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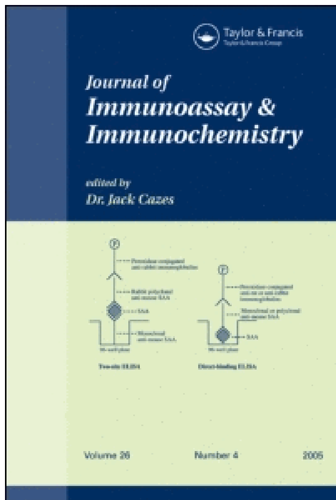

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
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To cite this Article Everitt, Einar and Varga, Mikael J.(1993) 'A Capture Enzyme-Linked Immunosorbent Assay for Virus Infectivity Titrations as Exemplified in an Adenovirus System', Journal of Immunoassay and Immunochemistry, 14: 1, 1 — 19
To link to this Article: DOI: 10.1080/15321819308019837
URL: http://dx.doi.org/10.1080/15321819308019837

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JOURNAL OF IMMUNOASSAY, 14(1&2), 1-19 (1993)

A CAPTURE ENZYME-LINKED IMMUNOSORBENT ASSAY FOR VIRUS INFECTIVITY TITRATIONS AS EXEMPLIFIED IN AN ADENOVIRUS SYSTEM

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA), employing a capturing antihexon monoclonal antibody specifically recognizing free hexons, was developed for quantitative infectivity titration of adenovirus in a microscale titration assay. The method is based on the quantitative assessment of the total excess production of the major structural protein late in infection in samples consisting of 10^5 virus-infected HeLa cells maintained as stationary suspension cultures. Results are obtained with a coefficient of variation of 10% within 50 hours after virus infection. The method was designed for monitoring substances interfering with viral replication, e.g., neutralizing antibodies or antiviral drugs. Since it measured the total antigen content associated with cells as well as antigens possibly released into the growth medium the general approach should be applicable to any viral system where a structural protein is synthesized in excess.

(**KEY WORDS:** adenovirus; infectivity titration; capture ELISA; hexon)

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INTRODUCTION

The original methods employed for titration of virus infectivity rely upon the production of visible or visualizable plaques, i.e. areas of local cell destruction following the radial diffusion of progeny viruses and the subsequent reinfection of neighbouring monolayer cells (1). One obvious advantage of the plaque technique is that the interpretation of the result always is straight forward and nondisputable. The rapidity of the plaque-formation is in essence determined by two factors: a) the length of the infectious replication cycle in the particular virus-cell system, and b) the efficiency of virus release from the infected cells. In the adenovirus system the plaque assay (2,3) suffers from the lengthy period of 8-10 days needed for plaque formation, although virus release may be enhanced to some extent by including 25 mM $MgCl_2$ in the growth medium and thereby yielding maximum plaque scores 2-3 days earlier (4). In all instances the requirements of healthy and intact monolayers are of utmost importance, together with the need for a "trained eye" in the process of plaque enumeration. An alternative to the actual plaque-counting procedure was the development of the fluorescent focus assay (5). In this assay the individually virus-infected cell is microscopically visualized by immunofluorescent staining within 48 h post infection. As in all plaque formation methods a large number of monolayer cell cultures are needed together with an enumeration process that is subject to personal judgement and variation. End-point cytopathic effect (cpe) assays may also be applied to the adenovirus system and results may be obtained within 3-4 days (3). The employment of tissue culture microtiter plates, proper staining procedures and automated readings would provide efficient handling of large sample series.

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In the present report we describe a method producing quantitative results on virus infectivity titrations within 50 h post infection. The method is particularly designed to measure the efficiency of substances inhibitory to virus replication and it is based on a quantitative immunological assessment of the excess production of the major adenoviral structural protein (the hexon antigen) in 1 ml stationary suspension cultures each of 10^5 cells.

MATERIALS AND METHODS

Cells and Virus

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HeLa cells were maintained in suspension cultures at densities of $2.5x10^5$ to $6x10^5$ cells per ml in Eagle's minimal essential medium modified for suspension cultures (S-MEM) (Flow Laboratories, Irvine, Scotland) supplemented with 3.5 % fetal bovine serum (FBS) (Flow Lab.) and 20 µg/ml of gentamicin (Biological Industries, Beth Haemek, Israel). The cells were routinely assayed for *Mycoplasma* contamination by a Mycoplasma T-C II kit (Gen-Probe, San Diego, Calif.).

The wilde type of human adenovirus 2 (Ad2) was propagated in HeLa cells in suspension cultures after a synchronous infection at an added multiplicity of infection (MOI) of 200 virions/cell. At 38 to 42 h post infection virus-infected cells were harvested by sedimentation, resuspended in 50 mM Tris-HCl buffer, pH 8.1, and ultrasonically disintegrated on ice. After solubilization of the homogenate in 1% n-butanol and centrifugation at $6{,}500xg_{av}$ for 20 min, virus was purified from the supernatant by two successive centrifugations in cesium chloride. The purified virus was extensively dialyzed (6), passed through a 0.22

μm Millex GV-filter, quantified spectrophotometrically (7) and stored frozen at -70°C.

Purification of the Ad2 Hexon Antigen

Hexon protein recovered from productively infected cells late in infection was isolated by anionic chromatography on DEAE-cellulose (8) and further purified by gel exclusion chromatography on Sepharose CL-6B as described previously (9).

Production of a Polyclonal Rabbit Antihexon Serum

Purified hexons were glutardialdehyde-fixed in the alkaline configuration in phosphate-buffered saline (PBS) at pH 7.5 and employed for immunization of rabbits as described previously (10). The serum obtained was used as the primary antibody in the present enzyme-linked immunosorbent assay (ELISA).

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Production and Purification of Monoclonal Antihexon Antibodies

Murine hybridomas producing monoclonal antibodies against the hexon were obtained by fusion of spleen cells from hexon-immunized Balb/c mice and mouse myeloma Sp2/0 cells (11). Fusions and cultivations were performed essentially as described by Köhler and Milstein (12), and Fazekas de St. Groth and Scheidegger (13). Hybridomas producing monoclonal antibodies against the hexon antigen were selected by screening the antibodies using an ELISA performed as described by Varga et al. (10). Positive cells were cloned at least twice by limiting dilution. The hybridomas were propagated as stationary suspension cultures and monoclonal antibodies were purified from the medium by affinity chromatography on a MASS protein A membrane (Nygene, Yonker, NY).

The antibodies were of the IgG_{2a} isotype with light chains of the kappa type as determined by a Mouse Typer ™ isotyping kit (Bio Rad Lab., Richmond, Calif.) The monoclonal antibodies were Ad2 type specific since they neither recognized free hexons of the Ad5 serotype (subgenus C, the same as Ad2), nor did they recognize hexons of the unrelated Ad3 of subgenus B.

Capture ELISA for Assessment of Free Hexons

High binding 96-well microtiter plates (Costar Europe Ltd., Badhoevedorp, The Netherlands) were coated with 50 µl per sample well of PBS containing 6 µg/ml of monoclonal antihexon antibodies. The plates were incubated for 1 h at room temperature and then at 8°C over night. Due to irregular results often obtained from the 36 periferal wells these were excluded in the assay. The plates were emptied and the wells were filled with 100 µl each of 5% fat free milk in PBS. After incubation for 3 h at room temperature, the plates were washed once in PBS plus 0.05% Tween 20 (PBST). Each well was then filled with 100 µl of PBS containing 1% bovine serum albumin (BSA) and 0.02% sodium azide and the plates were stored in sealed polyethylene bags at 8°C until used. Upon storage for one month such plates produced titration data indistinguishable from data obtained with freshly made plates.

All samples to be assayed consisted of 1 ml of culture fluids including 10^5 virus-infected cells. After ultrasonical treatment for 10 s at room temperature (using a Branson Ultrasonic disintegrator at a duty cycle of 0.5 and output control of 3) the samples had added to them Triton X-100 (Sigma Chemical Co., St. Louis, Mo) and antifoam (Antifoam Emulsion RD, Kebo AB, Stockholm, Sweden) from 10% and 1% stock solutions to give final concentrations of 0.5% and 0.02%, respectively. Depending on the anticipated results, such samples were

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first diluted between 20 and 40 times in PBS containing 1% BSA, 0.5% Triton X-100 and 0.02% antifoam.

Microtiter plates, blocked with 10% fat free milk in PBS and washed twice with PBST, were used to make serial two-fold dilutions of the test samples as well as of the hexon standards. Six two-fold dilutions were performed by sequential transfers in PBS/BSA/Triton X-100/Antifoam. From each dilution 50 µl aliquots were subsequently transferred to the coated and quenched ELISA-plates. Immediately before using the plates, these were washed once with PBST. The challenged plates were incubated for 2 h at room temperature, and then they were washed three times with PBST. To each well was subsequently added 50 µl of an antihexon serum (= primary antibodies) diluted 1:6,000 in PBST/1% BSA/0.01% antifoam. The plates were incubated for 1 h at room temperature, and then they were washed three times as above. Each well then received 50 µl of an anti rabbit IgG (= second antibody) conjugated with alkaline phosphatase (Sigma Chemical Co.) diluted 1:1,000 in PBST/1% BSA/0.01% antifoam. The plates were incubated for 1 h at room temperature and then they were washed three times as above. Finally, to all wells was added 50 µl of enzyme substrate (Sigma 104, 1 mg/ml in glycine-NaOH buffer, pH 9.8 containing 1 mM $ZnCl_2$ and 1 mM $MgCl_2$) and the plates were developed in the dark for 1 h at room temperature. Readings were performed at 410 nm with a reference at 490 nm using a Microplate Reader 600 (Dynatech Laboratories Inc., Chantilly, Va.).

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Mathematical Treatment of Data

Data obtained from the ELISA-reader were processed according to the following four parameter logistic function (14,15):

$$Y = [(a-d)/(1+(X/c)^b)]+d$$

where

Y is the reading value, i.e. the response,

X is the arithmetic dose, i.e. the dilution factor of the substance to be measured,

a is the background reading,

d is the maximum reading value,

c is the "effective dose 50" (ED_{50}), which is the dilution resulting in a response half way between a and d,

b is the slope factor, which determines the steepness of the curve.

In the present method the background reading (a) was the mean figure derived from three wells assayed with Mock-infected cells. The maximum value of the method (d) was likewise obtained from three wells assayed with 0.5 μg hexons per ml. By employing a non-linear regression analysis performed by a SigmaPlot program (Jandel Scientific, Corte Madera, CA) the parameters (c) and (b) were obtained. The hexon concentration of the sample was subsequently calculated based on the ED_{50}-value and its relation to the corresponding ED_{50}-value obtained from duplicates of standard curves with hexons in 2x6 wells starting at a concentration of 0.125 μg/ml.

A simple linear standard curve may be used with the limitation that the working range of the assay is reduced by one order of magnitude. This is because most of the measuring points of the unknown samples must fall within the range of the linear part of the standard curve. The advantage of the computerized processing of the measuring data is that all measuring points are employed even if most of them fall within the nonlinear part of the curve. If the standard curve model is utilized the curves of the unknown samples and the standard curve must be compared with each other at one selected point of absorbance in order to obtain correct and reproducible results.

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Virus Propagation in the Optimized Assay

A calculated number of HeLa cells was removed by sedimentation from a maintenance suspension culture and resuspended in stationary suspension culture medium (SS-MEM = S-MEM, non-essential amino acids (Flow Labs.) and 25 mM HEPES-buffer, pH 7.0) + 1% BSA to give a cell density of $1.5x10^7$ cells/ml, which at this stage was reestablished by enumeration in a Bürker counting chamber. Hundred microliter aliquots were distributed among a suitable number of 17x100 mm sterile, round-bottom cell culture tubes with caps (Becton Dickinson, Lincoln Park, NJ). Virions were added at desired MOIs and were allowed to attach at 37°C for 30 min on a shaking water-bath with orbital agitation. At the end of the attachment period 0.5 ml portions of SS-MEM supplemented with 3% FBS were added to the tubes, and from such diluted suspensions aliquots containing a counted number of cells in the range of $1x10^5$ to $1.5x10^5$ cells were removed and transferred to cell culture tubes containing SS-MEM with 3% FBS to give final volumes of 1 ml. The stoppered tubes were incubated at 37°C and harvested at 39 to 45 h post infection by mere submerging into a freezer at -70°C.

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RESULTS AND DISCUSSION

Specificity of the Capturing Monoclonal Antibody

Free hexons and purified virions were titrated on a hexon protein content basis in the optimized ELISA described in Materials and Methods. It is apparent that the monoclonal antibody selected for this assay preferentially reacted with free hexons in solution and only poorly recognized the corresponding epitopes of

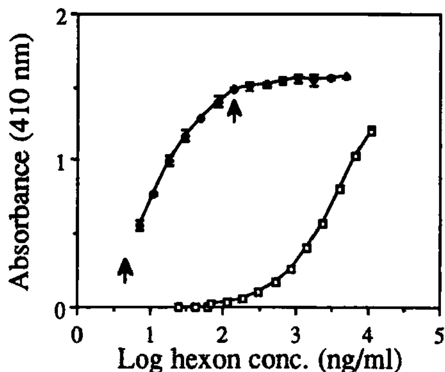

FIGURE 1. Quantitation of free and virion-associated hexons. The optimized capture ELISA as described in Materials and Methods was used to measure free hexons (◆) and hexons of purified virions (◇) on an equal hexon protein content basis. A figure of 140 µg of hexon per 10^{12} virions was used according to Maizel et al. (7,20). The arrows indicate the range of the standard curve in the optimized assay, and the vertical bars show the standard deviations obtained for the mean values of the measuring points from two separate determinations.

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virion-associated hexons, i.e. 300 times more virion hexons were needed to reach the same reading value as for free hexons within the linear working range of the assay (Figure 1).

HeLa and KB cells synthesize a vast excess of most of the structural proteins late in productive infection. Cells maintained in suspension as well as in monolayer cultures produce hexon antigen in the range between 2 and 4 µg per 10^5 cells (16). Due to the sensitivity of the ELISA this fact was considered when designing the method since a starting hexon concentration per sample of at the most 5 µg/ml was needed. In our laboratory the routine mass propagation of adenovirus in suspension cultures at a standardized density of 5×10^5 cells/ml will give 21,400±4,800 SD (n=12) purified virions per cell. Thus, when taking into account the total free hexon and virion yields of 2-4 µg and 2×10^9 virions (equalling 0.3 µg of hexons) per 10^5 cells, respectively, it is obvious that the 10-

fold excess of such hexons, together with the high antibody specificity, will provide that only free hexons are responsible for a positive signal in this immunoassay.

Thus, the present assay specifically quantifies the synthesis of the major late structural protein, whereas the fluorescent cell-counting procedure (5) monitors the number of initially infected cells harbouring the capacity to produce both hexons and virions after one cycle of replication. These aspects distinguish the present assay from the plaque counting techniques where the synthesized infectious progeny viruses are allowed to initiate reinfections of neighbouring cells to produce an area of cell destruction, i.e. the plaque. From this it follows that immunological methods may produce higher figures of the infectious titers since a certain percentage of the initially infected cells may produce noninfectious progeny virions or structural proteins without a concomitant virion assembly. This is not a drawback of the present method since it is designed to be used for the rapid and quantitative monitoring of e.g. neutralizing antibodies in epidemiological screening tests, or for large scale screenings of antiviral drugs. Any substance interfering with late transcription and the subsequent synthesis of a structural component most likely also affects the production of progeny viruses. However, especially when employed for the development of antiviral drugs such an assay should of course be checked with a method that measures the actual production of infectious progeny viruses.

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Antigen Yield as a Function of Cell Density

One of the aims of developing the present method was to accomplish quantitative infectivity titrations with the minimum use of mechanical equipment. We therefore investigated whether stationary suspension cultures would give

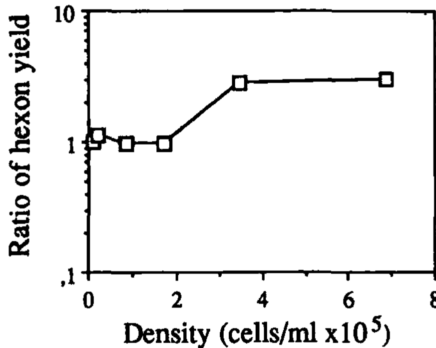

FIGURE 2. Ratio of the amount of hexons produced in agitated and stationary suspension cultures maintained at different cell densities. A calculated number of cells was virus-infected at an added MOI of 10 as described in Materials and Methods. At the end of the attachment period the cells were diluted 10-fold in SS-MEM and sedimented. The cells were resuspended in SS-MEM containing 3% FBS to give a density of 3.5×10^5 cells/ml and samples in duplicates were removed to create two parallel series of 1 ml cultures with densities between 0.1×10^5 and 6.9×10^5 cells/ml. One series was incubated on a roller drum and the other was incubated without agitation. At 41 h post infection the cultures were assayed for the total hexon antigen production as described in Materials and Methods. The calculated ratios, on a cellular basis, of the hexon titers recovered in agitated over stationary cultures are shown as a function of the cell concentration.

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hexon yields differing from cultures maintained under agitation. Thus, virus-infected cells at an added MOI of 10 were maintained at different cell concentrations in total volumes of 1 ml with and without agitation. It is apparent that up to a density of 2×10^5 cells/ml the hexon yields remained constant on a cellular basis irrespective of the mode of cultivation (Figure 2). At concentrations exceeding this level the hexon yields increased by a factor of 3 for the cells grown under agitation. An obvious reason for this could be a better access to nutrients upon agitation as the cell density increased.

To further study the hexon synthesis on a cellular basis, the yields were determined as a function of cell density of stationary cultures after infections at

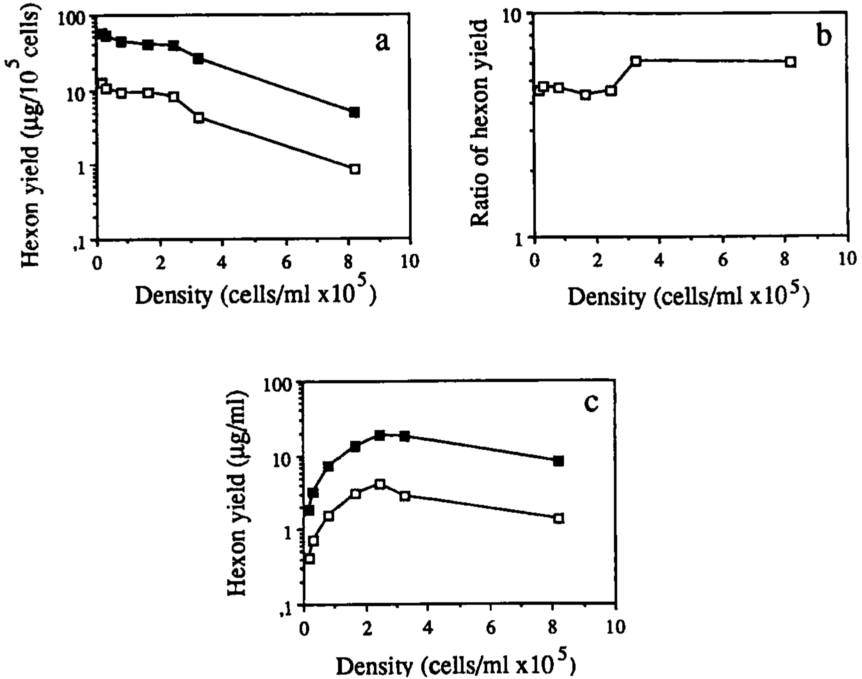

FIGURE 3. Hexon antigen production of Ad2-infected cells maintained as stationary suspension cultures at different densities. A calculated number of cells was virus-infected at added MOIs of 11 and 158 as described in Materials and Methods, and after attachment unadsorbed viruses were removed by sedimentation. The virus-infected cells were subsequently maintained at concentrations between $0.2x10^5$ and $8.2x10^5$ cells/ml and at 40 h post infection the accumulated hexon synthesis was determined as described in Materials and Methods. a) The total hexon yields on a cellular basis at MOIs 11 (□) and 158 (■) are plotted versus the cell densities. b) The hexon yield ratios for the 158 and 11 MOI-series are plotted versus the cell densities. c) The hexon yield per ml of total cell-medium homogenates at MOIs 11 (□) and 158 (■) are plotted versus the cell densities.

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added MOIs of 11 and 158. For densities in the range between $1x10^5$ and $2.5x10^5$ cells/ml the hexon yields on a cellular basis were reasonably insensitive to the cell concentration, but decreased drastically at densities of $3.3x10^5$ cells/ml and upwards (Figure 3a). When graphically representing the ratios of the actual hexon

yields on a cellular basis calculated for the two MOIs versus the cell densities, it was obvious that the ratios were almost constant between $0.2x10^5$ and $2.5x10^5$ cells/ml (Fig. 3b). When, from a technical point of view, considering the total hexon yield per ml of cell culture, a maximum production for both series was obtained at a density of approx. $2.5x10^5$ cells/ml (Figure 3c). Stationary suspension cultures of 1 ml at a density of approx. $1x10^5$ cells/ml were therefore chosen for the assay. This provides titration data of the hexon synthesis on a cellular basis, with reasonable insensitivity to slight variations in cell density, and thus only reflecting the added MOIs. These conditions would also allow for comparisons between separate experimental series performed on different occassions. Finally, the absolute concentrations of the hexon would fall within the proper range of the assay after 20 to 40 times dilutions of the starting samples.

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Hexon Yields as a Function of Added MOIs

For the purpose of performing sensitive titrations of substances inhibitory to the hexon antigen synthesis, which in most cases are likely to mirror effects on virus replication, an added lowest MOI was determined yielding a maximum hexon production. Thus, three parallel series were virus-infected with increasing MOIs. After attachment and subsequent dilutions, four repetitive samples from each MOI from one of the three series were removed to create an additional series of virus-infected cells. All samples were further incubated as 1 ml cultures. The cell cultures were subsequently assayed for hexon production as described in Materials and Methods. From a graphical representation of the data obtained from the three individually virus-infected series it is apparent that the hexon production reached a plateau at an added MOI of around 50 (Figure 4a). The mean coefficient of variation for the four repetitive samples derived from one and the same virus-infected tube was 9.4%, whereas the corresponding figure was 11.6%

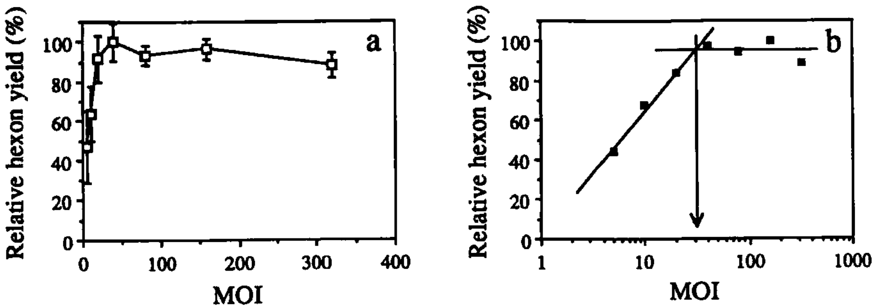

FIGURE 4. Hexon antigen yields in virus-infected cells as a function of added MOIs. A calculated number of cells was sedimented, resuspended and distributed among a suitable number of cell culture tubes in 100 µl volumes and at a density of $1.5x10^7$ cells/ml. The cells were virus-infected to create three parallel series at added MOIs between 5 and 320, and following incubation the cultures were assayed for total hexon production as described in Materials and Methods. a) The relative hexon yields are plotted versus the added MOIs. The vertical bars indicate the coefficients of variation for the mean values of the three separate experiments. b) From one of the three individually virus-infected series above four repetitive samples, from each MOI, were removed and incubated together with the three original series. The mean values of all determinations are graphically represented on a semi-log scale as a function of the cell density. The arrow indicates the extrapolated MOI-figure of added virions yielding a maximum hexon production.

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for the three parallel series each separately receiving the virus inoculum. As expected this simply demonstrates that the mere addition of virions to cells is a critical step, since repetitive removals from an already virus-infected cell suspension produced data of a somewhat higher degree of reproducibility. The accumulated technical errors inherited by the capture ELISA are indicated by the bars of standard deviation in Figure 1 and these are negligible. Replotting of all data on a semi-log scale gave upon extrapolation an approximate added MOI-figure of 30 for the break-point where no further increase in hexon yield was obtained in spite of the addition of higher MOIs (Figure 4b). This figure was subsequently applied as an operational infectivity unit producing a maximum

yield of hexons under the standardized conditions. Employing an added MOI of 30 ^{3}H-thymidine-labelled virions (88,000 cpm/10^{10} virions) per cell under the optimized conditions of the assay, the actual attachment level was shown to be 68%±2.7% (S.D.) for six repetitive attachment studies performed on one occasion.. The MOI-figure of the present assay for a maximum hexon production nicely fits with the previously obtained value in the corresponding titration assay using 30 times more cells in agitated suspension cultures and relying on rocket immunoelectrophoresis for the true quantification of progeny virions (17).

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Assessment of Virus Neutralization by Antihexon Antibodies

We have previously demonstrated that monospecific polyclonal antihexon antibodies are able to neutralize surface-bound Ad2 virions attached to HeLa cells in the cold (18,19). To verify the potential of the present method, HeLa cells were Ad2-infected at 4°C for 30 min at an added MOI of 60 virions/cell and at a cell density of $5x10^7$ cells/ml. These conditions would lead to an attachment level of 10-15% (19). After attachment the cells were freed of unadsorbed virus, serum-treated and washed as described in the legend of Figure 5. Such cultures were incubated and assayed for the total hexon production as described in Materials and Methods. It was shown that a 16-fold dilution of the antibodies still provoked a reduction in the hexon yield by 50% as compared with the untreated control (Figure 5).

In any other instance where the effects of a potentially interfering substance is to be investigated, this reagent may of course be supplied to the system at any suitable stage. Also depending on whether initial or long-term effects are under study, cells may be diluted or sedimented where appropriate, as long as 10^5 cells eventually are removed and grown in a total final volume of 1 ml of growth medium.

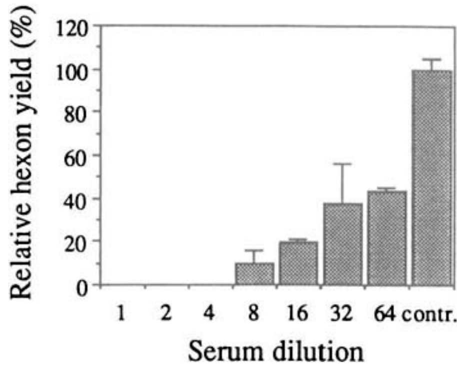

FIGURE 5. Assessment of the neutralizing capacity of an antihexon serum. A calculated number of cells was sedimented and virus-infected in the cold at an added MOI of 60. After attachment the cells were diluted 10-fold in ice-cold PBS and sedimented to remove unattached virus. The cells were resuspended in ice-cold PBS to give a density of $1x10^7$ cells/ml and then the cells were transferred in 100 µl aliquots to a suitable number of cell culture tubes kept on ice. Ten microliters of a serially diluted antihexon serum were added to the cells and these were further incubated at 4°C for 30 min. Then they were transferred to 37°C for a period of 10 min and after this incubation the cells were diluted 10-fold with PBS and sedimented at 300xg for 10 min to remove the neutralizing antihexon antibodies, which otherwise might interfere with the assay. The cells were resuspended in SS-MEM containing 3% FBS and $1x10^5$ cells were removed to create 1 ml suspension cultures. These were incubated and subsequently assayed for the total hexon production as described in Materials and Methods. The relative hexon yields are represented versus the dilution factors of the antihexon serum.

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In conclusion we believe that the present method may offer some advantage over the established plaque and fluorescent focus techniques when studying the inhibitory effects of antibodies and drugs on the replication of adenoviruses. The reasons for this are:

1) for each sample to be titrated, the method utilizes a limited and calculated number of cells in suspension culture, which are virus-infected under highly controlled conditions,

2) the method may easily be extended to assay large series of virus-infected cells for the purpose of screening the potential effects of antiviral drugs

or for surveillance of neutralizing antibodies in large scale epidemiological studies,

3) the immunological method provides quantitative and reproducible results within 50 h post infection, and

4) since the method assays the accumulated production of a structural protein associated with the cells together with viral proteins possibly released into the growth medium, the general approach should be applicable to any other similar viral system. Alternatively, with an appropriate set of immunological reagents, this microtitration assay may equally well be designed to measure the total amount of cell-associated and released progeny virions.

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ACKNOWLEDGEMENTS

The maintenance of the cell cultures by Blanka Boberg is gratefully acknowledged. We thank Ylva Blixt for a critical reading of the manuscript. This work was financially supported by grants from the Swedish Natural Science Research Council; Anders Otto Swärd's Foundation, Stockholm, Sweden; The Crafoord Foundation, Lund, Sweden; Carl Trygger's Foundation, Stockholm; and the Royal Physiographic Foundation, Lund.

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