A Quantitative Assay for Cytolysis Induced by Newcastle Disease Virus

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SUMMARY
We report here an assay for quantifying virus-induced lysis, in the absence of antibody and complement, produced within 2 h after adsorption. This technique makes use of {\textsuperscript{51}}CrO{	extsubscript{4}} release from cell monolayers pre-incubated overnight with the isotope. The release of {\textsuperscript{51}}Cr is specific for virus-induced lysis and is suppressible by 0-001 M-Ca\textsuperscript{2+}. This assay clearly distinguishes between wild-type Chinese hamster ovary (CHO) cells, clone K and a fusion-resistant mutant (CHO-15B), which was found to be resistant to virus-induced cytolysis. The stability of the association of isotope with monolayers of this cell type under the labelling conditions described makes this technique applicable to the study of the cytolytic effects of virus infection.

INTRODUCTION
Several viruses of the paramyxovirus group induce hemolysis and/or cell fusion mediated by the F glycoprotein in the virus envelope (Scheid & Choppin, 1974). Virus-induced hemolysis occurs in the absence of stabilizing Ca\textsuperscript{2+} ions. When Ca\textsuperscript{2+} is present, the activity of the F glycoprotein is seen not as cell lysis, but as fusion of infected cells (Okada & Murayama, 1966). Newcastle disease virus (NDV) has been extensively analysed and serves as a basis for studying the haemolytic activity of the paramyxoviruses.

In contrast to extensive studies reported on haemolysis (Bratt & Clavell, 1972; Clavell & Bratt, 1972), the mechanisms of NDV-induced fusion and lysis of cells in tissue culture have received relatively limited attention. The F glycoprotein appears to be associated with virulence in cell cultures and in avian species (Nagai et al., 1976). However, infected cells may remain viable and produce progeny virus for some time after extensive fusion has occurred (Compans et al., 1966). It has been suggested that fusion and cell death may occur by two separate mechanisms, namely accumulation of virus products (Alexander et al., 1973) or activation of lysosomes (Allison, 1967). The mechanism of the lytic activity of these viruses on susceptible cells in culture is also open to speculation. No cytolytic mechanism has yet been found in animal cell systems which might be deemed analogous to the role of lysozyme in the release of bacteriophage (Doerrmann, 1948).

A rapid and convenient quantitative assay for cell death has hitherto been lacking, the usual measurement being qualitative estimates of cytolysis based on direct microscopic observation (Bader & Morgan, 1961; Okada & Murayama, 1966), or quantitative estimates of cell killing based on reduction of cell cloning efficiency (Marcus & Sekellick, 1974; Toyama et al., 1977). Inhibition of host cell macromolecular synthesis, a relatively early event in virus infection, is significantly complicated by the diversion of cellular metabolic machinery to the synthesis of virus components and does not necessarily reflect, in a quantitative manner, the cessation of all cellular function.

Two methods frequently used to measure cytolysis induced by other agents are the uptake of trypan blue (Tennant, 1964) and release of {\textsuperscript{51}}Cr (Scaife & Brohee, 1967). Both methods are dependent on disruption of the permeability barrier at the plasma membrane.
$^{51}$Cr has been widely applied in immunocytotoxic assays, as in the characterization of serologically defined transplantation antigens (Wilson & Amos, 1972), or the detection of virus-specific antigens on infected cells (Smith et al., 1972; Glorioso et al., 1978) by measurement of antibody-dependent complement-mediated cytolysis. We report here the application of the $^{51}$Cr release technique to the measurement of cytolysis caused by NDV in the absence of antibody.

METHODS

Cell culture. Chinese hamster ovary (CHO) cells, wild-type clone K (hereafter designated CHO-wt), and mutant cells (CHO-15B) derived from the wild-type by resistance to ricin, were obtained through the courtesy of Kenneth Roozen of the University of Alabama, Birmingham. Cell stocks were maintained in monolayer cultures as previously described (Gallaher et al., 1973; Polos & Gallaher, 1979) except that Ham's F-12 medium was used instead of Eagle's minimal essential medium. For cytolysis experiments, cells were plated and used within 24 h, at a density of $8 \times 10^5$ cells/8 cm$^2$ dish.

Viral haemagglutination and haemolysis. Stocks of NDV strain F (N.J. – Roakin – 1946) were partially purified and assayed by haemagglutination microtitrations as described previously (Bratt & Gallaher, 1969; Bratt & Clavell, 1972; Clavell & Bratt, 1972). To determine haemolysis, freshly collected chicken erythrocytes were washed three times with phosphate-buffered saline without Ca$^{2+}$, pH 7.4 (PBS-A) (Dulbecco & Vogt, 1954) and adjusted to $10^8$ cells/ml. For $^{51}$Cr release experiments, erythrocytes were pelleted and 5 $\mu$Ci Na$^{51}$CrO$_4$ (New England Nuclear, Boston, Mass., U.S.A., 300 mCi/mg, sterile vanadium-free) were added to the pellet which was then incubated at 37°C for 1 h. The cells were washed by three to five cycles of dilution in PBS-A and centrifuged at 100 g until the level of supernatant radioactivity became stable at 1 to 2% of the cell-associated radioactivity. For assays of haemolysis, 0.5 ml of an erythrocyte suspension ($10^5$ cells/ml) was mixed with 0.1 ml chilled virus in PBS-A and allowed to react for 1 h at 4°C. Erythrocyte–virus mixtures were then resuspended in PBS-A prewarmed to 37°C (zero time) and incubated at 37°C for up to 1.5 h. Following incubation, the erythrocytes were pelleted at 100 g and the supernatants were removed. To measure the haemoglobin released, supernatant fluids were diluted with 3 vol. of PBS-A and absorbance at 540 nm was measured on a Beckman DB-GT spectrophotometer. To measure $^{51}$CrO$_4$ released, the undiluted supernatants were counted directly on a Beckman Biogamma counter.

Both haemolysis and $^{51}$CrO$_4$ release were expressed as the percentage of the maximum (100%) obtained by treating erythrocytes with 0.1 ml 0.03 M-NH$_4$OH in lieu of virus (Bratt & Clavell, 1972).

Chromium release from cells in culture. Monolayers of CHO-wt and CHO-15B cells were labelled with $^{51}$CrO$_4$ by addition of 5 $\mu$Ci to the medium (1 ml) in each plate. The monolayers were incubated for 10 to 12 h at 37°C. The medium was removed and the monolayers were washed three to five times with PBS until the level of spontaneously released radioactivity became stable. After cooling the cells with a final wash at 4°C, virus (0.1 ml) was added to the plate at the desired concentration and the monolayers were held at 4°C for 1 h. After virus adsorption, 1 ml PBS-A, pH 7.4, warmed to 37°C was added (zero time) and the plates were incubated at 37°C for up to 1.5 h. Following incubation, the supernatants were removed and centrifuged to pellet any suspended cells. Radioactivity in the supernatants was then measured in a Beckman Biogamma counter. In experiments designed to determine the time course of $^{51}$CrO$_4$ release from cells treated with NDV-F, spontaneous leakage from untreated control cells was measured at each time point.
Measurement of viral cytolysis

Fig. 1. Viral haemolysis of chicken erythrocytes. Freshly collected and washed chicken erythrocytes were incubated for 1.5 h with NDV-F, at the indicated concentrations. Haemolysis was measured by determination of haemoglobin (A540) and 51CrO4 (ct/min) in supernatant fluids.

Fig. 2. Time course of virus-induced 51CrO4 release from CHO-wt cells. Monolayers of CHO-wt cells were treated with NDV (40 000 HAU/ml). At the times indicated, radioactivity in the supernatant fluids was determined. Maximum radioactivity released by 0.03 M-NH4OH was 158 000 ct/min; total incorporated radioactivity was 190 500 ct/min. ●—●, Cells plus virus; ○—○, cells alone.

Calculations of the percentage release of chromium were made according to the following formula:

\[
\text{% Released} = \frac{\text{51Cr (ct/min) in maximum release} - \text{51Cr (ct/min) in spontaneous release}}{\text{51Cr (ct/min) in maximum release} - \text{51Cr (ct/min) in spontaneous release}} \times 100
\]

All values of 51Cr or haemoglobin release are the average of duplicate counts which varied by a maximum of 10 to 15%. As for erythrocytes, the maximum 51Cr release was determined by treatment of CHO cells with 0.1 ml 0.03 M-NH4OH in lieu of virus.

RESULTS

Release of 51Cr and haemoglobin from NDV-treated erythrocytes

We compared the release of 51CrO4 from chicken erythrocytes treated with NDV in the absence of Ca2+ with the release of haemoglobin (A540) by the techniques of Bratt & Clavell (1972). Fig. 1 shows that the two types of assay, when corrected for spontaneous leakage, are co-linear over a 100-fold range of virus concentration. The background of spontaneous release for both assays was 1 to 2% (not shown). The 51CrO4 released in 0.03 M-NH4OH (100% release) was 95% of the total isotope initially incorporated. By these criteria, measurement of haemolysis by haemoglobin release is indistinguishable from that based on 51CrO4 release.

Time course of cytolysis of CHO-wt cells

The optimum time for determining cytolysis in monolayer culture was established by the experiment represented in Fig. 2, which shows the time course of 51CrO4 release as data not
Yet processed by the formula in Methods. Release of $^{51}\text{CrO}_4$ was observed within 15 min after adsorption and after 1.5 h there was no significant change in the $^{51}\text{Cr}$ released, findings comparable to results obtained in previous studies of NDV-induced haemolysis (Bratt & Clavell, 1972). The rate of spontaneous isotope release from CHO-wt cells is also shown. In the absence of $\text{Ca}^{2+}$, an average of 1.4% of the absorbed $^{51}\text{Cr}$ was released/h, indicating that the label was firmly associated with the cells over this period. This low background compared favourably with the level of spontaneous release from erythrocytes. Lowest backgrounds were obtained with vanadium-free, $\text{Na}^{51}\text{CrO}_4$ of high specific radioactivity. The final ratio of $^{51}\text{CrO}_4$ released by treated cells to that released by control cells exceeded 10:1 and was greatest at 1.5 h when 56% of the total incorporated label, and 68% of that released by 0.03 m-$\text{NH}_4\text{OH}$, could be detected in the supernatants of NDV-treated cells.

**Comparison of cytolytic and haemolytic assays**

We compared the release of $^{51}\text{CrO}_4$ from erythrocytes by different concentrations of virus to release of $^{51}\text{CrO}_4$ from CHO cells which had been prelabelled overnight. As shown in Fig. 3, the degree of lysis of CHO cells determined by $^{51}\text{CrO}_4$ release compared favourably with enumeration of dead cells by uptake of trypan blue. Erythrocytes appeared to be more sensitive than CHO cells. The possibility that this was due to a higher inherent fragility of erythrocytes is supported by the experiment in Fig. 4. Spontaneous leakage of $^{51}\text{CrO}_4$ was measured after incubation of cells in PBS of various ionic strengths. Erythrocytes and cultured cells showed comparable $^{51}\text{Cr}^{}$ release at relative osmolarities of 0.5 to 1.0. However, at a relative osmolarity of 0.1 to 0.4, erythrocytes were four to seven times more fragile than cultured cells under our experimental conditions.

**Specificity of virus-induced cytology**

Since the haemolytic activity of NDV can be suppressed by interacting virus with erythrocytes in the presence of $\text{Ca}^{2+}$ (Bratt & Clavell, 1972), we measured release of $^{51}\text{Cr}$ from monolayer cells in 0.001 m-$\text{Ca}^{2+}$. As shown in Fig. 5(a), addition of $\text{Ca}^{2+}$ markedly inhibited the ability of NDV to induce $^{51}\text{Cr}$ release in CHO-wt cells. Additional evidence for the specificity of the release of $^{51}\text{Cr}$ was obtained by measuring lysis of mutant CHO cells. We have previously shown the mutant (CHO-15B) to be capable of attaching normal
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Fig. 4. Chromium leakage due to osmotic fragility. Chicken erythrocytes and CHO-wt cells were labelled with chromium and incubated for 1.5 h at 37 °C in PBS-A at ionic strength 0.1 to 1.0. Radioactivity of supernatant fluids from erythrocytes (●—●) and CHO-wt cells (O—O).

Fig. 5. Viral cytolysis of (a) CHO-wt and (b) mutant (CHO-15B) cells. CHO-wt and fusion-resistant CHO-15B cells were labelled, treated with virus and incubated in the presence or absence of 0.001 m-Ca²⁺. Supernatant fluid radioactivity of CHO-wt cells without Ca²⁺ (●—●); CHO-wt cells with Ca²⁺ (■—■); CHO-15B cells without Ca²⁺ (O—O); CHO-15B cells with Ca²⁺ (□—□).

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amounts of NDV, but to be resistant to NDV-induced fusion (Polos & Gallaher, 1979). Consistent with these phenotypes, the results shown in Fig. 5 (b) demonstrate that CHO-15B cells were resistant to NDV-induced cytolysis in either the presence or absence of Ca²⁺.

Additional evidence for the specificity of the observed cytolysis can be found in comparing the lysis of CHO cells induced by strains of NDV which differ in haemolytic activity. When grown in eggs, strain Italy-Milano 1945 (NDV-IM) requires 10-fold more virus, and strain Australia-Victoria 1932 (NDV-AV) 40-fold more virus, than NDV-F to induce comparable lysis of either CHO cells or erythrocytes (results not shown). Such strain specificities are consistent with the previous findings of Clavell & Bratt (1972).

DISCUSSION

The study of virus-induced cytolysis in tissue culture cells has been limited by lack of a reproducible and convenient quantitative assay. In the studies reported here, we have shown that the release of ⁵¹CrO₄ serves as an accurate measure of virus-induced cytolysis. The assay is rapid, easily performed, far less tedious than trypan blue exclusion and adaptable to cells either in suspension or in monolayer culture.

Use of NDV as a model system has demonstrated that cytolysis of monolayer cultures is parallel in all respects to the previous detailed studies of NDV-induced haemolysis by quantifying release of haemoglobin (Bratt & Clavell, 1972). Both haemolysis and tissue cytolysis occur within 1.5 h after adsorption of virus, and significant lysis does not occur in the presence of 0.001 m-Ca²⁺. While such conclusions have been stated for tissue cells before (Okada & Murayama, 1966), the only basis has previously been on qualitative microscopic observations. In both erythrocytes and monolayer cultures we have found that cells can be labelled to high specific activities, e.g. >10⁵ ct/min/10⁶ cells, and the isotope remains stably
associated with the cells for at least 4 h. Spontaneous leakage did not exceed 1%/h of the total incorporated as long as vanadium-free $^{51}\text{CrO}_4^-$, recently obtained from the supplier at high specific activity, was used.

Also, in parallel with haemolysis, which is known to be an all-or-none phenomenon with sudden and total release of all haemoglobin, virus-induced release of $^{51}\text{Cr}$ is due to nearly complete release from each cell affected by the viral cytolysin. This is supported by our findings that in erythrocytes the dose responses for release of haemoglobin and $^{51}\text{CrO}_4^-$ were superimposable, while in monolayer cultures the enumeration of dead cells by uptake of trypan blue was very similar to the relative release of $^{51}\text{CrO}_4^-$. The only significant difference between haemolysis and tissue cytolysis was an apparent higher sensitivity of erythrocytes to virus-induced lysis. Both a higher maximum release (80 to 90%), as well as higher release of $^{51}\text{CrO}_4^-$ at lower concentrations of virus, were found. This higher sensitivity could be due to either a difference in the virus interactions between erythrocytes and tissue cell membranes or simply a higher inherent fragility of erythrocytes to osmotic shock. The latter was documented in that erythrocytes were four to seven times more fragile than CHO cells to suspension in saline at ionic strengths 0.1 to 0.4 of that of PBS. Even though virus-induced $^{51}\text{CrO}_4^-$ release from monolayer cells did not exceed 50% in any of our experiments, we have noted that this is comparable to $^{51}\text{Cr}$ release in immunocytotoxic systems (Smith et al., 1972) and exceeds the $^{51}\text{Cr}$ release obtained by suspending CHO cells in distilled water for an equal period of time.

Sensitivity to NDV-induced cytolysis can be correlated with the interaction of the viral F glycoprotein with appropriate cellular receptors. Strains of virus deficient in inducing fusion were also deficient in inducing cytolysis (results not shown). Also, we have shown that a cell surface mutant, CHO-15B, previously determined to be insensitive to NDV-induced fusion (Polos & Gallaher, 1979), is also highly resistant to virus concentrations capable of inducing maximum lysis of wild-type CHO cells. The $^{51}\text{Cr}$ release we observed is therefore due to interaction of cell surfaces with a specific viral cytolytic component, rather than a reaction triggered by adsorption of virus or reaction with non-specific factors in the virus inoculum.

Sato et al. (1980) reported a similar assay for cytolysis of HeLa cells induced by Sendai virus. While documentation of the relationship of the observed cytolysis with the haemolytic activity of Sendai virus was lacking, it is likely that their assay is based on the same virus-associated activity as ours. The assays differ procedurally in that ours uses monolayer cultures rather than cells in suspension, dilute ammonia to determine maximum $^{51}\text{Cr}$ release rather than sodium dodecyl sulphate, and a prolonged labelling period at low (5 μCi) levels of $^{51}\text{Cr}$ rather than a short pulse label at high (40 μCi) $^{51}\text{Cr}$ concentration. We have reported generally lower backgrounds of spontaneously released $^{51}\text{Cr}$, which may relate either to methods used or sources of radioisotope.

The establishment of a quantitative assay for virus-induced cytolysis, together with the observation of low levels of spontaneous $^{51}\text{Cr}$ release, holds immediate promise in two areas. First, it should be possible to study the kinetics of cytolysis late in infection by a variety of non-enveloped as well as enveloped cytolytic viruses. While we have studied only ‘lysis from without’ due to a direct interaction of cells with high virus multiplicities (>100 p.f.u./cell) before significant virus replication (Doermann, 1948; Bratt & Gallaher, 1969), the assay should be applicable to the study of ‘lysis from within’ induced by low multiplicities at later times after virus replication as a cytopathic effect of productive infection. Thus, it may be determined, for instance, whether release of $^{51}\text{Cr}$ would precede or accompany a burst of progeny poliovirus or adenovirus; also, whether $^{51}\text{Cr}$ release accompanies or follows massive cell fusion. Second, studies on release of ions or fluorescent probes may permit characterization of the lesion produced by cytolytic virus interactions, just as the size and nature of the complement-mediated lesion in surface membranes was determined. Such
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information would add much-needed precision to our methods of investigating mechanisms of viral cytopathology.

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