Technique for Viral Neutralization Antibody Surveys in Primary Microcultures

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A simplified, microplate tissue culture procedure is described which facilitates neutralization antibody surveys on large numbers of sera. A comparison of the micromethod with that employing standard tube cultures indicates it to be as sensitive and far more economical.

Several investigators in recent years have suggested using microplate tissue culture systems as an economical, simple, and accurate method for the assay of neutralizing antibody (2, 8, 10). Since the original studies were employed on microplates which had to be detoxified and sterilized before use, workers were often unable to obtain consistent results. Variations in surface characteristics from plate to plate were common prior to the advent of equipment specifically treated for tissue culture. The introduction of sterile, nontoxic microplates has greatly enhanced the utility of this system in diagnostic virology.

In functioning as a combined National Institutes of Health-World Health Organization Simian Virus Laboratory (6), extensive studies have been underway at Southwest Foundation for Research and Education (SFRE) to define and elucidate the virological flora of primates. Surveillance of large numbers of primate sera for viral antibodies (5-7; S. S. Kalter, Proc. Nat. Acad. Sci. U.S.A., in press; S. S. Kalter and R. L. Heberling, in preparation; S. S. Kalter et al., Arch. Gesamte Virusforsch., in press) has been essential for a description of these epidemiological parameters. These studies generally involved the use of complement fixation and hemagglutination inhibition tests either by macro- or microprocedures. Neutralization testing was limited by the excessive amounts of materials required for the macrotechnique. This report describes a simplified technique employing established monolayers of primary microcultures which has facilitated the testing of large numbers of sera for neutralizing antibody.

Sterile, nontoxic disposable microculture plates are commercially available from Falcon Plastics Division (BBL, Oxnard, Calif.) or Linbro Chemical Co. (New Haven, Conn.). These plates are of rigid construction, individually wrapped, and consist of 96 flat-bottom wells per unit. Sterile, nontoxic lid plates and pressure-sensitive tape are also available. Microplates with lids may be held in a humid, 5% CO₂ atmosphere or sealed with the pressure tape and kept in an ordinary incubator. Masking tape may be wrapped around the edges of individual plates and lids, whereas multiple units with lids can be stacked and covered with Saran Wrap (Dow).

Primary baboon (Papio cynocephalus) kidney cells (BKC) were routinely employed in these procedures, although rhesus (Macaca mulatta) or African green (Cercopithecus aethiops) kidney cell cultures were occasionally substituted with satisfactory results. The trypsinized cell suspensions were prepared by methods previously described (3). Growth medium consisted of 0.65% lactalbumin hydrolysate and 2% inactivated newborn calf serum in Hanks buffered saline. Maintenance medium containing Earle’s minimal essential medium with 0.5% inactivated newborn calf serum was added after the cells achieved confluency, usually 4 to 6 days after seeding. All media contained 1 unit of bacitracin, 100 units of penicillin, 100 µg of streptomycin, and 100 µg of neomycin per ml. Spent media were removed by inverting the plate or by vacuum aspiration of individual wells.

Standard tissue culture tubes were seeded with 10⁴ to 1.5 x 10⁶ cells; microcultures were seeded with 2 x 10⁴ cells per well. Cell counts were based on the erythrocin B dye-exclusion method. In the micromethod, cells and media were easily dispensed with 1-ml Cornwall automatic syringes. Wells readily accommodate volumes of 0.2 to 0.3 ml, and under these conditions mineral oil overlies have not been found necessary.

Pools of simian adenovirus V-340 (9) SFRE reference stock and adenovirus isolate 6992, a candidate prototype strain isolated in this labora-
TABLE 1. Neutralizing antibody in captive primates: a comparison of methods

| Determination | Results of various tests for V340 antibody | Results of various tests for 6992 antibody |
|---------------|------------------------------------------|------------------------------------------|
|               | Johannesburg African Green (25)          | Inst. Merieux Patas (42)                 |
|               | Macro (TCID₆₀ 300)                       | Micro (TCID₆₀ 100)                      |
| Positive      | %                                        | %                                       |
| Negative      | 96                                       | 63 %                                    |
|               | Macrotitated (TCID₆₀ 100)                | Micro (TCID₆₀ 100)                     |
| Positive      | %                                        | %                                       |
| Negative      | 96                                       | 37 %                                    |

- Number of identical results between methods: 25/25 = 100%.
- Number of identical results between methods: 37/42 = 88%.
- Number of identical results between methods: 32/38 = 84%.
- Number of identical results between methods: 40/42 = 96%.

Baboon (Papio sp.) sera were obtained from the Institute Merieux, Lyon, France, as were the patas (Erythrocebus patas) sera. African green monkey sera were provided by the Poliomyelitis Research Foundation, Johannesburg, South Africa. Prior to testing, all sera were diluted 1:5 in phosphate-buffered saline at pH 7.2 and inactivated at 56°C for 1 hr.

In a sterile dilution tube, 0.3 ml of virus diluted to contain 100 TCID₆₀ per 0.1 ml was added to 0.3 ml of diluted serum. The mixture was allowed to react for 1 hr at room temperature, after which it was inoculated in 0.2-ml amounts into each of two BKC tubes. Control virus was titrated with 0.1 ml per tube, four tubes per 10-fold dilution. Tests were read 48 hr after cytopathology was observed in the virus control (100 TCID₆₀) tubes. Titrations were retained 7 to 14 days to determine the actual virus dose used in the test.

The technique which has been derived for the microneutralization test is basically a 1 to 3 miniaturization of the standard test used in this laboratory (Table 1). For the microtest, 0.1-ml quantities of both virus and serum were agitated vigorously for a few moments on a vortex mixer. After 1 hr at 25°C, 0.4 ml of maintenance medium was added to the reaction tube. This step provides a "transfer vehicle" for the virus-serum mixture as well as a medium change. Microplates were inoculated with ordinary 1.0-ml serological pipettes. The control titration incorporated a series of 10-fold dilutions, each of which is diluted 1 to 6 in medium and inoculated into four wells per dilution. Microtests were observed on an inverted microscope at low power (40 to 60×) and interpreted on the same schedule as the macromethod.

Incidence of antibody to V-340 in two groups of primates is shown in Table 1. Each series was tested by both the micro- and macrotechniques. For this comparison, the same lots of cells were employed, and duplicate tests were performed on the same day. Identical results were obtained in the African green sera, in which a single animal exhibited V-340 antibody. The patas sera also yielded commensurable results, with 37 of 42 identities between the two systems. Agreement factors of 84 and 96% were obtained when the two techniques were compared in testing patas and baboon sera for antibody to isolate 6992.

Experiments illustrating the reproducibility of the two neutralization systems were also performed (Table 2). Experiment 1 consisted of duplicate series of 42 baboon sera tested in different cell lots on different days; 40 of 42 results were identical. In experiment 2, the same sera were retested in both systems and resulted in 35 of 41 identities. In terms of reproducibility, the two macrotests were in 78% agreement and the microtests were in 81% agreement.

The data presented here are part of an extensive viral epidemiological study which is currently in progress. In this report they represent an effort to compare a simplified microtechnique.
for antibody surveys to standard tube-type procedures. Evaluations of a more statistical nature have been undertaken by others, and such reports concur in their acknowledgment of the reliability of the microculture system (2, 8, 10). This system is currently being used routinely in this laboratory for assays of adeno-, entero-, and herpesvirus neutralizing antibodies. These studies have consistently shown the microtitre tissue culture tests to be as sensitive as equivalent standard procedures.

Neutralization techniques described by Sullivan and Rosenbaum (10), which employ the micro-dilution loops, are highly satisfactory for assays with rapidly growing cell systems. It should be noted, however, that rotation of the newer "tuliptype" microdiluters tends to cause considerable scarification of the flat surface of the well. An interesting technique introduced by Catalano et al. (1) may eliminate this deficiency. The technique described herein precludes the use of dilution loops and permits one-step inoculation of microplates of established monolayers.

Advantages of the microculture system are analogous to those derived from applications of the widely used microtitre techniques in complement-fixation and hemagglutination tests. Assays of this type can be performed with \( \frac{1}{2} \) to \( \frac{1}{3} \) of the quantity of cells and media; \( \frac{1}{4} \) to \( \frac{1}{3} \) of the volume of reagents; \( \frac{1}{4} \) of the space; and \( \frac{1}{4} \) of the time taken in comparable manipulations of standard tube cultures.

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