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Cell Killing by Avian Leukosis Viruses

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Infection of chicken cells with a cytopathic avian leukosis virus resulted in the detachment of killed cells from the culture dish. The detached, dead cells contained more unintegrated viral DNA than the attached cells. These results confirm the hypothesis that cell killing after infection with a cytopathic avian leukosis virus is associated with accumulation of large amounts of unintegrated viral DNA. No accumulation of large amounts of integrated viral DNA was found in cells infected with cytopathic avian leukosis viruses.

Some avian leukosis viruses and all avian reticuloendotheliosis viruses can form plaques in chicken embryo fibroblasts (4, 5, 8, 10). Visualization of plaques requires specialized conditions such as treatment of cells with neutral red under agar (8). However, avian leukosis viruses of subgroups B, D, and F and reticuloendotheliosis viruses cause generalized cytopathic effects in infected avian cells during acute infection when virus spread and reinfection are allowed; this phenomenon does not require growth under agar or the presence of neutral red (15, 19).

After infection with a cytopathic avian leukosis virus, the cytopathic effects are first observed on day 2 or 3 after infection (acute phase) and persist until 8 to 10 days after infection. The cytopathic effects then disappear, and the cells resemble mock-infected cells and continue to multiply and release virus (chronic phase).

The cytopathic avian leukosis viruses cause characteristic changes in cells during acute infection; cells become granular, there is an increased number of rounded and floating cells, and there is a reduction in the number of cells attached to the culture dish relative to mock-infected control cultures or cultures infected with a noncytopathic avian leukosis virus (19).

A correlation exists between the reduction in cell number caused by the cytopathic avian leukosis and reticuloendotheliosis viruses and a transient accumulation of unintegrated linear viral DNA (9, 19). During acute infection, cells infected with cytopathic viruses contain 50- to 100-fold more unintegrated viral DNA than cells infected with noncytopathic viruses. During chronic infection with a cytopathic virus, the amounts of unintegrated viral DNA drop 50- to 100-fold from the maximal amounts found during acute infection. When virus spread is inhibited early after infection, there is a smaller reduction in cell number and no accumulation of large amounts of unintegrated viral DNA. These results are consistent with the hypothesis that the reduction in cell number and the accumulation of unintegrated linear viral DNA during acute infection are caused by early spread and superinfection by the cytopathic virus.

In the present work, we tested the hypothesis that the reduction in cell number in cytopathic virus-infected cultures is caused by cell death and detachment from the plate. We also tested the hypothesis that the cells that have large numbers of molecules of viral DNA die, leaving as survivors cells with small amounts of viral DNA. We found that infection with cytopathic avian leukosis viruses resulted in the actual killing of some cells in the population; these cells were recovered as nonadherent cells and were shown to contain proportionately more unintegrated viral DNA than live cells.

MATERIALS AND METHODS

Cells and viruses. Primary cultures of chicken embryo fibroblasts were prepared and characterized as previously described (13, 14, 19). The growth and assay of the avian leukosis viruses and spleen necrosis virus (SNV) used in this study were previously described (15, 16, 19).

Chicken embryo fibroblasts chronically infected with SNV were obtained as follows. Chicken cells in 100-mm petri dishes were infected with SNV and subcultured twice so that all cells were infected. After two passages (at which time the transient cytopathic effects had disappeared), the cells were plated in 100-mm petri dishes and used for superinfection.

Separation of dead and living cells. To harvest nonadherent cells, culture fluid was collected, and the plates were washed twice vigorously with TD buffer (0.14 M NaCl, 0.005 M KCl, 0.0007 M Na_2HPO_4, and 0.025 M Tris adjusted to pH 7.4 with HCl) to remove

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loosely attached cells. The harvested medium and the fluid from the washes were pooled and centrifuged. Adherent cells were harvested after trypsinization of the remaining cells on the culture plates.

Viability assays. The viability of the nonadherent and adherent populations of cells was tested in three ways.

(i) Renografin-Ficoll separation. The procedure for Renografin-Ficoll separation is similar to one used by Böyum (2) to separate living and dead cells. The cells to be tested (adherent or nonadherent) were suspended in standard medium at a concentration of 5×10^6 to 1×10^7 cells/ml. Then 5 to 10 ml of suspended cells was layered onto a 5-ml cushion of Renografin-Ficoll (1.077 g of Renografin per ml in 2.0% [wt/vol] Ficoll) and centrifuged at $350 \times g$ at room temperature for 15 min in an International centrifuge (model PR-2). Cells were recovered and counted with a Coulter counter.

(ii) Replating assay. After Renografin-Ficoll separation, cells were assayed for their ability to adhere to petri dishes and multiply. Cells from either the interface or the pellet of the Renografin-Ficoll cushion were suspended in standard medium and plated at densities of 5×10^5 to 5×10^6 cells per 60-mm petri dish (the usual cell density for plating chicken cells on a 60-mm dish is 6×10^5). Four hours after plating, fetal bovine serum was added to a final concentration of 3%.

(iii) Trypan blue exclusion test. A small volume of a suspension of nonadherent or adherent cells was mixed with an equal volume of the vital exclusion dye trypan blue (0.16% [wt/vol] in TD buffer), and the cells were counted in a hemacytometer.

Purification of total cellular DNA. Total cellular DNA was prepared from confluent monolayers of infected cells. DNA was extracted with phenol as described (19). The DNA was dissolved in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0), and the concentration was determined by measuring the optical density. When the DNA concentration was too low to measure by optical density, a spot test was performed (3). Serial twofold dilutions of a DNA standard and of the DNA to be measured were made in water containing 2 μg of ethidium bromide per ml. Then 0.005-ml portions of the DNA standard and the DNA to be measured were placed on Saran wrap and visualized with UV fluorescence. The DNA concentration of the unknown sample was determined (to within 15% accuracy) by comparing the UV fluorescence of the dilutions of the sample with the UV fluorescence of the dilutions of the DNA standard.

Separation of unintegrated and integrated viral DNAs. The procedure of Hirt (6) was used to separate high-molecular-weight DNA from low-molecular-weight DNA as described previously (19). Alternately, high-molecular-weight DNA was separated from low-molecular-weight DNA on 5 to 20% (wt/vol) NaCl gradients (0.01 M Tris-hydrochloride [pH 8.0]–0.005 M EDTA) (11). Total cellular DNA (100 to 200 μg) was loaded onto a 10-ml gradient and centrifuged at 35,000 rpm in a Beckman SW41 rotor for 6 h at 20°C.

Growth of Charon 21A clone λRAV-2 for DNA extraction. The Charon 21A clone containing the Rous-associated virus-2 (RAV-2) genome at its *SalI* site (λRAV-2, obtained from A. Skalka) (17) was grown at 30°C with shaking in *Escherichia coli* DP50 *supF* infected at a multiplicity of infection of 0.005 PFU/cell in 2× NZYDT growth medium. Phage were purified as described previously (11).

Preparation of nick-translated viral DNA hybridization probe. The RAV-2 insert from the λRAV-2 clone was isolated after digestion with the restriction enzyme *SalI* and purification by preparative agarose gel electrophoresis. Electroelution was used to elute the insert from the gel slice. Insert DNA was labeled in vitro by nick translation (12).

Gel electrophoresis, blotting, and molecular hybridization. Electrophoresis was carried out in 0.5 to 1% agarose slab gels (3 to 6 mm thick) containing 0.5 μg of ethidium bromide per ml. Electrophoresis, blotting, and hybridization with [^{32}P]cDNA or nick-translated ^{32}P-labeled viral DNA were performed as described previously (19) except for one modification: after electrophoresis, the agarose gels were treated in 0.25 M HCl for 15 min at room temperature to partially depurinate the DNA (18).

Autoradiography and densitometry scanning. Autoradiography was carried out at −70°C with Kodak X-Omat X-ray film as described (19).

DNA samples that were subjected to electrophoresis in parallel lanes, blotted onto the same nitrocellulose filter paper, and hybridized and autoradiographed were used to make measurements of the amounts of hybridizable DNA with a Joyce-Loebl microdensitometer. The exposure times used for scanning were such that the recorded densities were in the linear range of a standard dose-response curve. To obtain a measurement of number of molecules of unintegrated RAV-2 DNA, λRAV-2 DNA was used as a standard. λRAV-2 DNA was digested with *SalI* to generate a linear molecule of 5.0 megadaltons (Md). Serial two- to threefold dilutions of this molecule were subjected to electrophoresis in parallel with unintegrated viral DNAs from the Hirt supernatant fractions. The DNA was quantified as described above, and the numbers of copies of unintegrated viral DNA were determined with the λRAV-2 fragment used as a standard. To obtain a measurement of the number of molecules of unintegrated SNV DNA, a Charon 4A clone containing SNV (clone 14-44; 11) was used as standard. Clone 14-44 DNA was digested with *SacI* to generate a linear molecule of 4.6 Md. Unintegrated SNV DNA was subjected to electrophoresis in parallel with the standard DNA and quantified as described above.

Statistical analysis. Statistical analysis was performed by using the statistical program MINITAB at the Madison Academic Computing Center.

RESULTS

Infection with a cytopathic virus results in death and detachment of cells from the culture dish. Chicken embryo fibroblasts were infected with the cytopathic avian leukosis virus RAV-2, and nonadherent and adherent cells were harvested and counted at 2, 3, 4, 5, and 8 days after infection (Fig. 1). Three days after

infection and thereafter, the number of adherent RAV-2 infected cells was less than the number of adherent mock-infected cells. In contrast, by 3 days after infection and thereafter, there were more nonadherent RAV-2-infected cells than nonadherent control cells. At 4 days after infection, there were nine times more nonadherent RAV-2-infected cells than nonadherent control cells. At this time, approximately 25% of the RAV-2-infected cells were nonadherent, and there were pronounced morphological differences between the RAV-2-infected and control cultures (data not shown).

The viability of the adherent and nonadherent RAV-2-infected cells was determined by the trypan blue exclusion test (data not shown). Between 50 and 80% of the nonadherent cells were trypan blue positive. Approximately 10% of the adherent cells were trypan blue positive. Most of these were probably cells that would go on to become nonadherent.

Similar results were found with a technique that separates living and dead cells on the basis of their behavior when centrifuged through a Renografin-Ficoll cushion. After centrifugation, nonviable cells sediment through the Renografin-Ficoll cushion, whereas viable cells band at the top of the Renografin-Ficoll cushion. At 6 days after infection, nonadherent and adherent chicken cells infected with RAV-2 were harvested, loaded onto a Renografin-Ficoll cushion, and centrifuged. Most (60% or more) of the nonadherent cells were found at the pellet; no cells were observed at the interface (data not shown). Most (90%) of the adherent cells were found at the top of the Renografin-Ficoll cushion.

Replated cells from the pellet were not viable, even at 5 to 10 times the normal plating densities (data not shown). On the other hand, replated cells from the interface were able to attach and multiply.

These results show that separation of cells into nonadherent and adherent populations is an effective way to separate dead from live cells and that up to 25% of RAV-2-infected cells are dead. Therefore, we conclude that infection by cytopathic avian leukosis viruses results in the killing of some cells in the population. These cells become detached from the plate and can be recovered as nonadherent cells; adherent cells remain attached and either die later or go on to become chronically infected.

Autolysis of cellular DNA in nonadherent cells. Many of the trypan blue-staining cells observed had undergone extensive autolysis and appeared pycnotic or ghostlike. No cell nuclei could be observed in some cells. Further autolysis probably resulted in complete cell disintegration and probably explains the fact that the number of adherent and nonadherent infected cells was less than the number of adherent control cells.

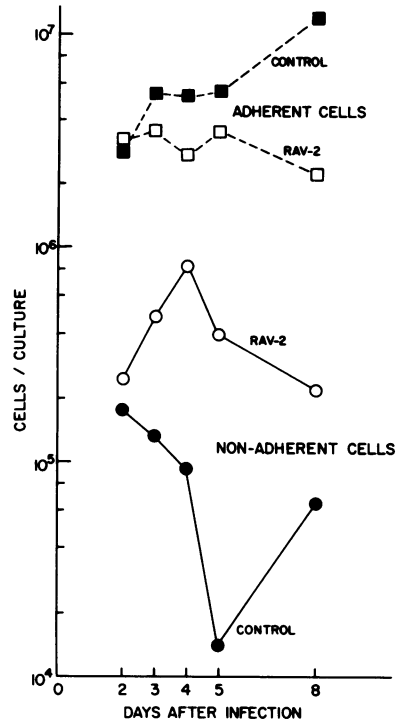

FIG. 1. *Number of nonadherent and adherent RAV-2-infected and control chicken cells. Chicken embryo fibroblasts on 100-mm culture dishes were mock infected or infected with RAV-2 at a multiplicity of infection of 0.25 PFU/cell. Nonadherent and adherent cells were harvested at 2, 3, 4, 5, and 8 days after infection, as described in Materials and Methods, and counted in a hemacytometer in the presence of trypan blue.*

The extent of autolysis in the nonadherent cells was studied by measuring the total cellular DNA recoverable from the nonadherent cells in relation to the amount of cellular DNA recoverable from adherent cells. The average of the amounts of total cellular DNA recovered from cells at 2, 3, 4, and 5 days after infection with RAV-2 was 1.4 pg/cell for nonadherent cells and 11.8 pg/cell for adherent cells. This significant reduction in the amount of DNA per cell recovered in the nonadherent cells relative to the amount in the adherent cells indicates that substantial degradation of DNA occurred in the nonadherent cells.

Figure 2 (lanes A and B) shows the extensive

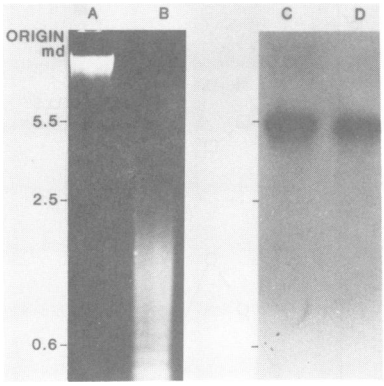

FIG. 2. *Agarose gel electrophoresis and autoradiogram of DNAs from RAV-2-infected nonadherent and adherent cells. Total cellular DNA was extracted from nonadherent and adherent RAV-2-infected chicken cells at 3 days after infection. The DNA was subjected to electrophoresis in a 0.5% agarose gel. In lanes A and B, the gel was stained with ethidium bromide, the DNA bands were visualized with UV fluorescence, and the gels were photographed. In lanes C and D, the DNA on the same gel was transferred to nitrocellulose filter paper and processed as described in Materials and Methods. Nick-translated RAV-2 [^{32}P]DNA was used as a hybridization probe. (A) Gel electrophoresis of total DNA from adherent RAV-2-infected chicken cells; (B) gel electrophoresis of total DNA from nonadherent RAV-2-infected chicken cells; (C) autoradiogram of DNA from adherent RAV-2-infected chicken cells; (D) autoradiogram of DNA from nonadherent RAV-2-infected chicken cells. Bacteriophage λ DNA was digested with EcoRI and served as a molecular weight standard.*

degradation of the total DNA from the nonadherent cells.

Amounts of unintegrated viral DNA in adherent and nonadherent cells. The hypothesis that cells that accumulate large amounts of unintegrated viral DNA die and become detached from the culture dish, leaving behind surviving cells that do not contain large amounts of viral DNA, was tested by measuring, after infection with a cytopathic virus, the amounts of unintegrated viral DNA in the nonadherent cells relative to the amounts of unintegrated viral DNA in the adherent cells. Figure 2 shows an autoradiogram of unintegrated viral DNA from the adherent (lane C) and nonadherent (lane D) RAV-2-infected chicken cells at 3 days after infection. Although extensive degradation of total cellular DNA was observed in the nonadherent infected chicken cells (Fig. 2, lane B), a clear band of viral DNA was observed in these cells (Fig. 2, lane D). The largest amounts of unintegrated viral DNA were found in the nonadherent cells at 3 to 4 days after infection (Fig. 3). At this time there were five to six times as many molecules of unintegrated viral DNA per microgram of cellular DNA in the nonadherent cells as in the adherent cells.

In the experiment described in Fig. 3, the number of molecules of unintegrated viral DNA was normalized to the amounts of cellular DNA recovered from the nonadherent or adherent

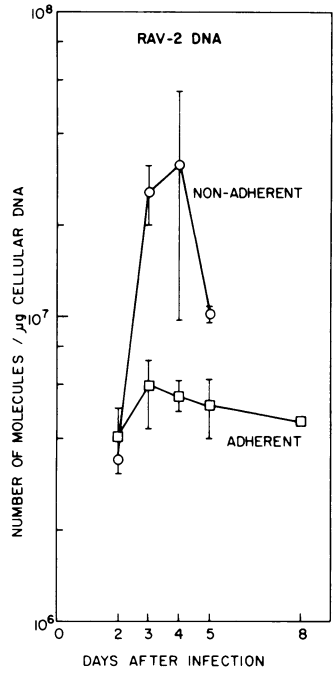

FIG. 3. *Amounts of unintegrated viral DNAs from nonadherent and adherent chicken cells infected with RAV-2. Chicken cells from the experiment described in the legend to Fig. 1 were fractionated by the Hirt extraction procedure. DNA was extracted, subjected to electrophoresis in a 0.7% agarose gel, and processed as described in Materials and Methods. Nick-translated RAV-2 [^{32}P]DNA was used as a hybridization probe. λRAV-2 DNA was digested with Sal-I and served as a standard for calculating numbers of molecules of unintegrated viral DNA. Twofold serial dilutions of the marker were subjected to electrophoresis in parallel with the DNA samples. The number of molecules of viral DNA was normalized per microgram of cellular DNA recovered. The amounts of viral DNA at each time point (with the exception of DNA from the nonadherent cells at 2 days after infection and the DNA from adherent cells at 8 days after infection) was measured on two or more separate gels, and the mean number of molecules per microgram of cellular DNA was plotted. Error bars span 2 standard deviations from the mean value. At 8 days after infection, the amount of cellular DNA in the nonadherent cells was very low; an accurate measurement of unintegrated RAV-2 DNA could not be made.*

cells. This normalization was performed because many cells in the nonadherent population had undergone extensive autolysis and had lost most or all of their DNA (see above). This normalization is valid only if unintegrated viral DNA is degraded to the same extent as total cellular DNA. The following experimental strategy was used to test this assumption. It had previously been shown that during chronic infection with SNV, a small but constant amount of unintegrated viral DNA (5.5 Md) persists for as long as the cells survive (9, 17). The amount of unintegrated SNV DNA in chronically infected cells was, therefore, used as a standard to determine the extent of degradation of low-molecular-weight DNA relative to total cellular DNA.

Chicken embryo cells, chronically infected with SNV, were superinfected with RAV-2. In parallel, normal, uninfected chicken cells were also infected with RAV-2. The numbers of nonadherent and adherent cells per culture were determined (S. K. Weller, Ph. D. thesis, University of Wisconsin, Madison, Wis., 1980). The results were similar to those presented above; there were fewer adherent RAV-2-infected cells (SNV chronic and normal chicken) than adherent, mock-infected SNV chronic chicken cells, and there were more nonadherent RAV-2-infected cells (SNV chronic and normal chicken) than nonadherent, mock-infected SNV chronic cells. The number of RAV-2-infected nonadherent cells peaked at 4 days after infection, at which time there were roughly 10 times more nonadherent RAV-2-infected cells than nonadherent mock-infected SNV chronic cells. At 4 days after infection, the number of trypan blue-positive cells in the two RAV-2-infected cultures comprised approximately 20% of the total cells and approximately 80 to 85% of the nonadherent cells. These results indicate that, as before, we were able to separate dead and living cells based on adherence to the culture dish.

Unintegrated RAV-2 DNA from the adherent and nonadherent cells was quantified by nucleic acid hybridization as described above (Fig. 4). The amounts of unintegrated viral DNA were normalized as before to the number of micrograms of total cellular DNA recovered. The numbers of molecules of RAV-2 DNA per microgram of cellular DNA in the RAV-2-infected chicken cells and in the RAV-2-infected SNV chronic cells are presented in Fig. 4A and B, respectively. The largest amounts of full-length unintegrated RAV-2 DNA were found at 3 to 4 days after infection in the nonadherent chicken cells and the nonadherent SNV chronic chicken cells (Fig. 4A and 4B, respectively). There were four- to fivefold more unintegrated RAV-2 DNA molecules per microgram of cellular DNA in the nonadherent RAV-2-infected cells than in the adherent RAV-2-infected cells (Table 1).

The numbers of molecules of SNV DNA in the nonadherent and adherent RAV-2-infected SNV chronic cells were also measured and were normalized per microgram of cellular DNA recovered (Table 2). The numbers of molecules of SNV DNA per microgram of cellular DNA in the nonadherent or the adherent cells were similar at 3 and 4 days.

The mean ratio of SNV DNA in nonadherent relative to adherent cells was calculated from separate measurements of the ratios taken at 3 and 4 days after infection (Table 1). The mean ratio, 1.4 ± 0.9, indicates that the amount of SNV unintegrated DNA in the nonadherent cells was approximately the same as in the adherent cells.

The mean ratio of unintegrated SNV DNA in nonadherent cells relative to that of unintegrated SNV DNA in adherent cells was compared according to a *t*-test in pairwise combinations with the mean ratios of RAV-2 DNA in nonadherent cells relative to RAV-2 DNA in adherent cells at 3, 4, and 5 days after infection (data presented in Fig. 3 and 4 and Tables 1 and 2). There were significant differences between the mean ratio of SNV DNA and the mean ratios of RAV-2 DNA at 3, 4, and 5 days after infection (P of 0.92, 0.96, and 0.99, respectively, by *t*-test). This result indicates that high-molecular-weight DNA was not degraded preferentially. Therefore, the normalization used in the experiment of Fig. 3 and 4 to determine the amounts of RAV-2 DNA in nonadherent and adherent cells was valid.

Amounts of integrated viral DNA in chicken cells infected with cytopathic and noncytopathic avian leukosis viruses. In the experiments described above, we demonstrated that there is a correlation between cell killing and the transient accumulation of unintegrated viral DNA. To determine whether there is also a correlation between cell killing and the accumulation of large amounts of integrated DNA, cells were collected at different times after infection with RAV-2 or RAV-1, and the number of molecules per cell of unintegrated and integrated DNA was measured by nucleic acid hybridization.

There were roughly 40 copies of unintegrated RAV-2 DNA per cell at 3 days after infection (Fig. 5). By 10 days after infection, there was an 80-fold decrease in the number of unintegrated RAV-2 DNA molecules per cell to approximately 0.5 molecules per cell.

At 3 to 4 days after infection, there was ap-

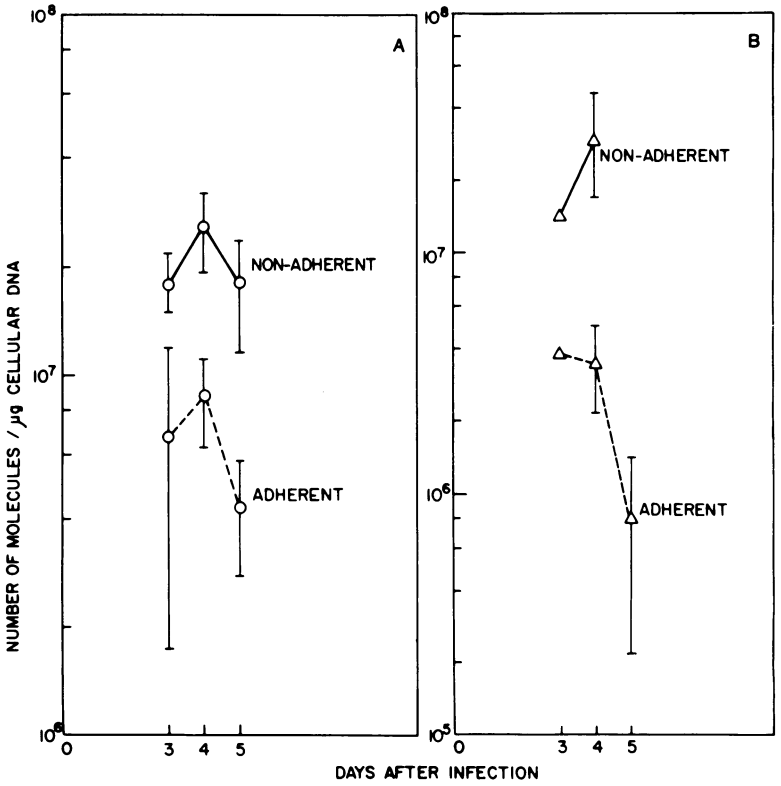

FIG. 4. *Amounts of unintegrated RAV-2 DNA in RAV-2-infected chicken cells and in RAV-2-infected SNV chronic chicken cells. Chicken embryo fibroblasts on 100-mm culture dishes were infected with RAV-2 at a multiplicity of infection of 0.15 PFU/cell. In parallel, SNV chronic chicken cells (prepared as described in Materials and Methods) were infected with RAV-2 at the same multiplicity of infection. Nonadherent and adherent cells were harvested at 2, 3, and 4 days after infection, as described in Materials and Methods. Total DNA was extracted from the nonadherent and adherent RAV-2-infected chicken cells (A) and RAV-2-infected SNV chronic chicken cells (B). The DNA was subjected to electrophoresis in a 0.5% agarose gel, which was processed, and the DNA was quantified as described in the legend to Fig. 3. The number of molecules of unintegrated RAV-2 DNA at each time point was measured on two or more separate gels, with the exception of the 3-day time points of the nonadherent and adherent RAV-2-infected SNV chronic chicken cells (B), which were measured once. The mean number of molecules of RAV-2 DNA per microgram of cellular DNA was plotted; error bars span 2 standard deviations from the mean value. In (B), at 5 days after infection, the amount of cellular DNA in the nonadherent cells was very low; an accurate measurement of unintegrated RAV-2 DNA could not be made. Symbols: (A) O——O, RAV-2 DNA in nonadherent cells; O-----O, RAV-2 DNA in adherent cells. (B) △——△, RAV-2 DNA in nonadherent cells; △-----△, RAV-2 DNA in adherent cells.*

proximately 1 molecule of unintegrated RAV-1 DNA per cell (Fig. 5). Thus, during acute infection there was approximately 40 times more unintegrated viral DNA in the RAV-2-infected cells than in the RAV-1-infected cells.

To quantify integrated viral DNA, the high-molecular-weight DNA (over 20 Md) was separated from low-molecular-weight DNA in sodium chloride gradients as described in Materials and Methods. When undigested RAV-1 high-molecular-weight DNA was subjected to electrophoresis and detected by hybridization (Fig. 6, lane A), all the viral DNA was found at the origin; no viral DNA was seen at the position of unintegrated viral DNA (5.2 Md). However, when undigested RAV-2 high-molecular-weight DNA was subjected to electrophoresis and hybridized, viral DNA was observed at the origin and at a position corresponding to unintegrated viral DNA (lane E). Thus, the high-molecular-weight RAV-2 DNA was contaminated with unintegrated viral DNA. To determine the amounts of integrated RAV-2 DNA, the amounts of contaminating unintegrated RAV-2 DNA were measured and subtracted from the total amounts of viral DNA measured, as de-

TABLE 1. *Mean ratios of viral DNA in nonadherent and adherent cells*

Days after RAV-2 infection	Mean ratio of RAV-2 DNA in nonadherent and adherent cells[a]	Mean ratio of SNV DNA in nonadherent and adherent cells[b]
3	4.2 ± 2.0[c]	
4	5.3 ± 3.1[d]	
5	4.2 ± 0.1[e]	
3 and 4		1.4 ± 0.9

[a] Calculated for each individual pair of measurements of RAV-2 DNA in nonadherent and adherent cells. Ratios from RAV-2-infected SNV chronic and RAV-2-infected normal chicken cells were pooled, and the mean ratio ± 1 standard deviation was calculated. Numbers shown are derived from the data presented in Fig. 3 and 4.

[b] Calculated for each individual pair of measurements of SNV DNA in the RAV-2-infected SNV chronic cells. The mean ratio ± 1 standard deviation was calculated from six separate measurements pooled from 3 and 4 days after infection. Numbers shown are derived from the data presented in Table 2. The ratios at 3 and 4 days were not significantly different.

[c] Average of four individual pairs of measurements, two from RAV-2-infected SNV chronic cells and two from RAV-2-infected normal chicken cells.

[d] Average of five individual pairs of measurements, two from RAV-2-infected SNV chronic cells and three from RAV-2-infected normal chicken cells.

[e] Average of two individual pairs of measurements from RAV-2-infected normal chicken cells.

TABLE 2. *Amounts of unintegrated SNV DNA in RAV-2-infected SNV chronic cells*

Days after RAV-2-infection	Type of cell	No. of molecules of unintegrated SNV DNA/μg of cellular DNA[a]
3	Nonadherent	$1.8 \times 10^6 \pm 1.2 \times 10^6$
	Adherent	$1.1 \times 10^6 \pm 2.2 \times 10^5$
4	Nonadherent	$1.1 \times 10^6 \pm 4.2 \times 10^5$
	Adherent	$1.2 \times 10^6 \pm 4 \times 10^5$

[a] Determined as described in Materials and Methods, using nick-translated SNV DNA (a gift of I. Chen) as a hybridization probe. The average of four or more separate measurements ± 1 standard deviation is shown.

scribed in the legend to Fig. 5. (The contaminating unintegrated viral DNA comprised 10 to 15% of the total viral DNA in the high-molecular-weight samples.)

There were 10 to 20 copies of integrated RAV-2 DNA per cell (Fig. 5). Thus, there was not a significant decrease in the amount of integrated RAV-2 DNA per cell as the cells progressed from the acute to the chronic phase of infection; this is in contrast to the observed 80-fold decrease in the amount of unintegrated RAV-2 DNA in the same time period. (We previously reported a fivefold drop in the amount of infectious viral DNA in the Hirt pellet fraction as the cells progressed from acute to chronic infection [19]; however, this drop was probably the result of the presence of contaminating unintegrated viral DNA in the Hirt pellet fractions.)

At 3, 4, and 10 days after infection with RAV-1, there were two to four copies of integrated RAV-1 DNA per cell (Fig. 5). There appear to be roughly 5 to 10 times more integrated RAV-2 DNA than integrated RAV-1 DNA per cell during acute infection, in contrast to the 40-fold

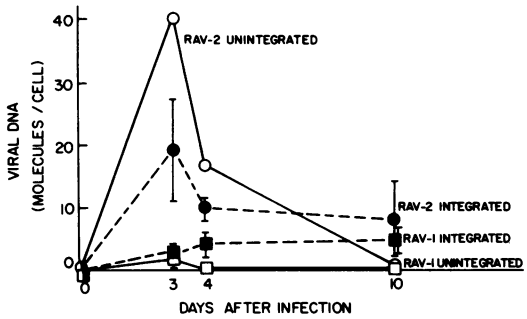

FIG. 5. *Amounts of unintegrated and integrated viral DNAs in RAV-1- or RAV-2-infected cells. Unintegrated and integrated viral DNA was quantified as described in the text. Nick-translated RAV-2 [^{32}P]DNA was used as a hybridization probe. The amounts of integrated DNA were determined as follows. The high-molecular-weight DNA was digested with EcoRI, and the amount of DNA in the 1.5-Md band was measured (see Fig. 5). λRAV-2 DNA was digested with SalI and EcoRI, and the 1.5-Md fragment served as a standard for calculating the number of molecules of total viral DNA. The unintegrated viral DNA in each sample of high-molecular-weight DNA was quantified by subjecting undigested DNA to electrophoresis. λRAV-2 DNA was digested with SalI and the 5.2-Md fragment served as a standard for calculating the numbers of molecules of unintegrated DNA. The amount of unintegrated viral DNA in each high-molecular-weight sample was subtracted from the total viral DNA in each sample in order to determine the amount of integrated viral DNA. The number of molecules of unintegrated and integrated viral DNA per cell was calculated from two or more separate measurements, and the mean values were plotted. Error bars for the numbers of molecules of integrated viral DNA per cell span 2 standard deviations from the mean value. Error bars are not presented for the numbers of molecules of unintegrated viral DNA per cell. The standard deviations were as follows: for unintegrated RAV-2 DNA, 18 at 3 days and 4 at 4 days; for unintegrated RAV-1 DNA, 1 at 3 days and 0 at 10 days. When no standard deviation is shown, only one measurement was made.*

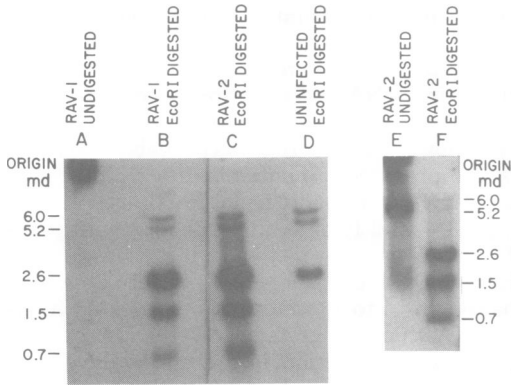

FIG. 6. *Autoradiogram of high-molecular-weight DNA from RAV-1- or RAV-2-infected cells before and after digestion with EcoRI. Chicken embryo fibroblasts in 100-mm petri dishes were exposed to RAV-1 or RAV-2 at a multiplicity of infection of 2.5 or 1.0 IU/cell, respectively. Total cellular DNA was extracted at 4 days after infection, and high-molecular-weight DNA was obtained after centrifugation in sodium chloride gradients. High-molecular-weight DNA was digested with EcoRI and subjected to electrophoresis in a 0.5% agarose gel. Five to 10 μg of cellular DNA was used per slot. The gel was processed as described in Materials and Methods. Nick-translated RAV-2 [^{32}P]DNA was used as a hybridization probe. (A) Undigested RAV-1 high-molecular-weight DNA at 4 days after infection; (B) EcoRI-digested RAV-1 high-molecular-weight DNA at 4 days after infection; EcoRI-digested RAV-2 high-molecular-weight DNA at 4 days after infection; (D) Eco-RI-digested uninfected chicken cell DNA; (E) undigested RAV-2 high-molecular-weight DNA at 4 days after infection; (F) EcoRI-digested RAV-2 high-molecular-weight DNA.*

observed difference in the amounts of unintegrated DNA in these cells.

DISCUSSION

In the present study, we demonstrated that infection with a cytopathic virus results in detachment of killed cells from the culture dish. We also demonstrated that the detached dead cells contain approximately five to six times more unintegrated viral DNA than cells which remain attached to the culture dish. The actual accumulation of unintegrated viral DNA in nonviable cells may be greater than the observed five- to sixfold accumulation, since many of the adherent cells eventually will die and probably have begun to accumulate unintegrated viral DNA. This result is consistent with the hypothesis that cells that accumulate large amounts of unintegrated viral DNA die and become detached from the culture dish, leaving behind surviving cells that do not contain large amounts of viral DNA.

In addition, we found no accumulation of large amounts of integrated viral DNA in cells infected with cytopathic avian leukosis viruses.

Virus production. Virus production by cells infected with noncytopathic viruses is 20- to 400-fold lower than virus production by cells infected with cytopathic viruses (17, 19; Weller, Ph.D. thesis, 1980). However, the levels of integrated viral DNA in cells infected with RAV-1 or RAV-2 differed only by 5- to 10-fold (Fig. 5). Thus, there does not seem to be a simple correlation between virus production and amounts of integrated viral DNA (1, 9).

Mechanism of cell killing. The mechanism of cell killing by cytopathic viruses is still unknown, but cell killing is correlated with an accumulation of 300 to 400 molecules of unintegrated viral DNA per cell. This accumulation of unintegrated viral DNA may be toxic to cells either by itself or as a result of RNA or proteins coded by it. We are currently performing experiments to test whether the unintegrated viral DNA can be used as a template for RNA synthesis. We have found no correlation between the amount of unintegrated viral DNA and the amount of virus production (17, 19; Weller, Ph.D. thesis, 1980), indicating that unintegrated DNA is not used as a template for virus production.

Alternatively, it may be that it is not the accumulation of viral DNA (or its products) that is toxic to cells, but rather the multiple superinfections could cause both cell killing and viral DNA accumulation. Further experiments will be necessary to elucidate the mechanism of cytopathogenicity by avian retroviruses.

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LITERATURE CITED

1. **Battula, N., and H. M. Temin.** 1978. Sites of integration of infectious DNA of avian reticuloendotheliosis viruses in different avian cellular DNAs. Cell **13**:387-398.
2. **Böyum, A.** 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. **21**(Suppl. 97):77-89.
3. **Davis, R. W., D. Botstein, and J. R. Roth (ed).** 1980. Rapid estimation of DNA concentration using ethidium bromide, p. 184-185. *In* Advanced bacterial genetics. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
4. **Dougherty, R. M., and R. Rasmussen.** 1964. Properties of a strain of Rous sarcoma virus that infects mammals. Natl. Cancer Inst. Monogr. **17**:337-350.
5. **Graf, T.** 1972. A plaque assay for avian RNA tumor viruses. Virology **50**:567-578.

6. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
7. **Ju, G., L. Boone, and A. M. Skalka.** 1980. Isolation and characterization of recombinant DNA clones of avian retroviruses: size heterogeneity and instability of the direct repeat. J. Virol. **33**:1026–1033.
8. **Kawai, S., and H. Hanafusa.** 1972. Plaque assay for some strains of avian leukosis virus. Virology **48**:126–135.
9. **Keshet, E., and H. M. Temin.** 1979. Cell killing by spleen necrosis virus is correlated with a transient accumulation of spleen necrosis virus DNA. J. Virol. **31**:376–388.
10. **Moscovici, D., D. Chi, L. Gazzolo, and M. G. Moscovici.** 1976. A study of plaque formation with avian tumor viruses. Virology **73**:181–189.
11. **O'Rear, J. J., S. Mizutani, G. Hoffman, M. Fiandt, and H. M. Temin.** 1980. Infectious and noninfectious recombinant clones of the provirus of SNV differ in cellular DNA and are apparently the same in viral DNA. Cell **20**:423–430.
12. **Rigby, P. W. J., M. Dickmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase. J. Mol. Biol. **113**:237–251.
13. **Temin, H. M.** 1967. Studies on carcinogenesis by avian sarcoma viruses. V. Requirement for new DNA synthesis and for cell division. J. Cell. Physiol. **69**:53–63.
14. **Temin, H. M.** 1968. Studies on carcinogenesis by avian sarcoma viruses. VIII. Glycolysis and cell multiplication. Int. J. Cancer **3**:273–282.
15. **Temin, H. M., and V. K. Kassner.** 1974. Replication of reticuloendotheliosis viruses in cell culture: acute infection. J. Virol. **13**:291–297.
16. **Temin, H. M., and V. K. Kassner.** 1976. Avian leukosis viruses of different subgroups and types isolated after passage of Rous sarcoma virus-Rous associated virus-0 in cells from different ring-necked pheasant embryos. J. Virol. **19**:302–312.
17. **Temin, H. M., E. Keshet, and S. K. Weller.** 1980. Correlation of transient accumulation of linear unintegrated viral DNA and transient cell killing by avian leukosis and reticuloendotheliosis viruses. Cold Spring Harbor Symp. Quant. Biol. **44**:773–778.
18. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. **76**:3683–3687.
19. **Weller, S. K., A. E. Joy, and H. M. Temin.** 1980. Correlation between cell killing and massive second-round superinfection by members of some subgroups of avian leukosis virus. J. Virol. **33**:494–506.