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A Rapid Virus Neutralization Assay for Newcastle Disease Virus with the Swine Testicular Continuous Cell Line

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SUMMARY. Five continuous cell lines, swine testicular (ST), human rectal tumor (HRT 18), fetal rhesus monkey kidney (MA104), bovine turbinate (BT), and quail tracheal (QT35), were evaluated and compared with chicken embryo fibroblasts (CEFs) for their ability to propagate B1 or Texas GB strains of Newcastle disease virus (NDV). The NDV Texas GB strain replicated in all the continuous cell lines used in this study. Only the ST and QT35 cells produced a cytopathic effect (CPE) similar to that produced in CEFs. However, the ST cell line remained attached while displaying CPE, whereas infected QT35 cells detached, as did the CEFs. The B1 strain of NDV replicated in ST cells, MA104 cells, and CEFs but with less CPE as compared with the Texas GB strain. Pretreatment with trypsin did not enhance CPE with either NDV strain at the level tested. Sera evaluated for neutralizing antibody titers to NDV were significantly higher in titer when the ST cell line was used and compared with CEFs. A high correlation was found between the microscopic examination and the tetrazolium dye (MTT) microassay methods for determining the viral neutralization endpoint, thus suggesting the ST cell line and MTT microassay could be used as an alternative to CEFs and microscopic examination for evaluating neutralizing antibodies titers to NDV.

RESUMEN. Prueba rápida de virus neutralización para el virus de la enfermedad de Newcastle utilizando una linea celular continua de testiculos de cerdo.

Se evaluaron y compararon cinco líneas celulares contínuas de testículo de cerdo (ST), tumor rectal humano (HRT 18), riñón de feto de mono rhesus (MA104), cornetes nasales de bovino (BT) y tráquea de codornices (QT35), con fibroblastos de embrión de pollo por su capacidad de propagar las cepas B1 y Texas GB del virus de Newcastle. La cepa Texas GB se propagó bien en todas las líneas celulares utilizadas en este estudío. Las células de testiculo de cerdo y las células QT-35 produjeron efecto citopatogénico similar al producido en fibroblastos de embrión de pollo. Sin embargo, las células de testículo de cerdo permanecieron adheridas cuando el efecto citopatogénico estaba ocurriendo, mientras que las células QT-35 infectadas se despegaron como lo hicieron los fibroblastos de embrión de pollo. La cepa B1 del virus de Newcastle se propagó en células de testículo de cerdo, en células MA-104 y en células de fibroblasto de embrión de pollo, pero mostró menor efecto citopatogénico comparado con la cepa Texas GB. El tratamiento previo con tripsina no aumentó el efecto citopatogénico con ninguna de las cepas de Newcastle en este estudio. Las muestras de suero evaluadas para titulos de anticuerpos neutralizantes para el virus de Newcastle tuvieron titulos significantemente más altos utilizando células de testículo de cerdo y comparándolas con los fibroblasto de pollo. Se observó una correlación alta entre los métodos de examen al microscopio y la micropueba con la tinción de tetrazolio para determinar la dilución final de la neutralización viral, sugiriendo que las células de testículo de cerdo y la microprueba con la tinción de tretazolio puede ser usada como alternativa a los fibroblastos de embrión de pollo y al examen al microscopio para la evaluación de los títulos de anticuerpos neutralizantes contra el virus de Newcastle.

Key words: Newcastle disease virus, virus neutralization, continuous cell line, MTT assay

Abbreviations: BT = bovine turbinate; CEF = chicken embryo fibroblast; CMF-PBS = calcium- and magnesium-free phosphate-buffered saline; CPE = cytopathic effect; DMEM = Dulbecco's modified Eagle's medium; EDTA = ethylenediaminetetraacetic acid; EID_{50} = mean embryo infective dose; ELISA = enzyme-linked immunosorbent assay; FBS = fetal

bovine serum; HI = hemagglutination inhibition; HRT18 = human rectal tumor; MA104 = fetal rhesus monkey kidney; MEM = minimum essential medium; MTT = 3-[4,5-dimethylthiazole-2-y1].2-5-diphenyltetrazolium bromide; NDV = Newcastle disease virus; QT35 = quail trachea; SPF = specific-pathogen free; ST = swine testicular; $TCID_{50}$ = mean tissue culture infectious dose; VN = virus neutralization; VN_{50} = 50% virus neutralization endpoint

The humoral immune response to Newcastle disease virus (NDV) is commonly evaluated by hemagglutination inhibition (HI), enzyme-linked immunosorbent assay (ELISA), agar gel precipitation, and/or the virus neutralization (VN) test (2). Conventionally, the HI test has been widely used and is an acceptable serologic technique for monitoring antibody levels against NDV in poultry (2). However, a lack of correlation has been reported between serologic responses, as measured by the HI test, and protection against virus challenge after vaccination with Newcastle disease vaccines (6). The HI assay has been reported to be unable to detect low levels of circulating antibody (6). Beard and Brugh (3) indicated that neutralizing antibodies to NDV in vaccinated birds could be demonstrated several months after vaccination by a variety of procedures even though the HI titers were negative. These results suggest that a more sensitive method for measuring the humoral antibody response is required for NDV to predict protective immunity.

VN is a test used frequently to determine the ability of serum antibodies to neutralize the infectious agent after vaccination (5). The most commonly used method to evaluate neutralizing antibody titers to NDV is based on the presence or absence of cytopathic effect (CPE) in chicken embryo fibroblast (CEF) cells (1,2). Such an assay requires a constant source of specific-pathogen-free (SPF) embryos and the preparation and maintenance of primary and secondary (CEFs) (4). Additionally, CEFs have a limited period of use. Typically, the neutralizing endpoint is determined by a microscopic examination of individual culture wells, and a quantitative estimation is made of the extent of CPE. Consequently, this methodology is time consuming and the results have an inherent subjectivity in endpoint determination (7).

Several microneutralization immunoassays have been described for quantitating neutralizing antibody titers (7,8,12,13) that have employed various methodologies for automation. Many assays have used an ELISA reader in place of microscopic examinations to obtain quantitative results. This saves time and labor and produces an objective determination of a neutralizing antibody titer endpoint.

The objectives of this study were to identify a continuous cell line capable of supporting NDV replication that could be used in a VN microassay and to adopt rapid methodology by utilizing ELISA reader technology.

MATERIALS AND METHODS

Media and reagents. Minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc., Gaithersburg, MD), and Hams F10–medium 199 mixture (1:1) were supplemented with 10% fetal bovine serum (FBS) (Atlanta Biological, Norcross, GA). Penicillin (100 IU/ml), streptomycin (100 μg/ml), and fungizone (0.5 μg/ml) were used to culture different cell lines. The maintenance media for propagating viruses or maintaining cells were the same as the growth media but without FBS. A calcium- and magnesium-free phosphate-buffered saline (CMF-PBS) was used for washing the monolayers. A solution of 0.05% trypsin supplemented with 0.53 mM ethylenediaminetetraacetic acid (EDTA) was used for cell passage. An MTT (3-[4,5-dimethylthiazole-2-y1].2-5-diphenyltetrazolium bromide; Sigma Chemical Co., St. Louis, MO) solution was prepared by dissolving 10 mg of MTT in 1 ml of CMF-PBS and solubilizing by sonication. The solution was then filtered through a 0.45-μm syringe filter and stored at 4 C in a lightproof bottle. The HC1-isopropanol (0.04 N HCl-isopropanol) solution was prepared by adding 40 ml of 1 N HCl to 1 liter of isopropanol. Then the HCl-isopropanol solution was stored at room temperature in a lightproof bottle.

Viruses. The lentogenic type B1, strain B1 (Intervet America Inc., Millsboro, DE), and the velogenic Texas GB (National Veterinary Services Laboratory, Ames, IA) strains of NDV were grown in 9-day-old embryonated SPF chicken eggs. Embryonated eggs were inoculated by the chorioallantoic route and incubated for 5 days at 37 C. The allantoic fluid was harvested and then clarified by centrifugation at 1000 × *g* for 30 min. The infectivity of the virus

was determined by establishing a mean embryo infective dose (EID_{50}) in embryonated eggs. The allantoic fluid was aliquoted and stored at −70 C.

Cell culture. CEFs and the following cell lines were used in this study: MA104 fetal rhesus monkey kidney, bovine turbinate (BT), HRT18 (human rectal tumor cells), swine testicular (ST), and QT35 (quail tracheal). CEFs were prepared from 9-day-old SPF chicken embryos (Hy-Vac Co., Gowrie, IA) and were cultured in DMEM containing 10% FBS. All CEFs were used at the third passage. The MA104, HRT18, and BT cells were grown in DMEM, whereas the ST cells were grown in MEM. The F10-199 medium was used to culture QT35 cells. All of these cells were grown to 80%–90% confluency and maintained in 25 cm^2 culture flasks (Corning Laboratory Sciences Co., Corning, NY) at 37 C in 5% CO_2. Cells were passaged every 4 days with trypsin-EDTA and placed into new 25 cm^2 culture flasks (2×10^4 cells) and incubated at 37 C in 5% CO_2.

Virus inoculation. Flasks containing monolayers of either CEFs or one of the continuous cell lines were washed twice with their respective serum-free media and were inoculated with 10^4 EID_{50}/ml (1 ml/flask) of NDV. After a 1-hr adsorption period at 37 C, the cell monolayers were washed twice with their respective serum-free media and 10 ml of maintenance medium was added. Additionally, NDV was pretreated with 10 μg/ml of trypsin for 60 min at 37 C and used as an inoculum at a final concentration of 0.5 μg/ml (10^4 EID_{50}/ml). Cells without virus and cells with 0.5 μg/ml trypsin were used as controls. The cell culture flasks were observed daily for 5 days for CPEs.

Serum samples. A number of serum samples, derived from SPF chickens previously vaccinated with NDV (type B1, strain B1), with different antibody titers were used in this study. The sera were heat inactivated at 56 C for 30 min and stored at −70 C until used. On the basis of the HI results, the sera were placed into low, medium, and high titer groups, respectively. Another eight serum samples were used to determine the effect of serum concentration on cell survival.

Virus titration in CEFs and ST cells. CEFs and ST cells were prepared in 96-well flat-bottomed tissue culture plates (Corning Laboratory Sciences Co.). Tenfold serial dilutions of the virus preparation were made in the respective FBS-free media. The virus titration ranged from 10^{-1} to 10^{-10}. One hundred microliters of each virus dilution was added per well with 12 replicate wells per dilution. The inoculated cultures were then incubated at 37 C for 3 days and examined for CPE. The mean tissue culture infective dose ($TCID_{50}$) was calculated according to the method of Reed and Muench (10).

VN assay. Twelve serum samples with different HI antibody titers were used for assessing the VN assay. Microscopic examinations were made to determine the amount of CPE. Alternatively, the tetrazolium dye, MTT, was used to measure the CPE. Fifty microliters of media was added into empty 96-well flat-bottomed tissue culture plates (Corning Laboratory Sciences Co.). Fifty microliters of the serum to be tested was added to the wells of the first column. Then, twofold serial dilutions were made across the plate to achieve a dilution range from 1:2 to 1:1024 (columns 1–10). A solution of 100 $TCID_{50}$ of the Texas GB strain of NDV, in a volume of 50 μl, was mixed with an equal volume of the diluted serum. The plates were incubated for 1 hr at 37 C in a humidified 5% CO_2 incubator. A 50-μl medium without serum was mixed with the virus suspension (column 11 of the plate) for the virus control, representing 0 neutralization. For the cell control, representing 100% neutralization, a medium without serum or virus was used (column 12 of the plate). One hour after incubation, 100 μl of the virus–serum mixture were transferred into 96-well culture plates containing ST cells or CEF monolayers and incubated at 37 C for 72 hr. At 69 hr of the incubation period, a microscopic examination of the plate was made before adding MTT. Then 10 μl of MTT (10 mg/ml) was added to each well and the plate was reincubated for 3 hr. At 72 hr of incubation, the plate was centrifuged at $1000 \times g$ for 10 min at room temperature. The supernatant was removed and 100 μl of 1 N HCl-isopropanol was added to each well to aid in dissolving the formazan crystals. The plate was shaken for 10 min on a plate shaker (mini-orbital shaker; Bellco Biotechnology, Vineland, NJ), and the cells were thoroughly resuspended by repeated pipetting with a micropipetter. The absorbance of each well was measured with a microtiter ELISA reader (Model EL310; Bio-Tek Instruments, Inc., Winooski, VT) at a wavelength of 550 nm.

All samples were tested in quadruplicate. The average of the absorbance was calculated for each serum dilution, the virus control, and the cell control wells. The absorbance value that was used to calculate the 50% virus neutralization endpoint (VN_{50}) for each serum was determined as follows:

[(average absorbance of cell control without virus)
+ (average absorbance of virus control)]/2

Then the average absorbance of each serial dilution was plotted for each sample tested. The serum dilution, which corresponded to a 50% neutralizing endpoint, as calculated above, was determined from the neutralization curve or by the method of Reed and Muench (10).

Serum effect on the MTT VN microassay. The purpose of this experiment was to determine whether the serum had an effect on the VN assay. Fifty microliters of medium was added to the first four rows

of the empty microtiter plate (Corning Laboratory Sciences Co.). An additional 50 µl of 1:2 diluted serum was added into the first well of each row. Two-fold serial dilutions were then performed across the plate to achieve a dilution range from 1:4 to 1:8190 (from column 1 to column 12). Subsequently, 50 µl of medium was added to the first four rows and 100 µl of medium was added to the remaining four rows. The serum dilutions were transferred into the CEFs or ST cell line in 96-well tissue culture plates and incubated at 37 C for 72 hr. At 69 hr of incubation, MTT was added and the VN procedure was performed as described above. A statistical analysis was conducted to determine if the serum had an effect on the cells.

Statistical evaluation. Statistical analyses were conducted with the SAS statistical software package (SAS Institute Inc., Cary, NC). Statistical significance was expressed as highly significant ($P < 0.01$), significant ($P < 0.05$), or not significant ($P > 0.05$). An analysis of variance (ANOVA) with Duncan's multiple comparison procedure was performed to determine the differences between neutralizing antibody titers as determined by the two methods (microscopic examination and MTT microassay) in both CEFs and ST cell lines. The correlation between neutralizing antibody titers for the two methods was evaluated by assessing the significance with the Pearson correlation coefficient (r), where $r > 0$ indicates a positive linear relationship and $r < 0$ indicates a negative relationship.

To ascertain whether the presence of serum had any effect in determining the VN_{50} in CEFs and ST cells, multiple linear regressions were used to relate absorbance values to dilution, allowing different serum samples to have different intercepts. The hypothesis of no serum effect was tested by comparing the fit of two regressions: one assuming no serum effect and the other allowing for a serum effect by on F-test. An ANOVA was also used to analyze the ST cell line.

RESULTS

Growth of Texas GB and B1 strains of NDV in CEFs and continuous cell lines. Microscopic examination of cell cultures infected with lentogenic B1 strain and the velogenic Texas GB strain of NDV revealed that the Texas GB strain replicated in all the continuous cell lines evaluated in this study. Replication of Texas GB strain produced distinctive CPE. However, only the ST and MA104 cell lines, in addition to the CEFs, supported the B1 strain of NDV. Less CPE was observed when compared with Texas GB strain. No CPE was detected when the B1 was inoculated into the other cell lines.

The CPE observed for the NDV strains was typical of that reported for NDV (4), whereby cell rounding and syncytia formation were present (Fig. 1). The extent of CPE induced by different NDV strains varied considerably (Table 1). The CPE appeared sooner with the virulent strain (Texas GB) than with the B1 strain. Typically, cell cultures that were infected with Texas GB displayed CPE between 48 and 72 hr after infection with the notable exception of HRT18 cells, in which CPE appeared after 96 hr. The greatest amount of cellular destruction was observed in CEFs, QT35, and ST cell lines infected with the Texas GB. In contrast, those cell cultures infected with the B1 strain (CEFs, ST, and MA104) showed less cell destruction, and at 96 hr after infection, much of the cell culture remained intact and not infected (Fig. 1).

Cell cultures infected with B1 and Texas GB that were pretreated with trypsin were not found to be different from the culture in which untreated viruses were used.

Titration of Texas GB strain of NDV in CEFs and ST. The Texas GB strain of NDV was titered in the CEFs and ST cell lines. The titers of Texas GB in CEFs were $10^{8.2}$ $TCID_{50}$/ml, whereas the titers in the ST were $10^{7.5}$ $TCID_{50}$/ml.

VN assay. Results of the comparison of NDV neutralizing antibody titers as determined by microscopic examination and MTT assay in both CEFs and ST cell lines are shown in Table 2. The neutralizing antibody titers of individual serum samples were statistically the same independent of cell line or method used. However, when all the samples were collectively compared (i.e., CEFs *vs.* ST cells) the neutralizing antibody titers in the ST cell line were significantly higher ($P < 0.05$) than those in the CEFs as determined by either the microscopic examination or the MTT microassay. The titers determined by the two methods (microscopic examinations and MTT microassay) in both CEFs and ST cell lines were highly correlated ($r = 0.967$ for CEFs, $r = 0.974$ for ST cell line).

Serum effect. The multiple regression data for both CEFs and ST cell lines indicated that

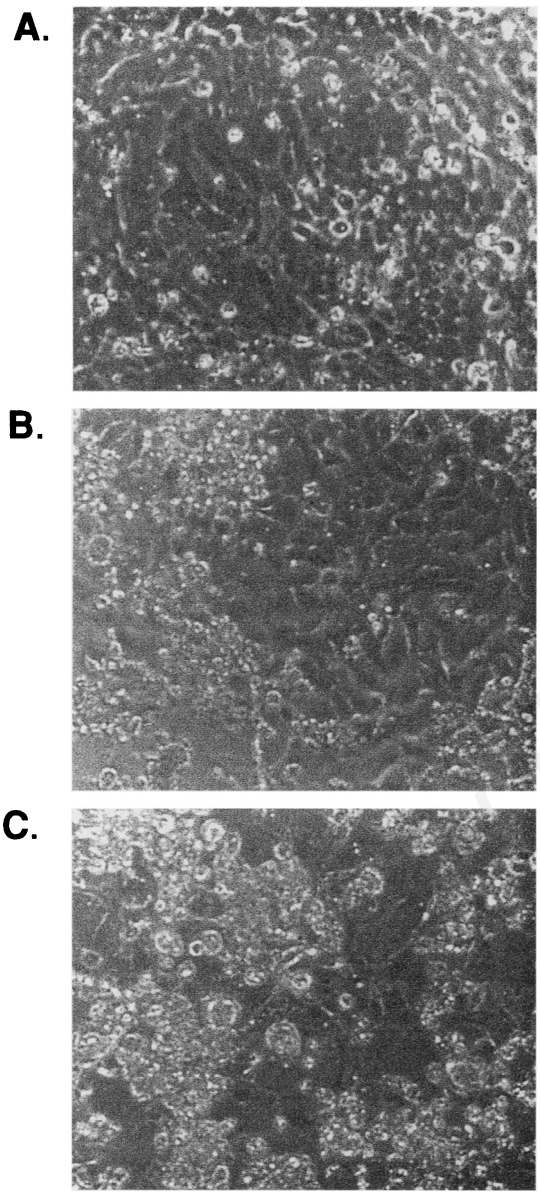

Fig. 1. Cytopathic effect of NDV in the ST cell line at 96 hr postinoculation. (A) Uninfected control monolayer (50×). (B) ST cell infected with B1, B1 strain of NDV (50×). (C) ST cells infected with Texas GB strain of NDV (50×).

the relationship between absorbance value and dilution was not the same for the cells with serum and cells without serum. The estimated relationship indicated that cells with serum were significantly lower than cells without serum at middle plate dilutions (see Fig. 2).

DISCUSSION

In the present study, different continuous cell lines were evaluated to identify a suitable continuous cell culture system that has the capacity to titer the NDV virus and to determine the system's potential for use in a rapid neutralization microassay. Use of the ST cell line afforded NDV replication with observable CPE of the Texas GB strain of NDV. Cytopathic changes were observed after 2 days of cultivation and were similar to those produced by CEFs. The ST cell line was convenient and easy to maintain in a 96-well plate cell culture system.

A comparison of the capacity of the Texas GB and B1 strains of NDV to replicate and display CPE revealed that the B1 strain replicated in MA104 cells, ST cells and CEFs but with much less CPE as compared with the Texas GB strain. In addition, the lentogenic B1 strain of NDV failed to produce detectable cytopathic effects in all other cell lines evaluated. Reeve and Poste (11) found that the capacity of different NDV strains to induce CPE in different cells was related directly to their virulence for chicks and fertile eggs. Trypsin pretreatment of NDV did not increase CPE in the method employed in this study, suggesting little, if any, effect on the NDV replication cycle of the B1 or Texas GB strain. However, Nagai *et al.* (9) reported that the presence of trypsin in the culture medium was required for efficient replication of the lentogenic strains of NDV in continuous cell lines. In their study, 2.5–10 μg/ml of trypsin was used to support lentogenic strains of NDV replication in various cells. This amount represents 5–20 times more than that used in the present study and may explain the difference in the results. However, Nagai *et al.* (9) could not apply the same procedure with all continuous cell lines (e.g., BHK21-F) because the cells could not be maintained as monolayers if such high levels of trypsin were present in the medium. Our experiences were similar, thus we elected to use a small amount of trypsin in order to maintain the cell monolayer. Unfortunately, the B1 strain did not display CPE under these conditions.

The ability of the ST cells to propagate Texas GB to high titer levels is an additional reason for utilizing the ST cell line in a VN assay. However, NDV grew to higher titers in CEFs than in the ST cell line. A possible explanation

Table 1. Cytopathic effect induced by the NDV strains B1, type B1 and Texas GB in CEFs and various continuous cell lines.[A]

Cell	B1	B1 + T[B]	Texas GB	Texas GB + T	C[C]	C + T
CEF	+	+	++++	++++	−	−
ST	+	+	++++	++++	−	−
MA 104	+	+	+++	+++	−	−
QT35	−	−	++++	++++	−	−
HRT18	−	−	+	+	−	−
BT	−	−	++	++	−	−

[A] + = less than 50% cytopathic effect, ++ = 60%–10% cytopathic effect, +++ = 70%–90% cytopathic effect, ++++ = 80%–100% cytopathic effect, − = no cytopathic effect.
[B] T = pretreated with trypsin.
[C] C = no virus, negative control cell culture.

for this titer difference between CEFs and ST cells may be differences in the sensitivity of the cells to the virus. NDV titers in the ST cell line might be increased by adapting NDV to the ST cell line by increasing the number of serial passages and/or by lengthening the incubation time, although the latter approach might reduce the sensitivity of the test because of nonspecific cell death.

Neutralizing antibody titers determined in the ST cells were significantly higher than their paired counterparts evaluated in CEFs. However, this difference was revealed only after all the data were analyzed collectively, when all neutralizing titers in the CEFs were compared with those determined in the ST cells. Therefore, although neutralizing antibody titers may have been higher in an individual serum sample when the ST cells were employed, the results were not significantly different for that individual sample. This subtle difference in neutralizing titers between the ST cells and CEFs may have been because of the difference in the way the two cell types support virus growth and the fact that the ST cell line has a longer life span than CEFs. The results of this study also revealed a high correlation between the VN assay with microscopic examination and the MTT

Table 2. Neutralizing antibody titers against NDV in 12 sera as determined by microscopic examination and MTT microassay in CEFs and ST cell line.

Groups	No.	HI[A]	Virus neutralization titer (reciprocal $\log_2$)			
			CEF-MICR[B]	CEF-MTT[C]	ST-MLCR[D]	ST-MTT[E]
Low titer	1	3	4	4.06	4.33	4.59
	2	3	3.5	3.66	4	4.36
	3	3	3.5	3.54	4	4.02
	4	3	3.5	3.56	3.66	4.7
Medium titer	5	6	7.67	7.63	8	7.24
	6	5	6	7.63	7.5	7.09
	7	5	6	5.78	7	7.54
	8	5	7.67	6.76	7.5	7.88
High titer	9	8	9.67	10.30	10.33	10.75
	10	8	9	9.16	10.33	10.05
	11	8	9	9.09	9.33	10.23
	12	8	8.67	9.52	9.33	10.34

[A] Hemagglutination inhibition titer.
[B] Virus neutralization titer in CEFs. The VN_{50} was determined on the basis of microscopic examination.
[C] Virus neutralization titer in CEFs. The VN_{50} was calculated on the basis of MTT microassay.
[D] Virus neutralization titer on ST cell line. The VN_{50} was determined on the basis of microscopic examination.
[E] Virus neutralization titer on ST cell line. The VN_{50} was determined on the basis of MTT microassay.

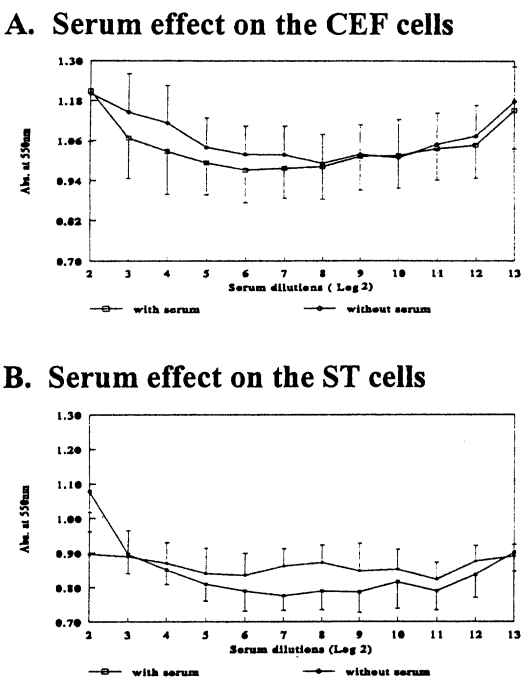

Fig. 2. The effect of the presence of serum in determining a VN_{50} in (A) CEFs and (B) the ST cell line. The lines represent the average absorbancies from eight serum samples. Error bars denote the standard deviation.

assay. However, no significant differences were found between the microscopic examination procedure and the MTT microassay procedure for determining the VN titer. These results support using the MTT assay as an alternative to microscopic examination. The MTT microassay would have distinct advantages for use with large numbers of serum samples. For example, the results are objective and lend themselves to automation because they are obtained with an ELISA reader instead of by visual examination. In addition, the data are easily transferred and analyzed by electronic means. This assay is less laborious and less time consuming, and the results are obtained in a short time.

In the present study, the VN_{50} used for determining the VN titer in the MTT microassay was ascertained by using the mean absorbance of the cell control (without serum) as representing 100% neutralization. This method has also been used by other investigators and has been an applicable method when measuring a large number of serum samples (12,13). However, Haddad (7) employed another approach for estimating the VN_{50} in which diluted serum without virus was added to the plate of the cells and used to calculate the percentage of VN for each serum dilution. Through this method, any serum effect that might influence the true value of VN_{50} was avoided. In the study by Haddad (7), the first dilution of each serum was used to represent the 100% neutralization value. This method seemed to be appropriate for positive sera; however, no explanation was given when a negative serum was used (i.e., the first dilution would not be 100% neutralizing). The results from the present study confirmed the existence of a serum effect. How the effect of sera would influence the true VN_{50} as determined in the study is not known from this study. In order to determine whether there is a significant effect, an experiment utilizing paired sera samples could be evaluated by using the estimated value (as in this study) and a method by which diluted sera without virus are incorporated into the plate design and used to determine a 100% neutralization and a 0 neutralization value. Comparison of the two procedures may be important in order to determine whether the true value of VN_{50} is significantly different from the estimated value.

In conclusion, the ST cell line was found to be a suitable alternative for CEFs for diagnostic and serologic assays. This cell line was easy to maintain, was more resistant to the toxic effects of serum, supported the growth of NDV, and lent itself to adaptation with the MTT/ELISA reader rapid methodology.

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