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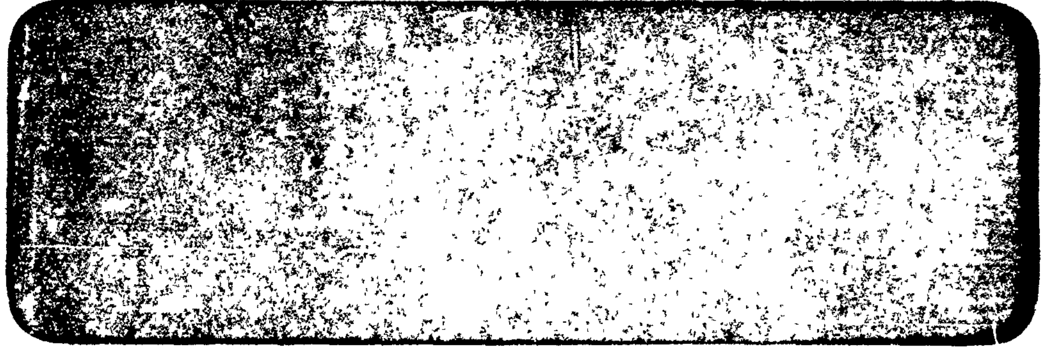

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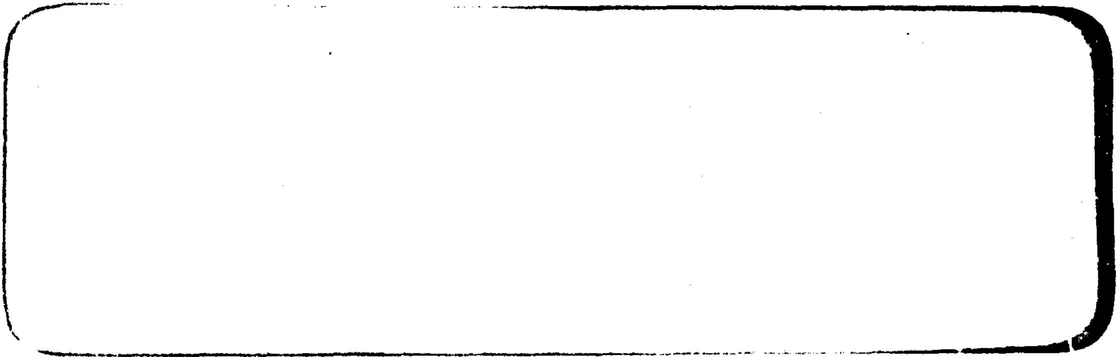

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Multiple Infection of Cell Monolayers by Virus Mixtures

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In studies of viral interference or mixed infections, difficulty is frequently encountered in distinguishing the specificity of cellular infections that result from the introduction of more than one virus onto cell monolayers. This may limit the type and number of agents that may be employed, particularly when the viruses evoke similar or indistinctive cellular responses. Quantitation of virus infectivity in the presence of another agent often involves intricate assay techniques. To facilitate experimental investigations at the cellular level, this communication describes a rapid and highly specific procedure for distinguishing and quantitating virus infectivity by selective immunofluorescent staining in conjunction with fluorescent-cell counting. A preliminary study of virus interference demonstrates the applicability of the procedure.

To effect multiple infection of cell monolayers, a mixture of yellow fever, variola, and psittacosis agents in equal volumes and in an approximate virus-cell ratio of 1:50 was inoculated in 0.2-ml volumes onto cover-slip cultures of McCoy cells. Centrifugation (19,000 to 29,000 × *g*, 15 min) was employed for adsorption of inoculum onto cover-slip cell cultures. Cell cultures were then incubated at 35 C from 16 to 24 hr in accord with previously established assays for each agent (N. Hahon and R. M. Nakamura, Virology **13**:203, 1964; N. Hahon, Appl. Microbiol. **13**:865, 1965; N. Hahon, J. Infect. Diseases **116**:33, 1966). After being fixed with acetone (−60 C), cover-slip cell monolayers were separated into three groups and selectively stained with one of three viral immune sera conjugated with fluorescein isothiocyanate. The infected cells were then counted. Multiple infection of cell monolayers was achieved, and no interference of growth occurred among the agents after simultaneous introduction of the agent mixture. The titer of virus controls was comparable to that of the corresponding agent in the mixture. Each agent, therefore, could be independently and quantitatively assayed in the presence of the others (Table 1).

TABLE 1. *Multiple infection of McCoy cell monolayers after inoculation with a mixture of three viral agents*[a]

Agent assayed	Titer[b]
Yellow fever	3.0×10^7
Yellow fever + diluent[c]	2.8×10^7
Psittacosis	6.4×10^7
Psittacosis + diluent	6.2×10^7
Variola	1.8×10^7
Variola + diluent	1.4×10^7

[a] The inoculum was a mixture of yellow fever, variola, and psittacosis agents

[b] Expressed as cell-infecting units per milliliter, determined by selective immunofluorescent staining and fluorescent cell-counting

[c] Mixture 199 plus 5% calf serum

Viral interference was demonstrated by altering the inoculation time of agents. When inoculation of the psittacosis agent and variola virus preceded by 20 hr the addition of yellow fever virus, the latter was excluded (Table 2). Neither variola nor psittacosis agents were excluded under these conditions by other agent combinations. Subsequently, it was determined that the psittacosis agent alone could completely exclude yellow fever virus, but that variola virus caused only a partial interference of growth. There was no reciprocity of interference.

To show that a virus having a rapid growth rate may be assayed simultaneously with viruses of slower rates, Venezuelan equine encephalomyelitis (VEE) virus was incorporated as the fourth agent in the virus mixture. At 1 hr after adsorption of inoculum, 1 ml of VEE hyperimmune serum was added to all cell monolayers. This procedure prevented the formation of VEE microplaques that appear within 16 hr after inoculation as well as the subsequent disorganization of cell monolayers by the virus. Thus, primary VEE-infected cells could be counted as early as 12 hr or as late as 24 hr (N. Hahon and K. O. Cooke, J. Virol., *in press*). The procedure for staining cell monolayers with conjugated immune serum was similar

TABLE 2. *Inoculation of McCoy cell monolayers with challenge virus preceded 20 hr earlier by virus mixture inoculum*

Initial agents	Challenge agent	Growth of agents		
		Yellow fever	Psittacosis	Variola
Yellow fever and variola	Psittacosis	+[a]	+	+
Yellow fever and psittacosis	Variola	+	+	+
Psittacosis and variola	Yellow fever	Excluded	+	+

[a] Number of fluorescent cells was comparable to that of control.

to that described earlier. The results indicated that the number of infected cells in cell monolayers inoculated with VEE virus alone was comparable to that of VEE virus in multiple-infected cell cultures. The presence of the fourth agent did not interfere with the growth of the other viruses. These preliminary experiments demonstrate the rapidity, versatility, and broad applicability of selective immunofluorescent staining in conjunction with fluorescent-cell counting for quantitative studies on multiple infection by virus mixtures.