The Common Cold: Titration of MR-1 Virus[1] in Embryonated Eggs

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Research with viruses producing diseases only in human hosts has been handicapped by the lack of a laboratory method for demonstrating the presence and amount of virus. The onset of typical disease in human volunteers following experimental inoculation often has been the only manner of detecting the presence of virus (*9*). Studies at the National Institutes of Health have been in progress to devise methods other than human-volunteer inoculation for measuring the amounts of common cold viruses. Thus far, attempts to demonstrate MR-1 virus by the production of disease in available laboratory animals, by hemagglutination (*2, 9*), by inhibition of hemagglutination by other viruses, by serological tests, and by interference with the multiplication of other respiratory viruses (*2*), have not been successful. The investigation of infected and noninfected allantoic fluids from chick embryos has, however, revealed certain chemical differences.

The only two components common to all viruses thus far isolated are protein and nucleic acid (*5*). Preliminary study of absorption at 275 mμ in the Beckman spectrophotometer showed high protein peaks in dialyzed MR-1–infected allantoic fluids from 10- to 16-day-old fertile eggs.[3] Although dialyzed normal allantoic fluids had very little absorption until the 13th day, thereafter the values varied so much in individual eggs that some normal fluids exhibited higher protein peaks than the infected fluids. It was evident, therefore, that this test did not afford a specific quantitative index of infection.

After numerous trials with dialyzed fluids seeking the determination of nucleic acid components by carbazole (*4*), diphenylamine (*7*), cysteine-sulfuric acid (*8*), and tryptophane-perchloric acid procedures (*3*), the only method found to differentiate infected from normal fluids was a modified tryptophane-perchloric acid (TPA) procedure. As the data of Table 1 indicate, allantoic fluid pools containing virus (demonstrated by the production of colds in human volunteers) had positive values, usually above .010, with this procedure, whereas pools often from eggs receiving the same inoculum but not infectious for human volunteers gave 0 or minus results. Minus TPA values occur presumably because the allantoic fluids contain substances which inhibit formation of color by the reagents. Because of the variabilities and technical difficulties involved in dialysis, it was desirable to obtain a more satisfactory method of freeing fluids from interfering substances.

TABLE 1

COMPARISON OF TPA VALUES AND COLDS PRODUCED IN HUMAN VOLUNTEERS BY ALLANTOIC FLUID FROM MR-1 INOCULATED EGGS

MR-1 virus-inoculated fluid	TPA value*	Symptoms	Human volunteers Incidence (#colds/#inoc.)	Severity
EP7 (7/7/47)	0.049	Colds	21/21	4+
EP7 (8/28/47)	0.034	"	14/16	3+
EP7 (12/2/47) Ultracentrifuged concentrate 5×	0.140	"	14/14	3+
EP8 (1/29/48)	0.032	"	6/8	3+
EP9 (3/17/48) Pool A	0.016	"	7/8	3+
EP9 (3/17/48) Pool B	0.058	"	5/7	3+
EP9 (6/24/48) Individual fluids with TPA over 0.244 pooled	0.425	"	8/8	4+
EP9 (6/24/48) Individual fluids with TPA about 0.010 pooled	0.006	"	3/8	1+
EP9 (2/12/48)	−0.031	none	0/7	
EP9 (3/2/48) Pool A	−0.005	"	0/8	
EP9 (3/2/48) Pool X	0.002	"	0/8	
EP10 (4/16/48) Ultracentrifuged concentrate 10×	-0.004	"	0/8	

*Determined on dialyzed fluids by the color procedure described in the text.

An adequate procedure was developed. Allantoic fluids from 13-[4] or 16-day-old embryonated eggs are harvested after chilling at ± 4° C overnight. Following the inoculation of thioglycolate medium for sterility tests, the fluids are frozen and stored at − 50° C. Each sample of fluid is thawed at the time of test, and duplicate 1-ml portions at 1° C are mixed with 2 ml of cold (− 50° C) methyl alcohol. After storage overnight at 1° C, the fluid is centrifuged (2,500 r.p.m.) for 20 min at 1° C. The precipitate is resuspended in 2 ml of distilled water and then mixed with 2 ml of M/5 calcium acetate solution.[5] Subsequent centrifugation (3,000 r.p.m.) for 20 min at 1° C sediments the precipitate used for the determination of the TPA value.

[1] MR-1 virus is the name given to the agent isolated and described by Topping and Atlas (*9*).

[2] We wish to acknowledge the valiant cooperation of over 400 adult male volunteers who have participated in these studies and the generous assistance of the Department of Corrections, District of Columbia, in making its facilities available for the human studies of the common cold.

[3] All dialyzed fluids reported were dialyzed against distilled water 4 days at +4° C with a minimum of 4 water changes/day.

[4] Fluids from 13-day-old eggs are preferred because they contain fewer interfering substances.

[5] The use of calcium acetate solution was suggested by Dr. Frederick Bell from unpublished data in experiments conducted by F. Bell, J. Wright, and K. Habel.

To the first (fluid control) of the two tubes from each sample of precipitated allantoic fluid is added 1.8 ml of distilled water plus 1.8 ml of 60% perchloric acid. To the second tube of each set is added 1.0 ml of distilled water, 0.8 ml of 0.375% *dl*-tryptophane, and 1.8 ml of 60% perchloric acid. After thorough mixing, the tubes are immersed in a boiling-water bath (95°–98° C) for 2 hrs. The tubes are cooled in tap water, and the optical density of each solution is determined in the Beckman spectrophotometer at 500-mμ wave length, using a 1-cm cell. For controls, two tubes containing distilled water in place of allantoic fluid (reagent controls)[6] and two tubes each containing 0.5 mg of desoxyribonucleic acid (DNA standards) are subjected to this procedure in each test. The TPA value is calculated by subtracting the reagent control reading plus the fluid control reading from the test reading.

By the use of the TPA method, infectious virus can be titrated in 6-day-old embryonated eggs. These are inoculated with each dilution of virus-containing material, incubated at 36° C, and harvested 7 days later. The TPA value of each allantoic fluid indicates whether or not the fluid contains virus. The infectious titer of a fluid, ID_{50}, is calculated (*6*) and indicates the dilution of virus which produces positive TPA values in half the inoculated eggs.

That positive TPA values demonstrate multiplication of the infectious agent is confirmed by the comparison of TPA values, titration in eggs, and colds produced in human volunteers by MR-1 virus-inoculated allantoic fluids (see Tables 2, 3). In the experiments shown in Table 3, individual fluids selected for negative TPA values from eggs inoculated with *undiluted* seed virus did not produce symptoms in human volunteers and showed no growth by titration in individual eggs. However, fluids with positive TPA values, even from eggs inoculated with dilutions up to 10^{-7} of the *same seed*, produced severe colds in human volunteers and titered in eggs up to $10^{-8.5}$.

TABLE 2

MR-1 virus-inoculated fluid					
Pool	TPA value	Titer in eggs	Human volunteers		
			Symptoms	Incidence	Severity
EP9 (7/7/48)	−.033	Untested	None	0/8	
EP8 (3/26/48)	−.030	"	"	0/8	
EP9 (4/6/48)	−.017	"	"	0/7	
EP11 (5/28/48)	.042	$>10^{-2}$	Colds	7/8	3+
EP11 (7/2/48)	.043	10^{-5}	Untested		
EP10 (8/30/48)	.205	Untested	Colds	8/8	4+

TABLE 3

Dilution of egg inoculation	MR-1 virus-inoculated fluid					
	Tested fluids	TPA Value	Titer in eggs	Human volunteers		
				Symptoms	Incidence	Severity
*10^{-2}	EP12C #27	−.012	Untested	None	0/4	
*Undil. to 10^{-1}	EP12C #8 and #20	.086	$>10^{-7}$	Untested		
†10^{-7}	EP13 #92 and #98	.055	Untested	Colds	2/8	2+
‡Undil.	EP12A #9 and #5	−.003	$<10^{-3}$	None	0/4	
‡Undil.	EP12A #1	−.005		"	0/4	
‡Undil.	EP12A #8	−.005		"	0/4	
‡10^{-2}	EP12A Pool Y	.060	$10^{-4.5}$	Colds	5/8	2+
‡1 : 2	EP12B Pool L	−.024	$<10^{-2}$	Untested		
‡Undil. to 10^{-2}	EP12B Pool H	.156	$10^{-8.5}$	"		
‡Undil. to 10^{-2}	EP12B Pool M	.011	$10^{-6.5}$	"		
§10^{-7}	EP13 #52 and #63	.069	Untested	Colds	8/8	4+

* Inoculum was EP11 (7/2/48).
† Inoculum was EP12C #8 and #20.
‡ Inoculum was EP11 (5/28/48) pool.
§ Inoculum was EP12B, Pool M.

Whether the substance responsible for the TPA value is a virus constituent or some by-product of infection in the fluid is being investigated. Since substances other than desoxyribonucleic acid (DNA) give color with the modified TPA procedure, positive values with this method do not necessarily represent DNA. Some evidence suggests that a virus constituent may be measured by the TPA value. The reacting substance may be present, however, before as well as after virus capable of multiplication can be demonstrated. Accordingly, for measurement of the actual MR-1 infectious capacity, titration in eggs utilizing the TPA procedure remains necessary.

[6] For reasons undetermined, the reagent controls may vary considerably from test to test.

Study of the manifold properties of MR-1 virus is facilitated by the TPA procedure. Under investigation is the reisolation of this virus from nasal washings (*1*) of volunteers with colds produced by egg passage MR-1 or by human-to-human transfer of the disease. With the TPA procedure, it may be possible to isolate and study agents other than MR-1 virus, without the necessity of laborious human volunteer studies.

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Electron Microfossils

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There are a number of areas, particularly in the Gulf Coast, where oil wells are drilled through thick sections of shale having few or no individual and unique characteristics as far as petrology and the usual paleontological markers are concerned. It is therefore often difficult to locate the well in the geologic section, except very roughly, and this complicates correlations from one well to another. Electron microscopy was one of the several techniques investigated in this laboratory in attempts to differentiate between portions of these shales. It was thought that it might be possible to observe differences in the microstructure of the shales, or that very minute microfossils might exist which would be sufficiently distinct to serve as markers.

Samples of cuttings were collected at the shale shakers of several wells, principally in the Chocolate Bayou field, approximately 30 miles south of Houston, Texas. Most of these samples were from the Frio, a nonmarine Oligocene formation. This is a gray-green, fine-grained, micaceous shale containing a little bentonite. In one well, the Phillips Petroleum Company No. 1 Robnett, this formation was encountered at 8,920′ and extends down past the last sample taken at 12,583′. Samples in the range from approximately 10,000′ to 11,000′ were taken at three other wells in this field. Samples were taken also at two wells in Upshur County, Texas, about 230 miles north of the Chocolate Bayou field. These were from the Eagle Ford formation, from the basal Upper Cretaceous. In addition, samples were examined from the Black Band shale, an iron ore mined in eastern Ohio from the base of the Conemaugh formation, of Pennsylvanian age. Thus, three eras are represented altogether—Cenozoic, Mesozoic, and Paleozoic, in that order.

The material was crushed, and the portion that would pass a 200-mesh sieve was mixed with water in such proportions that the water was just cloudy, the actual amount being determined by experiment and later judged by eye. It was found that results were better if the suspensions were allowed to settle briefly so that the larger particles were removed.

A number of distinctive objects were found. It is difficult to determine anything about the origin of some of

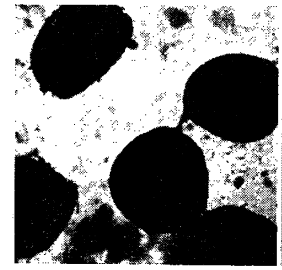

FIG. 1

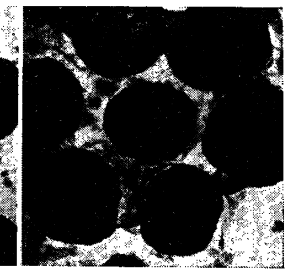

FIG. 2

them—whether they are plant, animal or mineral. The problem is complicated somewhat by the possibility of contamination of the samples by drilling mud and by air-borne materials. Bacterial contamination is known to occur, but the usual air-borne bacteria are easily recognized, for the most part by their relative transmittance to the electron beam. Objects not opaque to the electron beam were not considered likely to be fossilized.

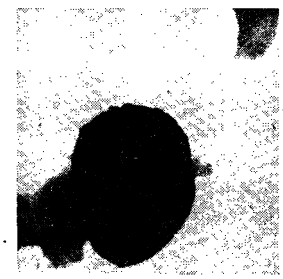

FIG. 3

FIG. 4

Some objects were seen which may be fossilized spores of bacteria, algae, or fungi. These are somewhat discoid in shape, some having smooth edges, as shown in Fig. 1, and some having crenulated edges. The clue that these may be spores is strengthened somewhat by the finding of one group of discoids still fastened together by some sort of a membrane, although the membrane may possibly indicate contamination by contemporary organisms (Fig. 2). These discoids were found in most of the samples. They were so prevalent in all formations studied that they would not be suitable as marker types, unless it is found on further work that they have a definite geologic limit somewhere in the section. No evidence of such a limit was found, although investigation of older formations may indicate one. These discoids are somewhat similar to the spores of contemporary fungi such as *Penicillium notatum* and *Penicillium digitatum*, shown, respectively,