Neutralization of Infectivity of Potato Yellow Dwarf Virus and Wound Tumor Virus Assayed on Vector-Cell Monolayers

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ABSTRACT

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At antiserum dilutions greater than 10^{-2} specific neutralization reactions could be expressed as a straight line relationship between the logarithm of the number of infected cells in vector cell monolayers and the logarithm of the serum concentration. Neutralization could be detected at up to a hundredfold greater antiserum dilution than the precipitin interface reaction against virus. At serum dilutions between $10^{-2.5}$ and $10^{-2.0}$, a marked increase in the slope of the neutralization curve was produced by antiserum but not by pre-immune control serum; the latter had no significant neutralizing activity at any dilution tested. The slope of the straight line was the same for the two potato yellow dwarf virus (PYDV) systems but different for the wound tumor virus (WTV) system. Tests revealed no cross neutralizations between antiserum to sowthistle yellow vein virus (SYVV) and PYDV or between antiserum to rice dwarf virus (RDV) and WTV. Cross neutralization occurred between two field forms of PYDV but cross neutralization was stronger in one direction than in the other. Heterologous immunofluorescent staining was less intense than control homologous staining and fluorescent antibodies to the two field forms of potato yellow dwarf virus stained about 1% as many infected cells heterologously as homologously. Cross absorption of conjugated antiserum with heterologous virus eliminated cross-reactive but not homologous staining.

Neutralization of animal virus infectivity has been studied extensively by use of the plaque assay technique. In contrast, only a few neutralization studies have been performed with plant viruses. Interference with the infectivity of tobacco mosaic virus (TMV) by antibody was determined by local lesion assay (22, 23). Rappaport and Siegel (22) studied neutralization of this virus in a solution with a virus protein concentration of 0.5 μg/ml. The insensitivity of the local lesion assay prevented measurement of virus activity at low virus concentrations. Moreover, interference with TMV inoculations by normal serum was shown to be important up to a serum dilution of 10^{-3} (22). This nonspecific non-antibody interference must be taken into account quantitatively if neutralization experiments which depend on virus assays on plants are to be correctly interpreted.

Specific neutralization of plant viruses which are persistent in their vectors and not mechanically inoculable to plants has been studied previously by two methods utilizing the vector. Black and Brakke (3) reported neutralization of wound tumor virus (WTV) by injection of mixtures of the virus and its antiserum into its leafhopper vectors that were later tested on plants for transmission of the virus. Gold and Duffus (14) used a membrane-feeding assay technique to demonstrate complete neutralization of infectivity of beet western yellows virus (BWYV) during incubation of virus with antisera. After incubation the treated virus was fed to the aphid vector through a membrane and the aphids were tested later on susceptible plants for ability to transmit the virus. Later, Duffus and Gold (12) used this technique to show that antiserum that completely neutralized BWYV had no effect on potato leaf roll virus. Rochow and Ball (25) also used this technique with the MAV strain of barley yellow dwarf virus and its aphid vector. These workers showed that tests for neutralization also could be performed by injection of the aphids with mixtures of virus and antiserum before the insects fed on test plants.

The development of vector cell monolayers (1, 6) and the use of immunofluorescence techniques for cell counting provide a degree of experimental control and accuracy for neutralization experiments with plant viruses which are propagative in their vectors that previously had not been possible with any plant virus. In the present study, attempts were made to demonstrate virus neutralization by antisera for WTV and potato yellow dwarf virus (PYDV) on their vector cell monolayers.

MATERIALS AND METHODS

Cell monolayers.—Since our research was done during 1968-1969, before AC20 and AS2 had become selected as

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preferred cell lines, AC16, AC17, AC18, AC21, and AS1, also established in our laboratory by Chiu and Black (6), sometimes were employed. Cells obtained from cultures younger than 4 days old were grown on 15-mm diameter coverslips. After seeding about $(2\text{-}4) \times 10^4$ cells in 0.1 ml of cell suspension over the entire area of each coverslip, the cells were allowed to attach to the glass surface by leaving the coverslips undisturbed for at least 2 hr. The coverslip cultures became nearly confluent after 24-48 hr of incubation at 30 C. Two to four coverslips were placed in each of several sealable glass petri dishes. At the time our work was done, the growth medium (6) was prepared with 17.5-20% fetal bovine serum (FBS).

Virus sources and inocula.—Vectorial strains (1, p. 74) of PYDV and WTV from plants of *Nicotiana rustica* L. and *Melilotus officinalis* L. Lam, respectively, were used as sources of inoculum. Two strains of PYDV (2) were studied: one strain (hereafter designated SYDV) is vectored by *Aceratogallia sanguinolenta*, the other strain (hereafter designated CYDV) is vectored by *Agallia constricta*.

In 1968-69, when our experiments were done, the use of purified preparations of PYDV as inoculum was unsuccessful, whereas crude extracts prepared aseptically from infected *N. rustica* stems, without Millipore filtration, produced many infections.

Effects of glycine and magnesium.—Before our research was done, growth medium, growth medium with a reduced FBS content (5%), or medium containing 0.5% bovine serum albumin instead of FBS had been used to suspend virus during inoculations (7, 9, 13). Magnesium chloride had been found to enhance virus titers (28) or, in combination with glycine, to stabilize virus particles (4). In several experiments with wound tumor virus and potato yellow dwarf virus, inocula prepared in three different solutions were compared at the same virus concentration. Virus suspended in a solution at pH 7.5 containing 0.1 M glycine, 0.01 M $MgCl_2$ (Gly-$MgCl_2$ solution) and 25 μg/ml DEAE-dextran resulted in ~4.5 times more infected cells than virus suspended in growth medium minus fetal bovine serum (FBS). Virus in the latter solution gave ~17 times more infected cells than virus in growth medium which contained FBS. Therefore, all inocula were prepared aseptically at 4 C without Millipore filtration in sterile Gly-$MgCl_2$ solution containing 25 μg/ml of DEAE-dextran.

Preparation of inocula.—Usually, to prepare inoculum of PYDV, about 2 g of stem pieces were selected from *Nicotiana rustica* plants in the acute stage of the systemic disease. These were weighed, surface sterilized with 70% ethanol for 3-5 min, rinsed with sterile distilled water, and then sliced into cross sections about 2-3 mm thick. These were triturated with mortar and pestle and about 1 g of sterile sand in 5 ml of Gly-$MgCl_2$ solution and DEAE-dextran. The crude extract was transferred to a 30-ml centrifuge tube. Another 5 ml of solution was used to rinse the mortar and pestle and was added to the tube. After centrifugation for 5 min at 6,620 *g* the supernatant (pH 5.8-6.2) was taken as a 1/5 dilution.

To prepare inoculum of WTV 2 g of selected clean root tumors from sweet clover were surface sterilized as above, rinsed, and then triturated with mortar and pestle and about 0.5 g of sterile sand in 10 ml of solution. The crude extract was transferred to a centrifuge tube and the mortar and pestle were rinsed with 10 ml of the above solution before it was transferred to the same tube. After centrifugation at 6,620 *g* for 5 min, the supernatant (pH 6.3) was reckoned as a 10^{-1} dilution.

Antiserum production.—The essential procedures for purifying these viruses before preparing antisera to them [namely, density gradient rate and quasi-equilibrium centrifigation followed by density gradient electrophoresis (3, 29)] have been modified in various subsequent studies. We followed the detailed procedure of Sinha and Reddy (26) in which density gradient zonal electrophoresis was omitted from the original purification process. This reduced the time and labor needed for purification and yielded a satisfactory antigen for the production of antiserum in rabbits. In neutralization tests the antisera were diluted to various concentrations in the same solution used for preparing inoculum. A clean dry pipette was used for each dilution. One ml of each dilution was added to an equal volume of the appropriate dilution of each virus and, without prior incubation, was allowed to react with the virus at 30 C during the inoculation period of 3 hr on the cell monolayers.

Preparation of fluorescein-conjugated antibody.—Infected cells in inoculated monolayers were detected by the use of the fluorescent antibody technique (10). Gamma globulin from antiserum was isolated by the method of Campbell et al. (5) and was conjugated with fluorescent dye essentially according to the procedure described by Spendlove (27). The procedure is described in detail below.

With constant stirring, 3 ml of saturated $(NH_4)_2SO_4$ solution was added, drop by drop, to a beaker containing 6 ml of serum at room temperature. The pH of the mixture was adjusted to 7.8 with 2 N NaOH. After stirring with a magnetic stirrer in a cold room (4 C) or in an ice bath for 2-3 hr, the mixture was centrifuged at room temperature (25 C) for 30 min in a clinical centrifuge. After the supernatant liquid was decanted the pellet was dissolved in saline (0.15 M NaCl) to restore the original serum volume. The precipitation with $(NH_4)_2SO_4$ and dissolving of the precipitate was repeated twice and the third precipitate was dissolved in saline to yield one-half of the initial serum volume.

Residual sulfate was removed by dialysis against saline in the cold (4 C) room. Dialysis tubing had been boiled in distilled water for 15-30 min and rinsed several times with distilled water before being filled with the solution of γ-globulin. Dialysis was continued against saline until sulfate ions were not detectable by adding a few drops of saturated $BaCl_2$ to several milliliters of any dialysate about to be discarded. For 3 ml of γ-globulin, dialysis against 2 liters of saline with two changes during 24 hr was usually sufficient. The γ-globulin retentate was then centrifuged at 4 C for 30 min at 1,230 *g* to remove insoluble material formed during dialysis.

To determine the globulin concentration, a sample of the solution was diluted with phosphate-buffered saline (PBS, 0.01 M phosphate, 0.15 M NaCl, pH 7.5) and its optical density read at 280 nm in a spectrophotometer. The protein concentration was calculated from the formula:

Protein concentration (mg/ml) =

$$\frac{\text{absorbance} \times \text{dilution factor}}{1.8}$$

where 1.8 = extinction coefficient of rabbit γ-globulin (20).

Crystalline fluorescein-isothiocyanate (FITC, Bioquest, Baltimore Biological Laboratory, Cockeysville, MD 21030) was used for conjugation. Usually, 15 mg of FITC per gram of protein is a satisfactory proportion for conjugation of rabbit globulin. After calculating the amount of FITC needed for the conjugation of the γ-globulin obtained, about 2.5 mg or more of FITC was dissolved in freshly prepared 0.1 M Na_2HPO_4 so that 0.6 ml would contain the exact amount of FITC desired for 1 ml of the concentrated γ-globulin solution.

With constant stirring, 0.6 ml of the FITC solution was added to a beaker containing 1 ml of the serum globulin. After adding 0.2 ml of 0.1 M Na_2HPO_4, the solution was adjusted with 0.04 N NaOH (approximately 0.2 ml) to pH 9.5, and then conjugation was allowed to occur for 30 min at room temperature without stirring. The conjugate was passed through a previously prepared G-50 beaded Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, NJ 08854) column (2 × 20 cm). The Sephadex column had been prepared by washing it three times with PBS to remove the fine particles, and then by soaking it in PBS for 6 hr or longer before packing. Elution was with PBS, and the collection of eluate fractions was started when the first visible band of colored conjugate eluted from the column. Collection was stopped with the last fraction from this first band. All fractions judged by their color to be sufficiently concentrated were combined and passed through a 0.65 μm (pore-size) Millipore filter to remove stained particulate matter. The conjugated antisera were stored frozen at −25 C either with or without lyophilization.

Various dilutions of conjugate between 1/5 and 1/80, or more, were prepared with PBS (pH 7.5) and each tested on infected and healthy cell monolayers to determine the optimal dilution which gave good specific staining and no nonspecific staining. A dilution of 1/60-1/80 of such a preparation was usually satisfactory for routine staining.

Inoculation, staining, and cell counting.—Coverslip cultures were washed twice with Gly-$MgCl_2$ solution and inoculated with 0.05 ml of WTV-inoculum, or 0.1 ml of PYDV-inoculum, by spreading it over the entire surface of the coverslip. Noninoculated healthy cultures always served as controls. At the end of the inoculation period, 3 hr at 30 C, inoculum was removed and the monolayers were washed five times with growth medium before covering the coverslip with 0.08 ml of medium. The inoculated cultures and controls were then incubated at 30 C for 27-30 hr for WTV (6, 7) and 48 hr for PYDV. In the neutralization experiments counts of infected cells were recorded as the average number in a microscope field (Fig. 1) or on a whole coverslip monolayer (Fig. 2, 3). For data on whole coverslips, diametral counting (19) of fluorescent cells was adopted.

Fixation, immunofluorescent staining, and fluorescent microscopic observation of inoculated monolayers were done essentially by the method of Chiu and Black (7). After fixation, and flooding the specimen with fluorescent antibody, staining was allowed to proceed at 37 C for 35-40 min in moist chambers. Tests had shown that a reaction temperature at 37 C was better than 25 C or 50 C. The stained specimens, mounted in PBS (pH7.5) with 60% glycerol usually were examined soon after mounting. However, they also could be stored at 5 C for as long as 2 mo before being examined. After conjugates had been used for staining they were saved, passed through 0.65 μm Millipore filters, and re-used several times.

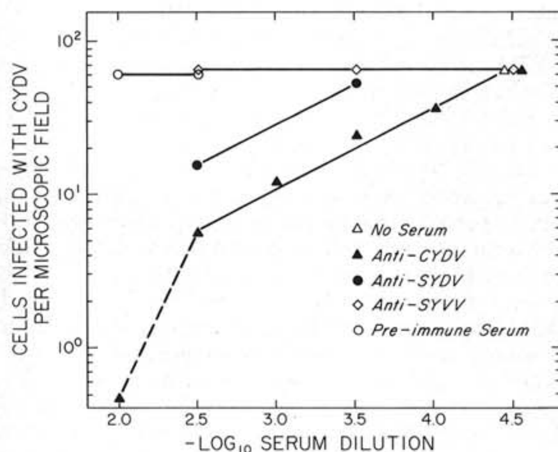

Fig. 1 Differential neutralization of the infectivity of the CYDV form of potato yellow dwarf virus by antiserum to CYDV and by antiserum to the SYDV form. The slope of the neutralization curve increased greatly between serum concentration $10^{-2.5}$ and $10^{-2.0}$. Antiserum to another plant rhabdovirus sowthistle yellow vein virus (SYVV), had no neutralizing effect although its precipitin ring titer against SYVV was $10^{-3.7}$.

RESULTS

Neutralization.—The effect of the antiserum concentration on neutralization was determined. For

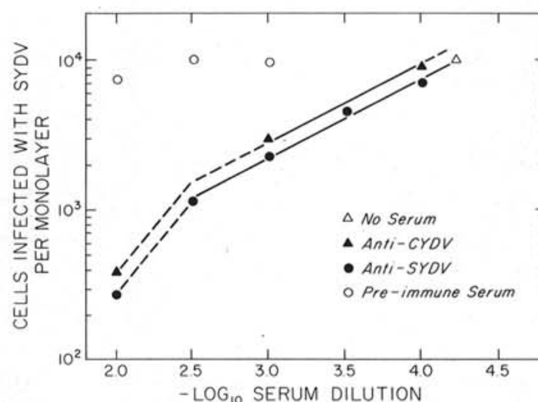

Fig. 2 Differential neutralization of the infectivity of the SYDV form of potato yellow dwarf virus by antiserum to SYDV and by antiserum to the CYDV form. The slope of the neutralization curve increased greatly between serum concentrations $10^{-2.5}$ and $10^{-2.0}$.

tests with SYDV AS2 cells were used and AC20 cells were used for tests with CYDV and WTV. To avoid any possible residual effect of antiserum on immunofluorescent staining, inoculated monolayers were washed five times with growth medium (instead of the usual two times) after removal of inoculum at the conclusion of the inoculation period; then incubation and immunofluorescent staining followed in the usual way. Pre-immune serum from each rabbit was used as a control on its antiserum. Also, other controls were included each of which was prepared by adding 1 ml of Gly-$MgCl_2$ solution instead of antisera, to 1 ml of virus solution.

Except for the dilution 10^{-2} of antiserum all other reacting dilutions, $10^{-2.5}$ to $10^{-4.5}$ for anti-PYDV, and $10^{-2.5}$ to $10^{-5.5}$ for anti-WTV, showed a degree of neutralization that can be expressed as a straight line when the log of the number of infected cells is plotted against the log of the serum concentration. The neutralization effect of the serum is detectable at lower concentrations of serum than is its precipitin interface reaction; the titers of the anti-WTV, anti-CYDV, and anti-SYDV sera were $10^{-3.5}$, $10^{-3.7}$, and $10^{-3.7}$, respectively, by precipitin interface tests against virus antigen, but were approximately $10^{-5.5}$, $10^{-4.5}$, and $10^{-4.3}$, respectively, by neutralization test (Fig. 1-3). Infectivities in the presence of pre-immune serum were not significantly different from those in the absence of antiserum.

In the reaction between SYDV and CYDV and their antisera, the number of infected cells was reduced by ~0.55 log unit for each log unit increased in antiserum concentration. The corresponding reduction in the WTV reaction was ~.75 log unit. The neutralization end point titer for anti-SYDV serum on CYDV was 0.8 log unit lower than that of anti-CYDV serum whereas the end point titer for anti-CYDV serum on SYDV was 0.2 log

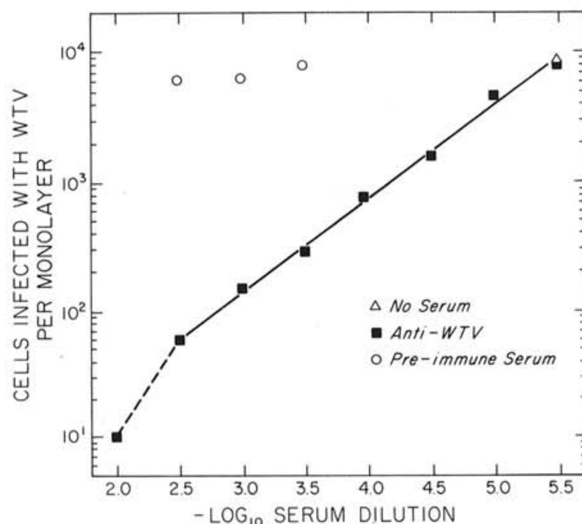

Fig. 3 Neutralization of wound tumor virus (WTV), a plant reovirus, by homologous antiserum. The slope of the neutralization curve increased greatly between serum concentrations $10^{-2.5}$ and $10^{-2.0}$. In a different experiment, not shown in Fig. 3, antiserum to another plant reovirus, rice dwarf virus (RDV), had no neutralizing effect on WTV although its precipitin interface titer against RDV was $10^{-3.6}$.

unit lower than that of homologous antiserum (Fig. 1, 2). In tests with all three antisera, the slope of the neutralization curve increased markedly between dilutions $10^{-2.5}$ and $10^{-2.0}$ (Fig. 1-3).

The two viruses, CYDV and SYDV, are related leafhopper-transmitted plant rhabdoviruses. Sowthistle yellow vein virus (SYVV) is an aphid-transmitted plant rhabdovirus (11); anti-SYVV serum obtained from D. Peters had no neutralizing activity against CYDV virus (Fig. 1), although its titer against SYVV was $10^{-3.7}$ in precipitin interface tests (21).

Wound tumor virus (WTV) and rice dwarf virus (RDV) are plant reoviruses. We are indebted to I. Kimura for an antiserum (18) with a titer of $10^{-3.6}$ in precipitin interface reactions with RDV. In other tests (not plotted in our figures) no neutralizing activity of the anti-RDV serum could be demonstrated against WTV.

As part of an experiment on CYDV, two additional tests were performed to check whether interference by residual antiserum left by inoculum on the inoculated cell monolayers might appear as neutralization in the assay by specific immunofluorescent staining. Three groups of three coverslip monolayers were prepared and all were inoculated with inoculum containing no antiserum. After inoculation, one group was treated with a 10^{-2} dilution of antiserum in Gly-$MgCl_2$ solution for 20 min at 30 C and another group with a 10^{-2} dilution of antiserum in growth medium for 2.5 hr at 30 C. After the usual incubation period and washings, the average number of stained cells per microscope field in these two groups was 66.8 and 64.9, respectively. The third group, which was not treated with antiserum after the inoculation period, gave a corresponding count of 65.8 which indicated that there was no false neutralization effect resulting from residual antiserum interfering with the immunofluorescent staining.

Specificity of immunofluorescent staining.—Immunofluorescent staining of cells infected by PYDV was markedly different from that of cells infected by WTV (8). For both SYDV- and CYDV-infected cells stained with fluorescent anti-SYDV or anti-CYDV sera, respectively, the staining at first was confined to the nucleus and began to appear in the cytoplasm later. In cells infected with WTV the staining was limited to the cytoplasm.

Antisera to SYDV and CYDV were reported to give serological cross reactions in precipitin interface tests (30) but to become specific for the homologous virus after absorption with the heterologous one. Specific staining of fluorescein-conjugated antisera to SYDV and CYDV was studied in a number of tests. In each test, two coverslip monolayers from a group of four seeded with a culture having a high proportion of cells infected with either SYDV or CYDV, were stained with homologous or heterologous antiserum. The heterologous antisera stained only 0.2 to 0.8% as many infected cells as did the homologous antisera. The intensity of staining with heterologous fluorescent antibody was noticeably less than that of the homologous control. This cross staining was completely eliminated without appreciable reduction of homologous staining by use of antisera absorbed with heterologous virus before staining (Table 1). The absorption was done by mixing the fluorescein-conjugated antiserum with an equal volume of purified

heterologous virus solution. After incubating the mixture at 37 C for 40 min it was centrifuged for 30 min at 12,600 rpm in a Servall SS-1 rotor. The supernatant liquid was then diluted appropriately for staining.

DISCUSSION

Perhaps because of the comparatively inefficient methods for assay for viruses on plants and also because of nonspecific non-antibody interference in such assays by normal serum, studies on neutralization of viral infectivity of plant viruses have been comparatively rare. Because both of these difficulties are absent when vector cell monolayers are employed for assay, it seems possible that neutralization studies will have a useful role in such systems. Our experiments show that neutralization experiments with plant viruses inoculated on vector cell monolayers can provide very precise results.

The fact that reaction curves between SYDV and CYDV and their antisera have the same slope and that the reaction curve between WTV and its antiserum has a different slope may be related to the fact that SYDV and CYDV are viruses of very similar size and shape whereas WTV is different in both respects.

The much greater increase in rate of neutralization by the increase in concentrations of antisera between dilutions $10^{-2.5}$ and $10^{-2.0}$ suggests that between these dilutions a second kind of reaction between virus and antiserum takes place. Because Rappaport and Siegel (22) reported that normal serum interferes with neutralization tests of viruses performed by infectivity assays on plants, any such change in rate might be obscured in such tests. It is worth noting that in our tests on vector cell monolayers, normal serum at dilution $10^{-2.0}$ interferes only slightly if at all (Fig. 1-3).

It was not until 2 yr after the completion of our research that a solution of histidine and $MgCl_2$ was found to be superior to glycine and $MgCl_2$ for the suspension of inoculum (16) and that DEAE-dextran was not necessary. This and other findings, especially substantial improvements in the purification of PYDV (15) and WTV (24) and more precise counting methods (17, p. 556 and p. 559) might be expected to allow significant improvements in future neutralization with these systems.

The serological relationship and distinction between SYDV and CYDV confirms serological properties of these viruses first reported by Wolcyrz and Black (29).

TABLE 1. Homologous and heterologous staining of cultured leafhopper cells infected with the SYDV and CYDV forms of potato yellow dwarf virus (PYDV)

Virus	Test	Cell line	Antiserum absorbed or nonabsorbed[a]	Number of cells stained with antisera to: CYDV	SYDV	Ratio[b] (%)
CYDV	1	AC18	nonabsorbed	8,181[c]	35[c]	0.43
	2	AC21	nonabsorbed	16,868[c]	94[c]	0.56
	3	AC20	nonabsorbed	3,091[c]	10[c]	0.32
	4	AC20	nonabsorbed	16,548[c]	127[c]	0.77
			absorbed	*[d]	0[e]	0
	5	AC20	nonabsorbed	4,063[c]	29[c]	0.71
			absorbed	*[d]	0[e]	0
SYDV	1	AS1	nonabsorbed	29[c]	1,429[c]	2.03
	2	AS1	nonabsorbed	59[c]	11,895[c]	0.50
	3	AS2	nonabsorbed	0.15[f]	36.7[f]	0.41
			absorbed	0[e]	38.7[f]	0

[a]Any absorption was by the heterologous PYDV virus.

[b](Number of cells stained with heterologous antiserum)/(cells stained with homologous antiserum) × 100.

[c]Counts were calculated as an average for a whole coverslip monolayer from counts of two diametral zones on each of two coverslips and multiplication by the diametral factor of 19.63 (18).

[d]There appeared to be as many cells stained by the absorbed antiserum as by its nonabsorbed control antiserum and no counts were made in these cases.

[e]The entire area of both coverslips was scanned without finding any stained cells.

[f]Counts were made on 20 microscopic fields selected at random on each of two coverslip monolayers and averaged to give the number of stained cells per field in the ×40 objective lens.

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