mg/ml.

Hartley guinea pigs of both sexes weighing 400-500 g were used. They were bled by cardiac puncture for collection of preimmune sera, then injected with 0.1 ml of the above emulsion into each hind foot pad. Additional injections were given as follows: Group I, 6 guinea pigs, at time 0 also received 0.1 ml intradermally under the neck. Group II, 9 guinea pigs, received 0.5 ml intradermally in 5 sites at week 1. Group III, 16 guinea pigs, 0.5 ml intradermally in 5 sites at week 1, and 0.25 ml intramuscularly at week 3. Group IV, 8 guinea pigs, received 0.2 ml intramuscularly at weeks 1, 2, 5, and 8. Group V received the same injections as Group IV plus 0.2 ml intramuscularly at week 11 and 0.2 ml intraperitoneally at week 14. Pigmented uvea was used for all groups, except Groups IV and V of which half received albino uvea. Animals were killed by exsanguination under anaesthesia 2-3 weeks after the last injection, except Group II animals which were bled at weeks 4 and 7, and exsanguinated at week 11. Control animals for each group were given similar injections of saline emulsified in Freund's complete adjuvant. Sera and tissues were saved from all animals for subsequent serological and histological examination.

Tissues used as serologic antigens were obtained from guinea pigs, rabbits and cows immediately after the animals were killed. Tissues were washed in sterile saline and stored frozen. For use the tissues were ground in a mortar with addition of saline diluent to make a 1:25 suspension (wt of tissue/volume). Supernatant fluids obtained by centrifugation at 2,000 rpm for 10 minutes were used in the test. The diluent used throughout for dilution of serologic reagents was barbital buffered saline containing optimal calcium and magnesium ions described for the complement fixation test(11).

Sera were examined for antibody by the complement fixation test (CF) described by Kabat and Mayer(11). Complement and hemolysin were titrated by the quantitative complement fixation procedure in a total reaction volume of 7.5 ml using 5×10^8 sheep red cells. The test itself was done by the 1/5 modification using doubling serial dilutions of serum contained in 0.4 ml, 0.5 ml of complement containing 5 $C'H_{50}$, and 0.4 ml of 1:25 dilution of antigen. The tubes were then incubated overnight in the refrigerator, following which 0.2 ml optimally sensitized sheep red cells were added. After 60 minutes' incubation in the water bath at 37°C, the tubes were compared visually with standard tubes showing 0, 25, 50, 75 and 100% lysis, which were read as 4+, 3+, 2+, 1+ and 0 respectively, showing the degree of turbidity. Hemolytic and anticomplementary controls of both sera and antigens were included with each run, in addition to complement deterioration test.

Eyes for histologic examination were fixed in 10% phosphate buffered formalin for 48 hours, then washed in running tap water for 8 hours, and placed in 70% alcohol. They were embedded in paraffin, 12 sections cut from different levels and stained with hematoxylin and eosin.

Results. Complement fixing antibody reacting in high titer with guinea pig uvea was produced in guinea pigs immunized with homologous uvea (Table I). Repeated immunization was important in achieving this response. Circulating antibody was detected in animals of Groups IV and V, which were repeatedly injected over a period of 2 or 3 months, but was not found in other groups of animals which were injected several times over a period of 3 weeks or less. The length of time during which animals were injected also seemed more important than the total amount of uvea given. Thus, antibody was produced in the 2 groups given a total of 50 or 70 mg uvea over a long term, but not in 2 groups given a similar total amount over a few weeks. Pigmented and albino uvea were equally effective in eliciting this response.

The antibody reacted specifically with some component of guinea pig uveal tissue (Table II). There was a slight suggestion of cross reaction with guinea pig lens, in serum dilutions of 1:10 or less, but there was no

TABLE I. Complement Fixing Antibody in Sera of Guinea Pigs Immunized with Homologous Uvea in Freund's Adjuvant.

Guinea pig No.	Serum dilutions* 10	20	40	80	160	320	640
17	4+	4+	4+	4+	4+	4+	4+
18	4+	4+	4+	4+	4+	4+	3+
23	4+	4+	4+	4+	4+	2+	±
24	4+	4+	4+	4+	4+	3+	1+
Controls							
Adjuvant, 4†	0	0	0	0	0	0	0
Pre-immune sera, 8‡	0	0	0	0	0	0	0

* Reciprocal of serum dilution showing reaction with 1:25 dilution of guinea pig uvea.
† Results of 4 individual animals.
‡ Pre-immune sera from the above experimental control animals.

evidence of reaction with other guinea pig tissues including liver, kidney, or normal serum. The antibody also failed to react with bovine or rabbit uvea. These results indicate that the antigen is not only organ specific but species specific.

Histologic examination of the eyes revealed inflammatory changes in the uveal tracts of 6 of 8 guinea pigs in Group IV, which were immunized over a 2-month period with either albino or pigmented uvea. These changes involved the choroid and ciliary body of 2 animals, the choroid of 3 animals, and the ciliary body of one animal. The iris was not affected. The reaction in the ciliary body was characterized mainly by focal infiltration of plasma cells without structural disarrangement of the tissue. In the choroid, however, areas of inflammation were thickened and showed infiltration with neutrophilic granulocytes and to a lesser extent with plasma cells.

TABLE II. Specificity of Complement Fixing Antibody in Sera of Guinea Pigs Immunized with Homologous Uvea.

Serology antigens*	Pooled sera† Pre-immune	Post-immune
Guinea pig uvea	0‡	640
" " lens	0	10§
" " kidney	0	0
" " liver	0	0
" " serum	0	0
Bovine uvea	0	0
Rabbit uvea	0	0

* 1:25 dilution.
† Sera pooled from 4 immunized animals shown in Table I.
‡ No fixation by 1:10 serum dilution.
§ Partial fixation by 1:10 serum dilution.

Inflammatory changes were always bilateral. One of 6 otherwise normal controls which received saline emulsion, showed unilateral inflammation of the ciliary body. No other lesions were seen in the control eyes.

In striking contrast, animals of Group V which received an even longer series of injections with uveal emulsion, failed to show inflammatory lesions in the eyes. However, animals of this group had untoward reactions following the last injection, given intraperitoneally, and several died. Survivors had no ocular lesions and significantly lower levels of CF antibody, ranging in titer from less than 1:20 to 1:80, than did animals with ocular lesions of Group IV, which had antibody titers of 1:160 to more than 1:640. The possibility that animals of Group V may have experienced protracted anaphylaxis resulting in lowered levels of circulating antibody is being investigated.

Animals on short term immunization schedules did not reveal ocular changes. Twenty-two experimental guinea pigs and 11 controls, which were injected for periods up to 3 weeks, showed neither ocular lesions nor demonstrable antibody. In Table III is a summary of results of both short and long term immunizations. These data show that lesions were produced only by long term immunization and were always accompanied by antibody production, but antibody was found in some animals without ocular lesions.

Discussion. By repeated immunization of guinea pigs with homologous uvea, we have produced antibody which reacts specifically with guinea pig uvea. The significance of

TABLE III. Short Term *vs* Long Term Immunization in Producing Anti-Uveal Antibody and Ocular Lesions in the Guinea Pig.

	Group	No. of injections	No. weeks injected	No. of animals with Lesions	No. of animals with CF antibody
Short term	I	1	1	0/6	0*
	II	2	2	0/9	0*
	III	3	3	0/7	0*
Long term	IV	5	8	6/8	8/8
	V	7	14	0/8	5/5

* Pooled sera from 4 animals tested.

these findings for immunogenic uveitis is 3-fold. They represent the first serologic demonstration of an immune response to homologous uvea in the guinea pig.‡ This response was elicited by a previously undescribed organ specific species antigen. Further, the results confirm the production of ocular lesions in the guinea pig by immunization techniques reported previously by Collins(9) and Aronson *et al*(5).

The organ specific antigen described here in guinea pig uvea is not the same as the uveal organ specific antigen previously described by Elschnig(1,2,3) and Woods(12, 13,14) in other mammalian eyes. One of us (4) has studied the latter antigen extensively, and concurred with the finding that it crosses species lines. This evidence of heterogenetic organ specificity, however, may be based on the similarity of determinant groups among different species of uveal antigens, rather than on the presence of a single antigen which is chemically the same in different species of animals. In contrast, the antigen found in guinea pig uvea was restricted to the guinea pig eye, and antibody produced to it did not cross react with uveal tissue of other species.

The negative serologic results of Collins (9) in attempts to detect this antibody in guinea pigs may be related to the relatively large amount of uveal antigen required to detect the response in the complement fixation test. Although this test is a very sensitive serologic procedure, the antigen is present in only small amounts in the tissue. Based on antigenic titrations, the antigen when diluted more than 1:50 usually gave diminished or negative complement fixation even though significant amounts of antibody were present.

A significant drawback to the idea of an immunogenic uveitis has been the inability to demonstrate both sensitizing antibody and ocular lesions in the same animal experimentally. Whether or not the circulating antibody demonstrated in this study was etiologically related to the ocular lesions was not determined. However, the production of both antibody and ocular lesions in the guinea pig provides a model for further study of effects of antibody and hypersensitivity on uveal tissue by passive transfer experiments and tissue culture techniques.

Summary. The immune response of guinea pigs to homologous uvea was studied, using various periods of immunization and the complement fixation test for detection of antibody. Injections over relatively long periods of time with uveal emulsions led to both antibody production and ocular lesions. Demonstrable antibody and ocular lesions were not found on short term immunization. The antigen herein described was both organ and species specific, in contrast to other uveal organ specific antigens previously described which are heterogenetic.

‡ Personal communication with S. B. Aronson indicated that he has found a precipitating antibody in guinea pigs reacting with homologous uvea.

1. Elschnig, A., *von Grafes Arch. Ophth.*, 1910, v75, 459.

2. ———, *ibid.*, 1910, v76, 509.

3. ———, *ibid.*, 1911, v78, 549.

4. Wacker, W. B., Dodd, M. C., *J. Immunol.*, 1961, v86, 690.

5. Aronson, S. B., Hogan, M. J., Sweigart, P., *Arch. Ophth.*, 1963, v69, 105.

6. ———, *ibid.*, 1963, v69, 203.

7. ———, *ibid.*, 1963, v69, 208.

8. ———, *ibid.*, 1963, v69, 379.
9. Collins, R. C., *Am. J. Ophth.*, 1949, v32, 1687.
10. ———, *ibid.*, 1953, v36 (part 2), 150.
11. Kabat, A. E., Mayer, M. M., *Experimental Immunochemistry,* 2nd Ed., Charles C Thomas, Springfield, 1961, p133.
12. Woods, A. C., *Arch. Ophth.*, 1917, v46, 8.
13. ———, *ibid.*, 1917, v46, 283.
14. ———, *ibid.*, 1917, v46, 503.

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Plaque Studies with Certain Group B Arboviruses. I. Japanese B Encephalitis Virus Strains on Hamster Kidney and Chick Embryo Tissue Culture.* (29522)

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Extensive work with Japanese B encephalitis virus (JBE) has been carried out in several tissue culture systems, beginning about 1954 and later reviewed by Diercks and Hammon(1,2). The sensitivity of hamster kidney tissue cultures (HKTC) by CPE was shown to be equal or superior to that of mice. Nagai(3,4) demonstrated the propagation of this virus in several kinds of tissue culture, mostly primary tissue culture from chick embryo, pig, monkey and mouse origin; but no CPE was observed.

Plaque formation by JBE has been demonstrated in chick embryo tissue cultures (CETC)(5,6,7,8), hamster kidney(9,10) and duck kidney tissue cultures(11). Wallis *et al* (12) reported certain factors influencing enterovirus and reovirus plaque formation which, it seemed, deserved investigation in respect to JBE virus plaque growth. These included the method of sterilization of neutral red to avoid cell toxicity and the choice of agars to avoid viral inhibitors. Attempts have been made to evaluate these and other factors influencing plaque formation of JBE and to compare the plaque characteristics of 3 different strains of JBE virus under a variety of conditions on HKTC and CETC.

Materials and methods. a. *Virus strains.* The Nakayama strain used had been through 32 passages in HKTC following 47 mouse passages(1,2); the $TCID_{50}$ of the frozen pool used was $10^{-7.5}$/0.1 ml. The OCT-541-wild (OCT-wild) strain(13) was isolated directly in HKTC from wild caught mosquitoes and was used in this study in its third passage; the $TCID_{50}$ was $10^{-6.5}$/0.1 ml. The OCT-541 attenuated (OCT-attenuated) strain was derived by and had the properties described by Rohitayodhin and Hammon(14, 15,16); the TCD_{50} of the pool used was $10^{6.5}$/0.1 ml.

b. *Monolayer tissue cultures.* Primary tissue cultures of HKTC or CETC were prepared in 3 oz prescription bottles. Trypsinization was by a modification of the method of Bodian(17).

Outgrowth medium for HKTC consisted of 4% calf serum, 1% vitamin stock (Microbiological Associates, 100× concentration), 0.02 g% $NaHCO_3$ (0.5% of a 4% stock solution), 0.0292 g% L-glutamine (1% of a 2.92 g% stock), and 92.5% of a 0.5% solution of lactalbumin hydrolysate in Hanks' balanced salt solution (HBSS) and 1% antibiotic mixture. CETC medium consisted of 4% calf serum, 0.06 g% $NaHCO_3$, 93.5% lactalbumin hydrolysate in HBSS and 1% antibiotic mixture. Glutamine and vitamin stock were omitted.

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