injection of 500 mU of lysine vasopressin were identical to those described above for PLV-2.

Intradermal injection of Lys-Vp mixed with patent blue V dye were also identical to those described for PLV-2 mixed with patent blue dye. The length of the blue streamers at the end of one hour varied between 6.5 cm and 30.0 cm (average 17.7 cm).

Comment. The dermal vascular responses observed for Lys-Vp were identical to those described for PLV-2. No differences in rates of development of pale and blue streamers or in duration of the response between injections of PLV-2 and Lys-Vp were observed.

Discussion. These experiments indicate that the rate at which streamers of patent blue V dye extend away from an intradermal injection site on the volar aspect of the forearm is accelerated by mixing either PLV-2 or Lys-Vp with the patent blue V dye. On the other hand, intradermal injections of other vasoactive polypeptides including bradykinin, oxytocin and angiotensin II did not accelerate the rate of blue streamer formation (unpublished observations). Since patent blue V dye traces the lymphatics, it is reasonable to assume that PLV-2 and Lys-Vp increased the rate of lymph flow. The mechanism by which the rate of lymph flow was increased is unknown. The relatively slight increase in volume of the bleb at the site of injection could not have been responsible for the increased rate of lymph flow since substances such as bradykinin and histamine which produce much more edema at the site of injection than PLV-2 and Lys-Vp do not accelerate the rate of blue streamer formation. Possible mechanisms by which PLV-2 and Lys-Vp may accelerate the rate of lymph flow include dilation of the lymph channels, increase in peristalic activity of the smooth muscle of the lymphatics and closure of lymphatico-venous communications.

Summary. Preliminary studies indicate that PLV-2 and Lys-Vp, 2 synthetic vasopressins, increase the rate of lymph flow in the superficial lymphatics of the forearm. The mechanism by which these polypeptides influence the rate of lymph flow is unknown.

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A Plaque Reduction Neutralization Test for Human Cytomegalovirus.* (29520)

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Research on cytomegalovirus infections has been handicapped by the lack of typing antisera for the virus as well as by the tedious nature of the presently available methods for carrying out neutralization tests. For routine work with the large number of cytomegalovirus strains that have been isolated in this and other laboratories, neutralization tests are not practical when based on the cytopathic endpoint(1), the immunofluorescent focus assay(2), or the intranuclear inclusion body assay(3) methods.

A plaque assay is described here which has proven much more convenient. It has been applied successfully to the neutralization test employing sera obtained from human adults as well as immune antisera made in monkeys.

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Materials and methods. Cytomegalovirus strains. Cytomegalovirus C_{87}, isolated from a human kidney culture, is the standard strain used in this laboratory(2). Strains BC_{13} and BC_{19} were isolated from the urines of normal children and L_{38e} from the urine of a sibling of a child with leukemia. Davis and Kerr strains were kindly supplied by Dr. Thomas H. Weller.

Cell-free virus was prepared by sonic disintegration of infected cells separated by trypsinization and resuspended in tris buffer. A human lung fibroblast culture in an 8-oz bottle showing 80-90% CPE was usually used; the cells were resuspended in 1 ml of tris in a lusteroid tube and treated for 40 seconds in a Raytheon sonic oscillator. The cell-free supernatant fluid, obtained after centrifugation at 1000 *g* for 15 minutes, was used. The exact amount of virus in such a preparation was not, of course, known. Experience indicated that it was usually between 10^5 and 10^6 PFU/ml.

Tube titrations of cytomegalovirus were done in human embryonic lung fibroblasts. Ten-fold serial dilutions of virus were made in tris. Each dilution was inoculated into 10 tubes, 0.2 ml per tube. After one hour adsorption at 37°C, 1.5 ml of medium (Eagle's, 10% calf serum) was added to each tube. The cultures were incubated stationary at 37°C, fluids changed twice each week, and microscopically examined for up to 18 days. Titers were calculated by the Kärber equation.

Plaque assay. Confluent human lung fibroblast (passage level between 4 and 16) cultures in 60 mm plastic petri dishes were used. These were initiated in a medium consisting of Eagle's solution, 20% calf serum, 0.22% $NaHCO_3$, penicillin and streptomycin. At the time of use, this medium was removed and 0.3 ml of tris buffer was distributed over the surface of each culture. Serial dilutions in tris buffer of the virus being titrated were added to the petri dish cultures, 0.2 ml per dish. Adsorption of 1 hour at 37°C, shaking at 15-minute intervals, was used. An overlay medium of Eagle's solution, 2% methyl cellulose(4,5), 10% calf serum, 0.22% $NaHCO_3$, penicillin and streptomycin was added, 7 ml per plate. The plates were incubated in a CO_2 incubator at 37°C. A second 7 ml overlay was added on top of the first after 7 days. Fourteen to eighteen days after inoculation of the plates, they were removed from the incubator and cooled to about 4°C in a refrigerator to make the overlay more fluid. The overlay was then sucked off, and enough methylene blue (0.03% solution) added to each dish to cover the cell sheet. The dead cells of the cytomegalovirus lesions stained much more deeply than the surrounding living cells. The dark blue plaques could be counted with the naked eye, or with the aid of a magnifying glass or low power dissecting microscope.

Antisera were prepared in monkeys by giving each animal a series of 6 inoculations, at 10-day intervals. On each occasion about 1×10^7 human lung fibroblast cells infected with cytomegalovirus were inoculated intramuscularly and intraperitoneally, 1 ml per site. One group of the monkeys received intact cells and the other, sonically disrupted cells. The intramuscular inocula of the first and last inoculations contained Freund's adjuvant.

Neutralization tests were done by mixing serial (usually 2-fold) dilutions of serum with equal volumes of virus suspension. It was not usually possible, using virus freshly obtained by sonication of infected cells, to obtain by dilution exactly 100 PFU/0.2 ml for the neutralization test. Therefore, challenge doses of virus used in different neutralization tests varied somewhat. All dilutions were made in tris buffer. Virus-serum mixtures were incubated for 1 hour at room temperature, after which they were assayed for plaques as described above, each petri dish receiving 0.2 ml of the virus-serum mixture. Serum titers were expressed as the final serum dilution producing 80% plaque reduction.

Results. Plaque assays. Several cytomegalovirus strains were assayed on human embryo fibroblasts by the plaque technique described above. Plaques were usually enumerated at the end of 14 to 18 days' incubation

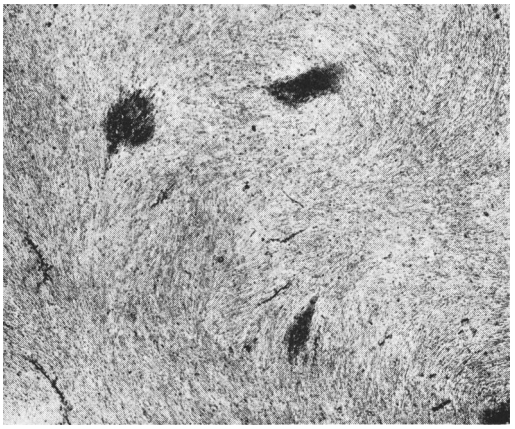

FIG. 1. Lesions of cytomegalovirus stained with methylene blue. (× 9)

at 37°C. The number of plaques did not increase after about 15 days. The staining and counting of plaques of a fast-growing (C_{87}) or a slow-growing (L_{38e}) strain at 5-day intervals between 15 and 30 days showed no change in the number of lesions (plaques), although they increased in size with the passage of time. The type of plaques obtained is shown in Fig. 1. Under the medium described, the cells continued to multiply and hence became closely packed, producing an irregular type of plaque. A macroscopic view of the plaques is shown in Fig. 2.

Plaques were also formed under an Eagle's/serum medium containing agar as the solidifying constituent. However, the agar overlay could not be readily removed for staining of underlying tissue, and was therefore abandoned in favor of the methyl cellulose overlay, which can be made more fluid by lowering the temperature.

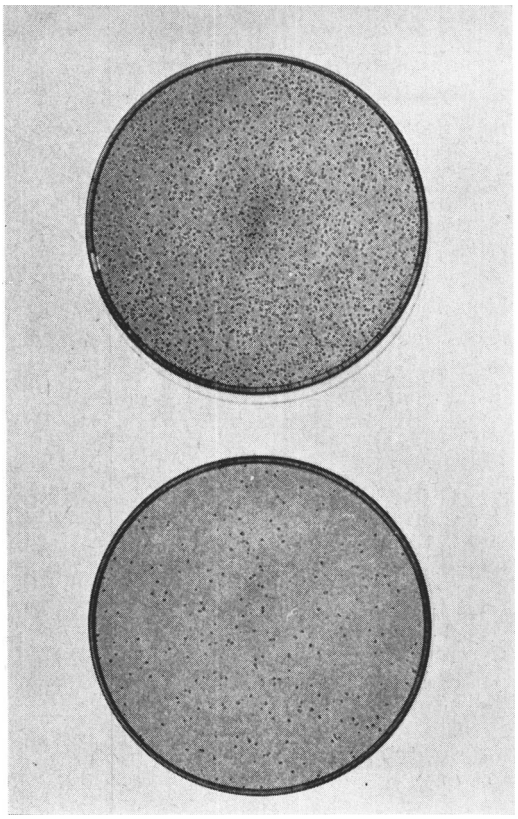

FIG. 2. Stained plaques of cytomegalovirus C_{87} (inocula of the 2 plates separated by a 10-fold dilution).

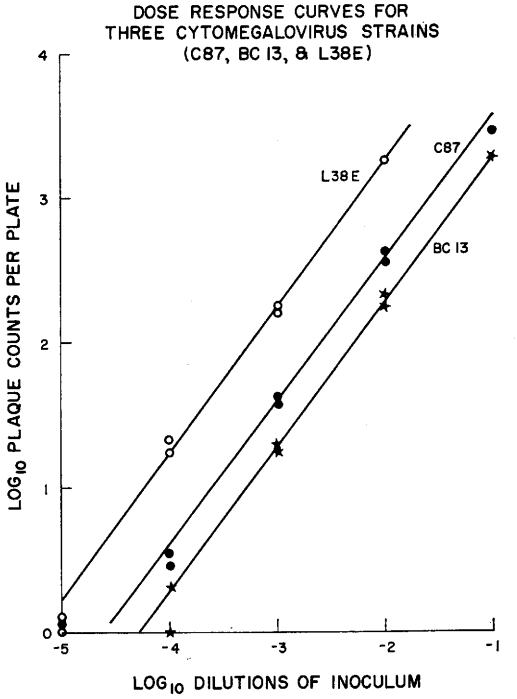

FIG. 3. Dose response curves for 3 cytomegalovirus strains (C_{87}, BC_{13}, and L_{38e}).

Dose response curves were obtained for several cytomegalovirus strains. As can be seen in Fig. 2 and 3, plaque counts varied directly with the dilution of the inoculum.

Parallel titrations of different strains by the plaque technique and by tube cultures indicated rather higher "PFU-titers" than "TCD_{50}-titers." The titers obtained by these

TABLE I. Parallel Titrations of Cytomegalovirus Strains by Plaque Test and Tube Test.*

Virus strain	Virus titers PFU/ml (log_{10})	TCD_{50}/ml (log_{10})
C_{87}	5.3	4.8
BC_{13}	5.9	4.5
L_{38e}	5.5	4.2
BC_{19}	5.6	4.8
Davis	6.7	5.2

* 18th day test readings.

TABLE II. Results of Neutralization Tests Employing Cytomegalovirus C_{87} and Human Sera.

Serum	Serum dilution*	Plaques/plate No.	Avg	% Plaque reduction	80% Serum titer*
M.J.	8	0, 0	0	100	
	16	0, 0	0	100	
	32	5, 1	3	94	64
	64	6, 8	7	85	
	128	29, 29	29	38	
	Control	55, 38	47	—†	
S.H.	10	3, 1	2	97	
	40	41, 49	45	41	16
	160	78, 92	85	0	
	Control	81, 71	77	—	
J.H.	8	80, 92	86	0	<8
	16	76, 84	80	0	
R.L.	8	89, 73	81	0	
	16	76, 91	84	0	<8
	Control	78, 84	81	—	

* Reciprocal of final serum dilution. † Not done.

2 tests with 5 strains of virus are shown in Table I. The difference in titers ranged from 0.5 log_{10} (strain C_{87}) to 1.5 log_{10} (strain Davis) values.

Neutralization tests. Seventeen individual adult human sera, as well as a pool of adult human sera,† have been tested against cytomegalovirus C_{87}. Table II shows in detail the results with 2 of the sera having the highest neutralizing capacity and 2 negative sera. The 80% plaque reduction values were used to express serum titers. Of the remaining 13 human sera not shown on the Table, one had a titer of 1:8, six had a titer of 1:10 and the rest were negative. The pool of adult human sera exhibited a titer of 1:8.

Antisera against strain C_{87} have been successfully prepared in monkeys. Results of repeated neutralization tests with strain C_{87} and 4 homologous antisera are presented in Table III. Monkeys no. 2579 and 2580 were immunized with intact infected cells, and monkeys no. 2581 and 2582 with sonically disrupted infected cells. As seen from the Table, the virus challenge dose was usually about 100 PFU of virus. However, the use of doses as low as 28 PFU or as high as 179 PFU did not seem to introduce significant variation in the results obtained.

Preinoculation sera of 2 of the animals (2580 and 2582) did exhibit some plaque reduction when tested at a dilution of 1:4, but did not show detectable neutralization when diluted 1:8. All 4 animals developed the capacity to neutralize 80% of the challenge virus at a serum dilution of 1:32 to 1:256. Monkeys 2581 (titer 1:200) and 2582 (titer 1:256) seemed to have responded to immunization better than the other 2 monkeys. These 2 animals had been immunized with cell-free virus which might have been the factor in their higher titer.

A preliminary attempt to apply the plaque reduction test to the antigenic analysis of some of the cytomegalovirus strains is presented in Table IV. Strains C_{87}, Davis and Kerr were tested with anti-C_{87} monkey serum 2582. From the results obtained, it appears that strain Kerr is slightly different from strain C_{87}. At a 1:256 dilution, 89% of the homologous virus was neutralized, while the same degree of neutralization of Kerr strain was obtained with an 8-fold higher serum concentration (90% neutralization at 1:32). Strain Davis seems to be the same as C_{87} and somewhat different from Kerr, which is also in agreement with Weller's findings(1).

Experiments are underway with sera prepared against several of the strains and taken at different periods during immunization. The neutralization tests, as described above, as well as neutralization kinetics experiments are being employed.

Discussion. Numerous human cytomegalovirus strains have been isolated in this laboratory from several sources: from urines of children with cytomegalic inclusion disease, children with acute leukemia, and normal

† Hyland pool used for the fluorescent focus assays (2) and kindly supplied by Dr. Fred Rapp.

TABLE III. Homologous Neutralization Titers with Sera Obtained from Monkeys Immunized with Cytomegalovirus C_{87}.

Monkey No.	Test No.	PFU challenge dose	Pre-inoc. serum, % plaque reduction at dilution: 4	8	80% Immune serum titer*
2579	1	100	0	—†	32
2580	1	147	54	—	32
	2	140	44	0	64
2581	1	179	—	—	200
2582	1	179	—	—	256
	2	28	68	—	256
	3	140	16	0	256

* Reciprocal of final serum dilution. † Not done.

TABLE IV. Cross-Neutralization Test with Anti-C_{87} Serum and Cytomegaloviruses C_{87}, Davis and Kerr.

Monkey serum 2582 tested at dilutions:	Virus strains C_{87} Avg PFU per plate	C_{87} % Plaque reduction	Davis Avg PFU per plate	Davis % Plaque reduction	Kerr Avg PFU per plate	Kerr % Plaque reduction
4*	0		0		0	
8	0		0		0	
16	0		0		0	100
32	0		0		7	90
64	0	100	0	100	36	50
128	1	100	16	70	62	14
256	16	91	29	46	78	0
512					76	0
Control	179		54		72	

* Reciprocal of final serum dilution.

healthy children, as well as from kidneys obtained at autopsy from babies with cytomegalic inclusion disease or congenital heart malformation. It became necessary to determine whether the strains isolated from children with the classical disease were antigenically identical or different from those isolated from children with other disease entities or from normal children in the same community. The use of tube neutralization tests employing sera of infected individuals as described by Weller(1) would have rendered this task virtually impossible. The plaque assay described here has proven useful for antibody detection in human or immune monkey sera. A plaque assay has been recently described for mouse cytomegalovirus(6), but the mouse strain produces plaques early so that the conditions for the mouse virus assay could not be applied to the slow-growing human strains.

The failure of previous workers to produce antisera against human cytomegalovirus in animals might have been due to the rather insensitive tests used for antibody detection. With the plaque reduction neutralization test, antibody production was demonstrated in monkeys immunized with whole or disrupted cytomegalovirus infected cells.

The rather low levels of neutralization obtained with both the human sera and hyperimmune monkey sera may be a true reflection of the amount of neutralizing antibody, but may, on the other hand, result from the high virus particle/PFU ratio described for this virus(7). In one of the preparations used here, the ratio of particles (including enveloped and non-enveloped forms) to PFU was about 100,000:1.‡ Such non-infectious virus could well "block" the neutralizing antibody.

Summary. A plaque technique for human

‡ Particle counts were kindly performed by Dr. K. O. Smith.

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.

The technical assistance of Mrs. Catherine Green is gratefully acknowledged.

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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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