Micro-Neutralization Test for Identification of Rhinovirus Serotypes.* (31345)

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Since origination of the microtitration method by Takatsy(1) and its further development by Sever(2), it has been adapted to many uses, including tissue culture and virus titration and neutralization(3,4). The present report describes modification of this method for the initial serotype screening of rhinoviruses using neutralization testing in a system of intersecting serum pools.

Only neutralization testing has been practical as a means of rhinovirus serotype identification, and the discovery to date of over 70 distinct rhinovirus types has made studies of rhinovirus epidemiology extremely cumbersome. Titers of rhinovirus hyperimmune antisera generally have been lower than those obtained for antisera to other picornaviruses, and rhinovirus antisera pools thus have relatively high total serum concentrations. This results in a cytotoxicity which is more pronounced in conventional plexiglass or plastic plates, and which made necessary the development of suitable glass containers for the micro procedures.

Materials and methods. A strain of diploid human embryonic lung cells (WI-38)(5) was selected for use because its sensitivity permits optimum rhinovirus growth without prolonged virus adaptation. Cells were obtained from a commercial source‡ in either tubes or flasks.

Sixty-six hyperimmune animal antisera were prepared or obtained as previously reported(6). Sixty of these were combined into 3 schemata of 20 intersecting pools each by the method of Schmidt *et al*(7), as adapted to rhinovirus identification by Schieble and Lennette (personal communication). Each pool was constructed to contain 20 units of each of 4 or 5 antisera. Final serum concentrations of the pools ranged from 10% to 40%. Six low-titered antisera were combined in 2 non-intersecting pools containing 3 antisera each. Antisera were not heat inactivated. Rhinovirus strains were isolated during a longitudinal study of industrial employees(8), and were characterized as rhinoviruses by previously described methods(6). Most viruses were in the second to fourth WI passage, and had infectivity titers of 10^2 to 10^4 $TCID_{50}$ per ml.

Testing was done in 6 mm diameter soda-lime glass precipitin tubes cut to 12 mm lengths and inserted into plastic plates§ from which the bottoms of the wells had been removed by clippers (Fig. I, A). Duplicate tubes containing mixtures of .025 ml of undiluted virus and .025 ml of each serum pool were incubated at 33°C for 2 hours under conditions of motion, humidity, and CO_2 atmosphere as described below. At the end of this time, .10 ml of trypsinized WI-38 cells (120,000 per ml) in 10% uninactivated newborn calf serum and 90% basal medium Eagle (diploid) (BME)‖ were added to each tube. Virus control tubes received .025 ml of Hanks' balanced salt solution, .025 ml of undiluted virus and .10 ml of WI cell suspension. The "micro racks" were then covered with aluminum foil and attached by rubber bands to a roller drum (0.2) rpm tilted backwards 35 degrees from the vertical axis (Fig. 1, B). This was incubated at 33°C in an atmosphere of high humidity with 2% CO_2 and air. After 2 hours tubes were emptied by one vigorous

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§ Plastic and plexiglass plates and micro pipets were obtained from Cooke Engineering Co., Alexandria, Va.

‖ Grand Island Biological Co., Grand Island, N. Y.

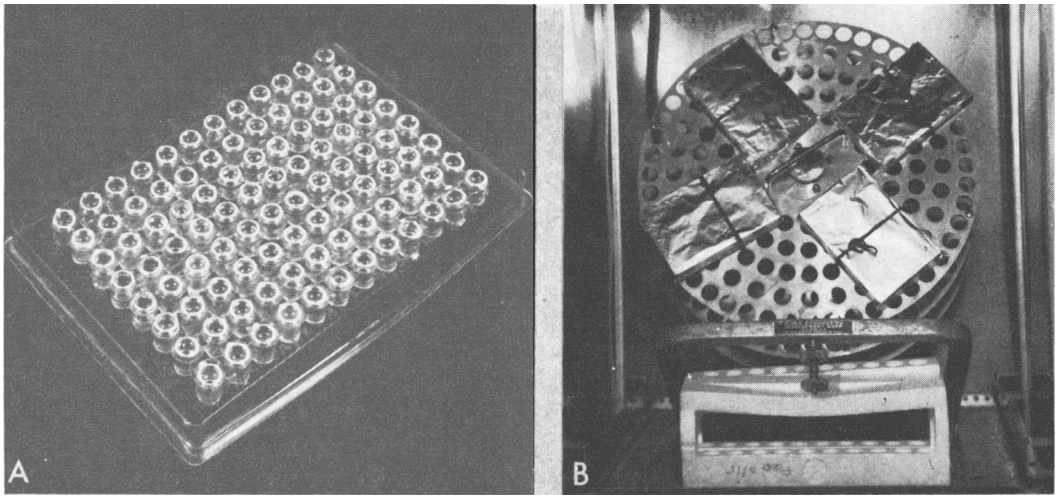

FIG. 1. Rhinovirus micro-neutralization test apparatus. (A) plastic plate holding small glass tubes; (B) roller drum to which is attached aluminum foil covered "microracks."

hand motion, re-fed 5% uninactivated newborn calf serum-BME, and returned to the incubator. The aluminum foil covering was temporarily removed, and tests were read daily on a Leitz inverted microscope. Tubes were considered positive when 50% or greater cytopathic effect (CPE) had occurred, although tests with slow-growing rhinovirus strains were sometimes judged complete when neutralization was apparent but before the CPE in the control wells had involved 50% of the cell sheet. Minimal CPE eventually appeared in one or more of the wells in 12% of confirmed positive tests. Such virus breakthrough has also been observed with the macro system. Tubes in incompleted tests were emptied and re-fed (5% calf BME) after 3 days.

Results of micro-neutralization testing were compared indirectly to results obtained earlier with 100 consecutive neutralization tests using the same intersecting pool system with diploid cells grown in standard 12 × 125 mm screw cap tubes. For this comparison, a "test" constituted running one virus against one schemata of intersecting serum pools or against one non-intersecting serum pool in the case of low titered antisera. Such indirect comparison is justified since tentative serotype identifications made by either method received final verification by standard neutralization testing using 20 units of the individual heat inactivated (56°C for 30 minutes) antiserum incubated with 100 $TCID_{50}$ of virus for 2 hours at room temperature.

Results. Cells sheeted rapidly in the glass containers and cytopathic effects were readily discernible (Fig. 2, A and B). One hundred and thirty-one of 154 (85%) attempted micro-neutralization tests were successfully completed, a rate similar to that obtained for successful testing with the macro-neutralization method in this laboratory (Table I). The frequency of testing failures due to nonspecific tissue cell degeneration was the same for both methods. With both techniques, instances of such tissue cell degeneration tended to occur in successive tests, and apparently reflected abnormalities in the lots of WI cells used.

Instances of testing failures due to insufficient virus were partially related to such things as titer of virus in specimen harvest, duration of storage, and exposure to freezing and thawing. In general, specimens had undergone more use prior to the micro testing. Test duration with the micro method was shortened by the increased amount of virus used and also because 18% of tests were judged complete prior to 50% destruction of cells. Seventy-three per cent of successful micro tests were finished by 4 days, and all were completed by 6 days. With increasing experience, greater numbers of micro tests

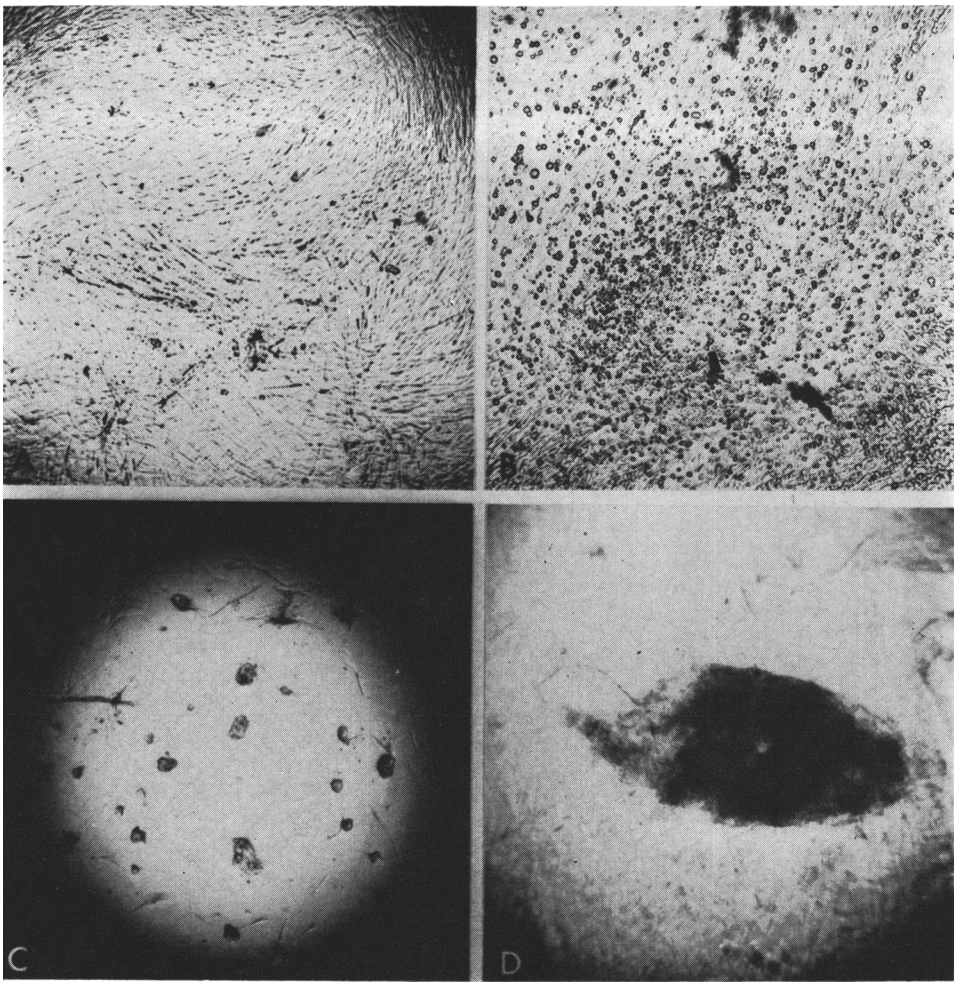

FIG. 2. Rhinovirus micro-neutralization tests with WI-38 cells (A) virus neutralization, glass container, 35×; (B) virus CPE, glass container, 60×; (C) cytotoxicity associated with calf hyperimmune antisera pools in plexiglass container, 41×; (D) granular precipitate associated with guinea pig hyperimmune antisera pools overlying intact cell sheet, plexiglass container, 45×.

showing virus inhibition were judged complete at an earlier stage.

Twenty-seven rhinovirus strains were neutralized in the macro screening tests and 54 in the micro ones. The relative number of provisional identifications cannot be compared since testing by the two methods was performed with different rhinovirus isolates. Results of pool testing with macro and micro techniques compared to the results obtained with definitive single serum testing showed similar accuracy. The serotype indicated was confirmed for 93% of the macro tests and for 96% of the micro ones.

An analysis of tissue cell costs revealed that 100 successful micro tests cost approximately 9 times less than 100 successful macro tests. The greatest saving was the increased volume and speed of execution with which rhinovirus serotyping tests could be performed.

Discussion. Rhinovirus epidemiological studies using standard serotyping methods have become increasingly difficult as the number of reported serotypes has risen. Adaptation of a micro system to rhinovirus neutralization testing offers a way of identifying rhinovirus serotypes with greatly reduced outlaws of time, space, and materials.

TABLE I. Pooled Antisera Rhinovirus Neutralization Testing with Macro and Micro Methods.

	Macro			Micro		
	No.	%	Cumulative %	No.	%	Cumulative %
Tests* attempted, including repeats	112			154		
Tests completed successfully	100	89		131	85	
Cause of testing failures						
Tissue cell degeneration	8	7		12	8	
Insufficient virus	4	4		11	7	
			100			100
Time for test completion in days						
2				32		24
3	4		4	51		63
4	12		16	13		73
5	24		40	25		92
6	32		72	10		100
7	9		81			
8	7		88			
9	6		94			
10	4		98			
11	2		100			
Accuracy as determined by confirmation of virus neutralization with standard testing using a single antiserum:						
Single serum/Serum pools	25/27	93		52/54	96	
Tissue cell costs per 100 tests	———	$1,600.00	———	———	$175.00	———

* Test constitutes running one virus against one schemata of intersecting serum pools or against one non-intersecting serum pool.

Some of the problems encountered in evolving the micro system evaluated above will be mentioned briefly. Rhinoviruses were tested using the WI cell strain in which they were isolated, thereby avoiding the necessity for adaptation to a continuous cell line such as HeLa cells. Peculiarities of rhinovirus growth such as the need for motion necessitate certain adaptations which are not necessary for similar testing with other agents. Motion provided by several apparatuses,¶ with or without modifications, permitted growth of rhinovirus strains with varying degrees of success. Certain types of motion allowed rhinovirus growth in macro but not in micro systems and vice versa. Problems encountered in some instances due to excess heat production were solved by placing the apparatus motor outside of the incubator. Tissue cell destruction sometimes resulted from turbulence in the medium created by certain kinds of motion. Fulfillment of the motion requirement was achieved by returning to the use of a standard roller drum. Originally it was supposed that mounting micro containers on a roller drum would result in loss of the contents due to spillage. This did not occur, however, because of surface tension.

Another major problem was tissue cell toxicity resulting from the interaction of plastic or plexiglass containers and animal sera used for cell nutrition or virus inhibition. This was most marked with hyperimmune animal antisera used in schemata of intersecting pools. WI cells alone could be grown in plexiglass plates with standard procedures and several media; growth was also possible in plastic plates pre-treated with ethyl alcohol (70% for 2 hours) for detoxification. However, exposure of WI cells to animal (bovine, guinea pig and goat) hyperimmune antiserum pools in these containers caused major cytotoxic changes. In addition, certain lots of calf serum used in growth medium caused toxic changes not seen with glass containers.

The toxicity resulting from calf serum ap-

¶ Universal oscillating shaker, Yankee fixed speed rotator, Lab Tec aliquot shaker, Vortex Jr. mixer.

peared immediately and was characterized by clumping of the cells which never spread to become confluent (Fig. 2, C). This toxicity was not removed by dialysis of calf serum against buffered saline. The cell destruction associated with hyperimmune antisera of other animal species (guinea pig and goat) was delayed in appearance, occurring after 8 to 12 hours. It appeared to be associated with a granular precipitate which formed during virus-antiserum incubation, but which did not prevent the initial establishment of an intact underlying cell sheet (Fig. 2, D).

Halving of the final antiserum concentration of the pools by using 10 units of each antiserum did not reduce toxicity enough to permit successful testing in plastic. Omitting heat inactivation of the antisera was beneficial, but residual toxicity still proved too great in non-glass containers. As a substitute to an unsuccessful search for glass microtitration plates, "tiny tubes" were devised. These proved satisfactory as glass containers with the plastic plate serving as a rack. This allowed partially successful testing using antiserum pools, but toxicity was not completely eliminated. A final modification which diminished the amount of granular precipitate and controlled both types of cytotoxicity was to refeed the cells with fresh medium 2 hours after their addition to the tubes. It was feared that the process of removing the virus-serum mixture might lead to cross-contamination, but in practice this has not been a problem. Apparently any virus spread in this manner is insufficient to produce a significant amount of CPE before the test is completed. Refeeding of tests done in non-glass containers has not consistently prevented toxic cell destruction.

Other investigators attempting micro-neutralization tests have reported similar cytotoxicity when using human serum in high concentration(9). This toxicity was found to reside in the pre-albumin fraction of serum, and was effectively removed by ammonium sulfate precipitation. This or some absorption procedure may hold promise as a means of reducing the toxicity of antisera used to create pools for micro-neutralization testing.

Summary. A micro method for rhinovirus neutralization has been described using glass containers and early refeeding of cells to control cytotoxicity associated with highly concentrated antisera pools. Satisfactory motion for the growth of all rhinoviruses tested was supplied by slight modification of a standard roller drum. The micro technique compared favorably with one using standard screw cap tubes, and permitted a large saving in material costs and time.

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