

in Salk poliovaccine, in which the antigen exists in concentrations (0.1–1.0 $\mu\text{g}/\text{ml}$) at which adsorption might be expected to result in severe or complete elimination of potency. The known potency of the vaccine suggested that the suspending medium (medium 199) contained an adsorption preventative. Medium 199, slightly modified by omission of the pH indicator and a number of components of doubtful stability, was tested and proved fully effective. Further tests of the components of the medium at appropriate concentration revealed that the sole active component was Tween 80,¹ which is present in medium 199 at a concentration of 20 $\mu\text{g}/\text{ml}$. The complete medium prevented the adsorption of poliovirus at a dilution of 1:10 and permitted the adsorption of a trace of elutable antigen at 1:40. In comparison, the end point of effectiveness of Tween 80, alone, was approximately 0.3 $\mu\text{g}/\text{ml}$ (Table 2). In the test system used, these end points may be regarded as identical.

These results add poliovirus antigen to the substantial number of biologically active substances that have been shown to be subject to changes in potency due to adsorption to container surfaces. The phenomenon has been reported in connection with insulin (4), acid prostatic phosphatase (5), tuberculin (6–8), and fowl plague and vaccinia viruses (9). Such adsorption may be especially troublesome in virus research. It appears that only the customary use of medium 199, containing Tween 80, as a diluent for the preparation of samples, has permitted meaningful poliovirus assays and safety tests; assays performed in this laboratory using saline or phosphate buffers as diluents were shown to yield misleading results due to adsorption losses. It is possible that in many studies of viruses and virus antigens, losses due to adsorption to container surfaces may account for otherwise inexplicable assay, stability, filtration, and purification difficulties.

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Preparation of Fluorescein-Labeled Coxsackie A14 Antibodies from Immune Mouse Ascitic Fluid¹

Diagnostic techniques concerned with group A Coxsackie viruses are complicated by a limited host range. The possible application of fluorescent antibody (FA) techniques toward this end would have distinct advantages over procedures involving suckling mice, but these require adequate quantities of antibody of high titer, which preferably should be induced in the homologous host in order to avoid nonspecific reactions. Herrmann and Engle (1) immunized mice

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with viruses and then employed sarcoma 180 cells to produce ascitic fluid that contained viral antibodies. Lieberman *et al.* (2, 3) reported the production of large quantities of bacterial antibodies in mouse ascitic fluid induced by antigen-adjuvant mixtures and Kasel *et al.* (4) produced complement-fixing, neutralizing, hemadsorption-inhibiting, and hemagglutination-inhibiting viral antibodies in mouse ascitic fluid. Recently Levinthal *et al.* (5) reported the use of fluorescein-labeled immune mouse ascitic fluid globulin in FA studies of polyoma infection in mice. The present report demonstrates that immune mouse ascitic fluid can also be a convenient source of antibody for FA studies of a Coxsackie virus.

Twenty adult male Webster mice were

TABLE
COMPARISON OF STAINING OF LLC-MK₂ CELLS
INFECTED WITH COXSACKIE A14 AND NORMAL
UNINFECTED LLC-MK₂ CELLS

Dilution of conjugate	Staining reaction ^a	
	Infected cells	Normal cells
Undiluted	4+	2+
1:2	4+	0
1:4	4+	0
1:8	3+	0
1:16	3+	0
1:32	2+	0
1:64	1+	0

^a Reactions were graded 4+, 3+, 2+, 1+, and 0 according to the brilliance of the observed fluorescence.

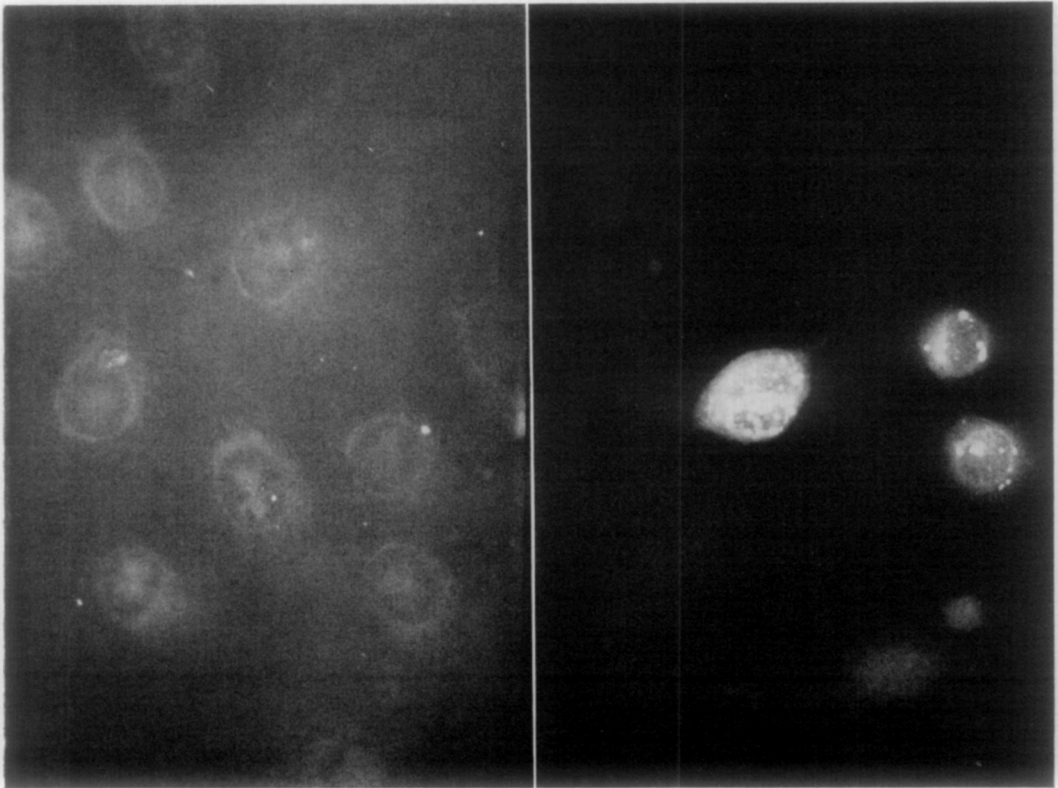


FIG. 1. LLC-MK₂ cells stained with fluorescein-conjugated globulin derived from immune mouse ascitic fluid. Magnification: $\times 400$. A. Normal cells. Note weak perinuclear (blue) autofluorescence. B. Cells 12 hours after infection with Coxsackie A14. Note particulate distribution of fluorescence. One heavily infected cell appears to have fluorescent aggregates which are over, not in, the nucleus.

immunized and ascites was induced by two series of virus-adjuvant injections given according to Lieberman's recommended schedule (3). The inoculum ($10^{7.2}$ LD₅₀ per milliliter) was prepared by emulsifying equal parts of either complete or incomplete Freund's adjuvant and a 20% suspension of Coxsackie A14 virus prepared from infected infant mouse muscle. The Coxsackie A14 virus, representative strain No. 52113, was obtained from the American Type Culture Collection and had undergone six transfers in infant mice since its isolation. Incomplete Freund's adjuvant consisted of 1 part Arla-cel A and 9 parts Drakeol 6 VR whereas the complete adjuvant contained *M. butyricum* in addition to the emulsifying agent and oil. Because the first series of injections utilizing incomplete adjuvant failed to induce ascites, a second series was given with complete Freund's adjuvant. Half of the mice developed ascites 7-14 days after the third injection and yielded 1-10 ml per tap. Ascitic fluid was collected once a week by paracentesis, pooled, centrifuged, and stored at -20°C. Over a 2-month period approximately 150 ml of fluid was harvested from ten mice.

The globulin fraction of 10 ml of ascitic fluid was isolated by half saturating the fluid with ammonium sulfate. The precipitated globulin was dissolved in distilled water and dialyzed until free of sulfate. The globulin solution was conjugated with fluorescein isothiocyanate as described by Marshall *et al.* (6) except that a dye:protein ratio of 1:80 (0.0125 mg dye/1 mg protein) was used as recommended by Griffin *et al.* (7). The conjugate was fractionated by the cellulose anion exchange method of Riggs *et al.* (8).

A 15-ml aliquot obtained by elution with 0.125 M NaCl in 0.0175 M phosphate buffer, pH 6.3, was characterized in terms of fluorescein:protein ratio (F:P), electrophoretic mobility, and staining capacity. The F:P ratio was determined by the method of Goldwasser and Shepard (9), and electrophoresis was done on cellulose acetate paper strips. This globulin conjugate had a F:P ratio of 14.1×10^{-3} and the same electrophoretic mobility as gamma globulin. The

staining capacity was determined with normal and infected coverslip preparations of the continuous monkey kidney line, LLC-MK₂ (10), to which this virus had been recently adapted in this laboratory. Normal and 12-hour infected coverslip cultures were washed, fixed in acetone, dried, stained with twofold dilutions of the conjugate for 1 hour at 37° in a moist chamber, washed, mounted in Elvanol (11), and examined with a Zeiss GF 425 microscope. The light source was an Osram HBO 200 lamp and the filter system was a UG5 exciter filter in combination with a GG4 and an OG4 barrier filters. The conjugate titration results presented in Table 1 indicate that this preparation may be diluted at least 1:4 for use. Tissue powder absorption for the removal of nonspecific staining was unnecessary when the conjugate was diluted 1:2 or more. The staining reaction of the 1:4 dilution with normal and infected cells is shown in Fig. 1.

Since 10 ml of the ascitic fluid yielded a 15-ml conjugate fraction which may be diluted 1:4, the 150 ml of ascitic fluid harvested from 20 inoculated mice should provide approximately 900 ml of the diluted conjugate. This simple technique provides relatively large volumes of fluorescein-labeled globulin that is free of nonspecific staining reactions.

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Studies on Japanese B Encephalitis Virus

IV. Plaque Assay of Japanese B Encephalitis Virus in a Stable Line of Porcine Kidney Cells¹

Japanese B encephalitis (JBE) virus has been shown to produce plaques under an agar overlay medium in chick embryo cell (1-3), duck kidney cell (4), and hamster kidney cell (5) cultures, but not so far in any stable cell lines. The destruction of porcine kidney stable (PS) cells following inoculation by JBE virus (6) suggested their use for the plaque assay.

The bottle cultures of PS cells were initiated with approximately 15×10^4 cells per milliliter, then incubated for 5 days at 37°C with one change of medium. The growth medium consisted of 10% calf serum and 0.5% lactalbumin hydrolyzate in Earle's balanced salt solution.

When cell monolayers were established the medium was removed and the cells were washed twice with Earle's balanced salt solution. Cultures were inoculated with 0.2 ml of appropriate dilutions of the virus [the Mukai strain (7) serially passed in PS cells] and placed at 37° for 2 hours for adsorption. As shown in Fig. 1, maximal adsorption was obtained in 120 minutes' incubation at 37°. Unadsorbed virus fails to initiate plaques after the addition of agar.

The cultures were overlaid with a me-

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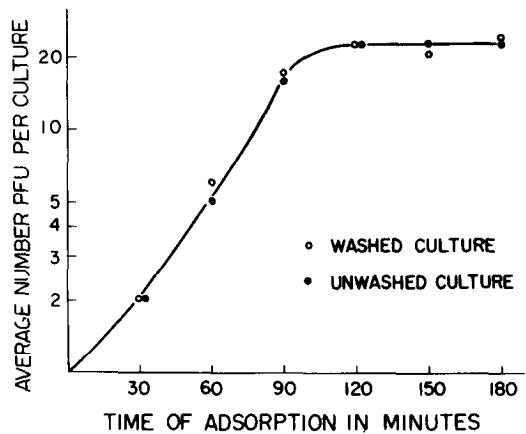


FIG. 1. Relationship between plaque count and time of adsorption at 37°C.

dium made by mixing equal parts of 2.0% agar in distilled water and the following constituents: Earle's solution (10 times concentrated without either phenol red or NaHCO_3), 18 ml; sterile distilled water, 38.8 ml; bovine albumin (5.6% stock solution), 5.4 ml; NaHCO_3 (7.5% stock solution), 8.0 ml; 5% lactalbumin hydrolyzate in 0.001 N NaOH, 18.0 ml; yeast extract (10% stock solution), 1.8 ml; antibiotics (1 ml of penicillin, containing 40,000 units/ml; and 0.2 ml of streptomycin containing 200 mg/ml). After solidification the bottles were turned over and placed at 37°.

Plaques increased in number only until 5th or 6th day. On the 7th day, neutral red (1:2000 in 0.5 ml of phosphate buffer solution) was added and the bottles were kept in the dark at 25° for 2 days.

The plaques produced were clear and approximately 4-6 mm in diameter. A linear relationship was observed between the number of plaques and the virus concentration, a result indicating that each plaque was produced by a single virus particle (8). Plaque formation was effectively prevented by specific rabbit antiviral antiserum. The plaque method and the tube method of titration gave similar end points.

For cloning, neutral red (1:36,000 per milliliter final concentration) was incorporated into the first overlay medium in which case plaques were scarcely visible.