It is possible that the syndrome we have observed is due to a severe deficiency of an unidentified essential nutrient present in soybean oil. However, such deficiency symptoms have not been obtained by feeding purified fat-deficient diets to chicks, even in prolonged experiments(11). Therefore, if we are dealing with a deficiency, high lipid diets such as we have used may induce the paralysis by increasing the requirement for the missing nutrient. At present, however, we cannot exclude the possibility of a paralysis-inducing toxic factor present in the reconstituted triglycerides.

Summary. Feeding mixed triglycerides synthesized from soybean oil fatty acids as the sole non-protein energy source in a simplified diet produced severe growth retardation, incoordination, and a crippling leg paralysis in young chicks. The possibility that these effects may be due to the lack of an unidentified essential nutrient is discussed.

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1. Sunde, M. L., Poultry Sci., 1956, v35, 350.
2. Donaldson, W. E., Combs, G. F., Romoser, G. L., Supplee, W. C., ibid, 1957, v36, 807.
3. Rand, N. T., Scott, H. M., Kummerow, F. A., ibid., 1958, v37, 1075.
4. Renner, R., Elcombe, A. M., Fed. Proc., 1963, v22, 490.
5. Brambila, S., Hill, F. W., ibid., 1964, v23, 550.
6. Dam, R., Leach, R. M., Jr., Nelson, T. S., Norris, L. C., Hill, F. W., J. Nutrition, 1959, v68, 615.
7. Carew, L. B., Jr., Renner, R., Hill, F. W., Poultry Sci., 1959, v38, 1183.
8. Carver, D. S., Johnson, E. L., ibid., 1953, v32, 701.
9. ———, ibid., 1954, v33, 543.
10. Carew, L. B., Jr., Hopkins, D. T., Nesheim, M. C., J. Nutrition, 1964, v83, 300.
11. Marion, J. E., Edwards, H. M., Jr., ibid., 1963, v79, 53.

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Studies on Experimental Infection of Weanling Mice with Reoviruses.* (29989)

THOMAS R. HOBBS AND CARMINE C. MASCOLI† (Introduced by M. C. Dodd)
Department of Microbiology, West Virginia University School of Medicine, Morgantown

The role of reoviruses in human and animal disease has been studied by many workers, but is not yet well defined(1). There have been several well documented cases of enteric disease in children where no other agent was isolated, the most notable of which involved 3 separate outbreaks of respiratory illness and enteritis in a Washington, D. C. nursery(1). Lerner *et al* reported evidence of reovirus type 2 infection in 7 children, 6 of which had exanthems(2).

Type 2 reovirus has been isolated from patients with steatorrheic diarrhea and from apparently healthy school children(3).

These viruses have been isolated from a variety of animal species including cattle, dogs, monkeys, chimpanzees, wild and laboratory mice and man(4,5,6,7), in some cases with overt clinical illness(1,5,8,9).

The purpose of this study was to determine whether or not respiratory illness could be established in mice *via* the intranasal route of administration, to study the clinical, gross and histological pathology and finally to attempt to demonstrate the virus by fluorescent antibody (FA) procedures, correlating such procedures with conventional virus detection methods.

Experimental animals. Young rabbits and adult roosters were used for reovirus antibody production. Weanling White Swiss mice (3-4 weeks old) were used as the test animals.

Virus. The viruses employed in this study were obtained from the American Type Culture Collection (ATCC). They were reovirus type 1 Lang Strain(13), passed 26 times in primary monkey kidney (PMK) cells; reo-

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† Present address: Merck Institute for Therapeutic Research, West Point, Penna.

virus type 2 Strain D-5 Jones(14), passed 16 times in PMK cells; reovirus type 3 Abney Strain(15), passed 17 times in PMK cells. They were stored in PMK maintenance medium at —70°C.

Preparation of antireovirus fluorescein conjugated serum. Antisera were obtained from roosters which had received a series of 4 weekly injections of (1:1) reovirus type 1-adjuvant mixture (Algivant—Colab Laboratories Inc., Chicago Heights, Ill.). Antisera to all 3 types of reovirus were also produced in young rabbits with a series of 5 weekly virus-adjuvant injections. The sera used had high hemagglutination inhibition titers (1:128 or more). All sera were absorbed with mouse liver powder. The specificity of the antisera was checked by cross hemagglutination inhibition tests on all the reoviruses and were further checked against standard antisera obtained from the Communicable Disease Center, Atlanta, Georgia. The globulins were fractionated and conjugated with crystalline fluorescein by the Riggs method(10). Protein content of the precipitate was determined by the standard biuret method on a B. & L. "Spectronic 20" spectrophotometer and a ratio of 1 to 40 dye to protein was used in conjugation. Fluorescein conjugated anti-chicken and anti-rabbit globulins for the indirect fluorescent antibody technique were purchased from Micobiological Associates, Bethesda, Md.

Infection of animals. Mice were lightly anaesthetized with ether and 2 to 3 drops of a high titered ($TCID_{50}$) virus suspension were placed directly in front of the external nares. The animals were then placed in cages and later sacrificed at varying intervals.

Fluorescent antibody techniques on frozen sections of mouse lungs. Lungs and trachea were removed en-bloc, sections were cut for subsequent fluorescent antibody (FA) studies and other portions were ground for virus isolation. Frozen lung sections were prepared by dissecting the lungs and placing each section in a 12½ mm × 1½ mm Lusteroid tube containing melted and cooled gelatin. The material was frozen and the tubes were split longitudinally to remove the gelatin block. Six to eight micron sections were cut on a Cryostat freezing microtome and placed on alcohol cleaned slides containing a thin film of egg albumin. The sections were then stained by the direct and/or indirect FA method. Excess dye was removed by 2 phosphate buffered saline (PBS) washings, followed by a distilled water rinse to remove the saline. The slides were mounted in buffered glycerol, pH 7.0, and examined under ultraviolet light (Leitz Ortholux Microscope, HBO 200 light source, 2 mm UGI exciter filter and Euphos Garner barrier filter, E. Leitz, Inc., New York).

Hemagglutination-fluorescent antibody modification. Throat washings were taken from the mice immediately prior to sacrificing the animals. The washings were collected by introducing 0.25-0.5 ml sterile Hanks' balanced salt solution into the trachea with a capillary pipette, flushing the respiratory passages thoroughly and recollecting as much of the washing as possible. These washings were then used in a hemagglutination-fluorescent antibody modification as first described by Baratawidjaja with influenza(11). A human type O erythrocyte suspension was mixed with an equal volume of throat washings and allowed to stand for one hour. A smear of the suspension was then placed on alcohol cleaned slides, dried and fixed in acetone for 10 minutes. The smears were then stained by the conventional indirect fluorescent antibody procedure, mounted in buffered glycerol and examined as above.

Experimental results. Ten healthy weanling mice were inoculated intranasally with reovirus type 1 as described earlier. Three of the 10 developed moderate to severe respiratory symptoms within 3 days. Several control mice inoculated with media alone displayed no symptoms. On the fifth day all 3 symptomatic mice died and on the seventh day the remaining 7 were sacrificed.

Supernates of homogenates of portions of the lungs of all 10 animals were inoculated into Leighton tube PMK cultures and the cultures observed for cytopathology. After 7 days' incubation, fluids were removed from the culture tubes and titered for hemagglutinins. Hemagglutination inhibition tests were run on the isolates using standard type-specific antisera. The hemagglutination and hemagglutination inhibition tests used here

TABLE I. Correlation of Symptoms and Isolation of Virus from Mouse Lung Homogenates.

Animal No.	Symptoms evident	Deaths	C.P.E. in P.M.K.	Hemagglutination activity	Hemagglutination inhibition by reo type 1 antisera diluted 1:256
1	No	No	....	....	
2	"	"	....	....	
3	Yes	Yes	....	1:32	Complete inhibition
4	No	No	....	....	
5	Yes	Yes	+	1:32	" "
6	No	No	....	....	
7	"	"	+	....	
8	"	"	....	....	
9	Yes	Yes	+	1:64	" "
10	No	No	+	1:16	" "

were done according to the method of Kalter (16). The results of these procedures are shown in Table I. Cytopathic agents were isolated from the 3 animals which died and from one asymptomatic mouse. All 4 isolates showed hemagglutinin activity which was specifically inhibited by reovirus 1 antisera.

Coverslips were withdrawn from those tubes showing cytopathology and stained by the direct fluorescent antibody method. Results are summarized in Table II and can be seen to correlate fairly well with those of conventional procedures of isolation and identification. Fig. 1 shows a comparison of control and virus infected cultures by this method.

In the second of the mouse experiments, direct demonstration of the virus in tissue by fluorescent antibody techniques was attempted. Virus infection was accomplished as before, but in this experiment all 3 reovirus types were used with 10 animals per type. Twenty-four hours after inoculation and at daily intervals thereafter 2 mice infected with each reovirus type were sacrificed. Lungs were processed, as before, for virus recovery and frozen sections were made for FA studies. Table III shows the correlation between time of recovery of reovirus and positive FA results, as well as confirmation of virus identity by hemagglutination and hemagglutination inhibition tests. In all instances, the results of FA procedures correlated precisely with those of conventional procedures. Controls in the FA procedure consisted of:

1. Infected lungs treated with normal serum.

2. Normal lungs treated with immune serum.

The fluorescence seen in infected lungs was well located in bronchial and alveolar lining cells (later in infection) and was brilliant in character. Control slides of normal lungs

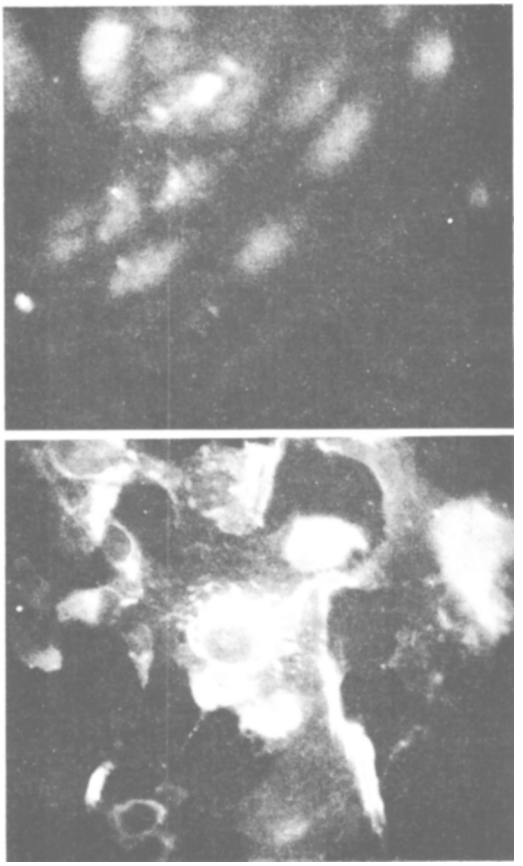

FIG. 1. Immunofluorescence of reovirus infected primary monkey kidney cell cultures. Uninfected control is above; infected below.

TABLE II. FA Results on Reovirus Infected PMK Cells.

PMK culture inoculated with mouse lung homogenate	Labeled rabbit antisera	Results
#3*	Anti reo 1	Excellent fluorescence
#3	Normal serum	Poor to no "
#5	Anti reo 1	Excellent "
#5	Normal serum	Moderate " †
#9	Anti reo 1	Excellent "
#9	Normal serum	Poor to no "
#10	Anti reo 1	Weakly fluorescent
#10	Normal serum	" "

* Refers to animal No. from Table I.

† This control slide showed a significant difference in degree of fluorescence from its positive counterpart, but displayed considerably more fluorescence than the other control slides.

treated with immune sera showed an overall dark bluish appearance which could not be mistaken for true fluorescence. This is shown in Fig. 2. Grossly infected lungs exhibited areas of consolidation and hepatization. Exudate was moderate to heavy and in stained sections, fluoresced brightly. Evidence of gross pathology was seen as early as 48 hours after infection and before clinical symptoms.

In the third experiment, only reovirus type 1 (Lang Strain) was used. In addition to the reisolation of the virus from mouse lung homogenates, and direct fluorescence of virus in lung tissue, throat washings were taken for use in the modified FA-red cell technique (11).

Table IV shows the composite results obtained by the various identification procedures on lung specimens and throat washings.

Antisera were adsorbed with human type A and B, Rh positive erythrocytes before

TABLE III. Comparison of Virus Identification by Means of CPE and HA-HAI Tests in PMK Cultures with FA Tests on Infected Mouse Lung.

	24 hr post infection		48 hr post infection		72 hr post infection	
Reovirus	Specimen 1	Specimen 2	Specimen 1	Specimen 2	Specimen 1	Specimen 2
Type 1						
CPE in PMK cells	+	+	+	+	+	+
Hemagglutination from infected PMK supernates	+	—	+	+	+	+
Hemagglutination inhibition by specific antisera	+	—	+	+	+	+
Fluorescent antibody on mouse lung	+	—	+	+	+	+
Type 2						
CPE in PMK cells	+	+	+	+	+	—
Hemagglutination from infected PMK supernates	+	—	+	+	+	—
Hemagglutination inhibition by specific antisera	+	—	+	+	+	—
Fluorescent antibody on mouse lung	+	—	+	+	+	—
Type 3						
CPE in PMK cells	+	—	—	—	+	—
Hemagglutination from infected PMK supernates	+	—	—	—	+	—
Hemagglutination inhibition by specific antisera	+	—	—	—	+	—
Fluorescent antibody on mouse lung	+	—	—	—	+	—

TABLE IV. Correlation of Virus Detection Procedures.

	Specimen No.									
	1	2	3	4	5	6	7	8	9	10
24 hr:										
Throat washing										
Direct HA with throat washings	—	—	—	—	—	—	—	—	—	—
FA	—	—	—	—	—	—	—	—	—	—
Virus isolation	+	+	+	+	—	—	—	—	—	—
Lung sections	+	+	+	+	—	—	+	—	—	—
48 hr:										
Throat washings										
HA	+	+	+	+	—	+	—	—	+	—
FA	+	+	+	+	—	+	—	+	+	+
Virus isolation	+	+	+	+	+	+	—	+	+	+
Lung sections	+	+	ND	+	+	ND	+	+	+	+
72 hr:										
Throat washings										
HA	+	+	+	+	+	ND	ND	ND	ND	ND
FA	+	+	+	+	+	ND	ND	ND	ND	ND
Virus isolation	+	+	+	+	+	ND	ND	ND	ND	ND
Lung sections	+	+	+	+	+	ND	ND	ND	ND	ND

ND = Not done.

use. The FA-red cell modification procedure proved to be a rapid, sensitive and highly specific means of reovirus identification. Control slides showed an almost complete absence of color, whereas on positive slides, the red cells fluoresced a brilliant yellow green. These results are shown in Fig. 3. Correlation of the results of all procedures is shown in Table IV. Modified RBC-FA and hemagglutination tests were negative after 24 hours, but virus was isolated and lung FA sections were positive. In the 48-hour specimens, there was good correlation between the results of virus isolation, lung section FA, hemagglutination and the FA-red cell modification and by 72 hours, the results of the various procedures utilized to demonstrate the virus were in better agreement.

Discussion. Certain workers have established reovirus infection in cattle by intranasal instillation(4); however, no clinical evidence of disease was noted. Other investigators have established clinical illness in mice by intraperitoneal injection(9,12). The results of conventional virus isolation and identification procedures, fluorescent antibody techniques and the pathology observed in this study show conclusively that a respiratory infection was established in mice.

Virus was also shown to be present and actively propagating in animals devoid of clinical illness. Such findings are characteristic of enteroviruses.

Areas of pathology in the lungs correspond to areas of detectable fluorescence throughout the progress of the infection.

Exudate which was expressible from the consolidated areas of the lungs was found to consist largely of white blood cells, tissue debris and mucus. This material stained nonspecifically with fluorescein dye. The FA-red cell and HA procedures indicate that virus is released from respiratory epithelial cells approximately 48 hours post infection.

The FA-red cell modification test had the distinct advantage of rapidity, ease of performance and high specificity. In addition, this procedure was more sensitive than other FA or hemagglutination procedures.

Summary. A mild to severe respiratory infection was established in mice with reoviruses. In certain animals, the virus was detectable in the respiratory tissues although no evidence of illness was observed. In other animals, respiratory illness was evident early, became overt terminating with the death of

the animals. Fluorescent antibody techniques both direct and indirect, correlated with other methods of virus detection. The FA-red cell technique proved to be a rapid, sensitive and highly specific method of reovirus

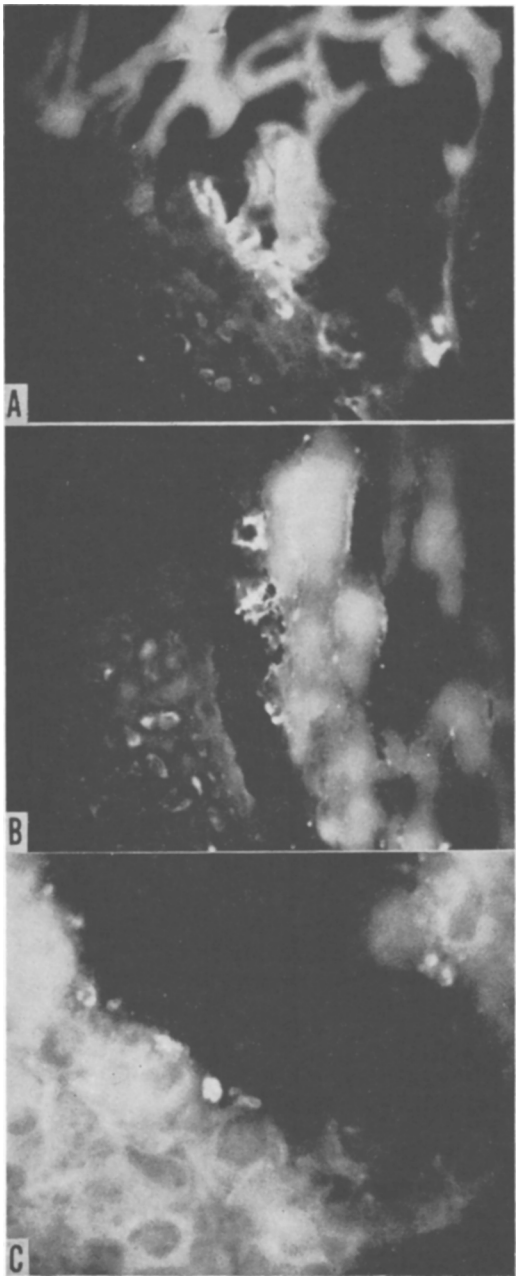

FIG. 2. A & B: Immunofluorescence of reovirus infected mouse lungs (125×). Lungs from mice infected with reovirus type 1 and stained with reovirus type 1 fluorescein conjugated antisera. C: Immunofluorescence of reovirus infected mouse lungs (125×).

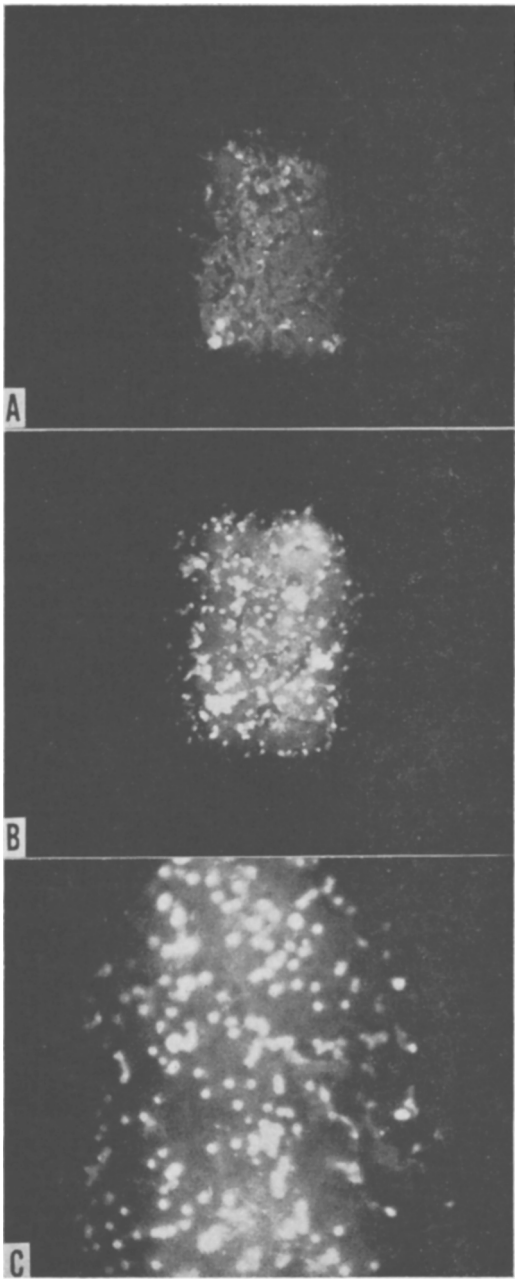

FIG. 3. A & B: Modified FA-hemagglutination technique. Erythrocytes untreated with throat washings are shown at the top. Cells treated with throat washings are seen in the lower photograph. C: This photograph is a higher magnification of erythrocytes treated with throat washings and processed by the FA-hemagglutination method (270×).

detection and would seem to be applicable for detection of other hemagglutinating viruses.

1. Sabin, A. B., Science, 1959, v130, 1387.
2. Lerner, A., Cherry, J. A., Klein, J. O., J. Clin. Invest., 1962, v41, 1379.
3. ———, New Eng. J. Med., 1962, v267, 947.
4. Rosen, L., Abinanti, F. R., Am. J. Hyg., 1960, v71, 250.
5. Lou, T. Y., Wenner, A., ibid., 1963, v27, 293.
6. Hartley, J. W., Rowe, W. P., Huebner, R. J., Proc. Soc. Exp. Biol. and Med., 1961, v108, 390.
7. Rosen, L., Ann. N. Y. Acad. Sci., 1962, v101, 461.
8. Stanley, N. F., Med. J. Australia, 1961, v2, 815.
9. Stanley, N. F., Dorman, D. C., Ponsford, J., Australian J. Exp. Biol. and Med. Sci., 1953, v31, 147.
10. Riggs, J. L., Seiwald, R. J., Burckhalter, J. H., Downs, C. M., Metcalf, T. G., Am. J. Path., 1958, v34, 1081.
11. Baratawidjaja, R. K., Hewson, A., Labzoffsky, N., Can. J. Microbiol., 1963, v99, 563.
12. Stanley, N. F., Nature (London), 1961, v189, 687.
13. Ramos-Alvarez, M., Sabin, A. B., Proc. Soc. Exp. Biol. and Med., 1954, v87, 655.
14. Ramos-Alvarez, M., Ann. N. Y. Acad. Sci., 1957, v67, 326.
15. Rosen, L., Hovis, J. F., Mastrota, F. N., Huebner, R. J., Bell, J. A., Am. J. Hyg., 1960, v71, 258.
16. Kalter, S. S., Procedures for Routine Laboratory Diagnosis of Viral and Rickettsial Diseases, Burgess Publishing Co., Minneapolis, Minn., 1963.

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