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# Target Cells for Avian Myeloblastosis Virus in Embryonic Yolk Sac and Relationship of Cell Differentiation to Cell Transformation

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The yolk sac of the 12-day chicken embryo retains the blast stage progenitors to cells of the myeloid lineages with a very low level of contamination by more mature myeloid cells which have begun to express the characteristic myeloid cell markers. Both in vivo and in vitro experiments have supported the hypothesis that target cells for the BAI-A strain of avian myeloblastosis virus are contained within the myeloid lineages. An assay system for avian myeloblastosis virus was developed which utilizes this yolk sac cell system and which appears to be more sensitive than previous published assays. In addition, the kinetics of a liquid culture transformation system is presented in which at least 4% of the yolk sac cell population was transformed in a relatively synchronous fashion at 2 days after infection. The morphological transformation preceded an increased rate of cell proliferation. Cell separation procedures provided a 10- to 20-fold enrichment of target cells and demonstrated that the target cell population copurifies with macrophage colony-forming cells which are the committed progenitors to the macrophage lineage. In combination with earlier work, this work demonstrated that cells committed to the macrophage lineage at all stages of differentiation may serve as target cells for infection by avian myeloblastosis virus.

The acute, transforming avian leukemogenic viruses generally exhibit a restricted set of potential target cells. In vitro studies have demonstrated that avian myeloblastosis virus (AMV) is the most restricted of this group and represents the only one which fails to transform fibroblast cultures (14). When this virus is injected into chickens (2) or quail (23), it produces the well-described myeloblastic leukemia. All other neoplastic responses which have been observed after the injection of the AMV complex have relatively long latent periods (8) and are known to be also induced by the MAV-1 and MAV-2 helper viruses present in the virus stock (2, 21). Transformation in vitro by AMV appears to occur in tissues which contain immature hemopoietic cells or high levels of myeloid cells (1, 16). The cells derived from in vivo tumors and the cells derived from in vitro transformation of hemopoietic cells are relatively homogenous and represent similar, if not identical, phenotypes. Thus, in vitro systems appear to provide a reasonable copy of in vivo processes, and the target cells appear to be restricted to cells in the myeloid lineage.

Most cell populations which can be utilized as target cells contain a mixture of myeloid cells at various stages of cell differentiation or maturation (1). This is particularly true of the most commonly utilized bone marrow cells (13, 15). In their early experiments, Moscovici et al. suggested that the adherent macrophage population can serve as target cells (20, 21). These results have been confirmed more rigorously by us (12). Although the results with bone marrow cell populations raise the possibility that less mature cells may also serve as target cells for infection by AMV, the exact characterization of the target cells has remained unclear. The use of cell separation procedures has refined the description of these target cells; however, the low efficiencies of the assays employed limit the interpretation of the conclusions reached (13, 15). An additional complicating factor is that successful infection and successful transformation were generally measured together as a composite. Little attempt has been made to investigate the possible differential sensitivities to infection alone. It is known that infection depends on continued cell proliferation (7, 12) and that for macrophages, this process is controlled by specific growth factors (27).

The 12-day chicken embryo yolk sac contains a high concentration of hemopoietic progenitor cells. For cells in the myeloid lineages, this population consists almost entirely of cells at the blast cell stage, before the expression of any myeloid-specific markers (5; E. M. Durban, Ph.D. thesis, University of Pennsylvania, Philadelphia, 1980; E. M. Durban and D. Boettiger; manuscript in preparation). About 5% of the nonerythroid cells in fresh primary cultures express the earliest markers of either the granulocyte or the macrophage lineage. In vitro, in the presence of chicken serum factors, this cell population undergoes a single, relatively synchronous wave of cell differentiation: granulopoiesis reaching a peak at 3 days and macrophage differentiation reaching a peak at 5 days. There does not appear to be any significant progenitor cell self-renewal in this system. The nature of this population makes it useful in analysis of whether myeloid cell progenitors are susceptible to AMV.

## MATERIALS AND METHODS

**Cells and viruses.** Twelve- to fourteen-day SPAFAS chicken embryo yolk sacs were dissected free of other embryonic membranes. The yolk sacs from four to eight embryos were pooled, washed with TD buffer (138 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.56 mM $Na_2HPO_4$, 19 mM Tris) until the majority of the yolk was removed, and the sacs were minced with scalpels. The minced yolk sacs were further dispersed by using an E-C Cellector with 100-mesh screen; this procedure produced a good single-cell suspension. The cells were washed and layered on a Ficoll-Hypaque gradient (Lymphocyte Separation Medium; Litton Bionetics) consisting of a layer of undiluted Ficoll-Hypaque and a layer diluted to 50% with TD buffer. The gradients were centrifuged for 15 min in a clinical centrifuge at top speed. The remaining yolk

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and cellular debris remained on top of the 50% Ficoll-Hypaque, and erythroid cells were pelleted at the bottom. The interface fraction was taken and cultured in BT88 medium (22) containing 10% tryptose phosphate, 5% calf serum (Flow Laboratories), and 5% chicken serum (GIBCO Laboratories) (referred to as complete BT88) at a density of $2 \times 10^7$ cells per ml.

AMV was provided as 0.5-g pellets by Life Sciences Inc., under a contract from the Division of Cancer Cause and Prevention, National Cancer Institute. Each pellet was suspended in 10 ml of TD buffer by using a Dounce homogenizer. Suspended virus was frozen at −70° or diluted 1:10 in medium with 5% calf serum and filtered through a filter (0.22-μm pore size) for sterilization. The virus was further diluted to $10^{-2}$ and portioned for storage at −70°C. All virus dilutions were calculated based on the initial virus suspension at 10°C. The actual virus concentration in the undiluted stock was calculated to be between 10- and 100-fold that found in leukemic chicken plasma.

**Agar colony assay.** Fresh yolk sac cultures were plated in 35-mm dishes at $2 \times 10^7$ cells per dish and infected with various dilutions of AMV stock at 2 to 6 h. Cells were plated in the agar suspension at 2 to 24 h after infection. Agar assay plates contained 200, 40, or 10 μl of the infected cell suspension per 35-mm agar suspension dish. On day 1, 200 μl corresponded to ca. $2 \times 10^6$ cells. The agar medium consisted of 1 ml of bottom agar containing 18.5% each of RPMI 1640 and tryptose phosphate (Difco Laboratories); 38.5% Hams medium F10; 12% each of calf serum (Flow Laboratories) and chicken serum (GIBCO); and 0.46% agar (Difco). The top agar contained 1 part cells suspended in complete BT88 (used for growing the yolk sac cultures), diluted as necessary with RPMI 1640, and 4 parts bottom agar. Top agar (1 ml) was added on top of the bottom agar, and cultures were incubated at 41°C. Cultures were fed with 1 ml of bottom agar on day 5 or 6, and colonies were counted by using a dissecting microscope on days 10 to 15. Transformed colonies were distinguished from normal colonies by their compact morphology versus the very disperse morphology of normal macrophage colonies in this system (see also references 11 and 16). This distinction was confirmed by picking representive colonies for microscopic analysis. For fetal calf serum agar used in some experiments, all chicken serum in the media mixtures was replaced with fetal calf serum (Flow Laboratories). The AMV focus assay was performed as described by Moscovici and Moscovici (22).

**Percoll gradient separation.** Percoll (Pharmacia Fine Chemicals) (20 ml) was diluted with 20 ml of double-strength F10 medium and centrifuged in a Sorvall SS-34 rotor for 40 min at 16,000 rpm to generate the gradient. Freshly prepared yolk sac cells, $8 \times 10^8$ cells in 8 ml of medium, were layered on top of the gradient and centrifuged in a clinical centrifuge for 15 min at 2,000 rpm. Fractions were collected by puncturing the tube. The densities of the fractions were determined by comparison to a parallel gradient which contained density marker beads (Pharmacia). Fractions collected from the gradient were counted, cytocentrifuge slides were prepared to determine cell types, and a portion was plated at $2 \times 10^7$ cells per ml and infected with 0.1 ml of a $10^{-3}$ dilution of stock AMV.

**Assays for cell morphology and cell proliferation.** **(i)** For autoradiography, cultures initially plated at $2 \times 10^7$ cells per ml were plated at various times with 10 μCi of [$^3$H]thymidine per ml for 3 h at 41°C. The cells were washed with phosphate-buffered saline by centrifugation, and cytocentrifuge smears were prepared. The cells were fixed in 70% ethanol for 20 min, washed, and coated with Kodak NTB 3 emulsion. After a 2- to 3-week exposure, the preparations were developed, soaked for 12 h in 70% ethanol, and stained with MacNeal stain.

**(ii)** The mitotic index defines the percentage of cells in mitosis at a given hour. This was scored on MacNeal-stained cytocentrifuge preparations for nonadherent cells and on MacNeal-stained cells in situ for adherent cells.

## RESULTS

**Characteristics of the agar colony for AMV.** The assay system we used was based on the original observations of Dodge and Moscovici (11) on the production of agar colonies by AMV-infected and normal uninfected cells derived from in vitro cultures of yolk sac cells. In their experiments, conditioned medium from primary chicken embryo cultures was used as a source of colony-stimulating activity (CSA). In our hands, pretested lots of normal chicken serum proved to be a better source of CSA for macrophages. Titration of the chicken serum lot used determined that 10% chicken serum produced the highest number of normal macrophage-type colonies from normal yolk sac cells.

A linear relationship between the number of colonies per plate and the number of cells plated was obtained (Fig. 1). This result suggested that the level of CSA (in the chicken serum) was not limiting. The maximum number of transformed colonies ranged from 100 to 300 per $10^6$ cells in different experiments. This is about 10-fold higher than that reported by Graf et al. (16). The difference may be explained by the higher level of CSA used in our assay system (4).

This colony assay was modified to serve as a titration assay for virus stocks similar to that devised previously for bone marrow-derived cells (16). Parallel plates of freshly prepared yolk sac cells were seeded at $2 \times 10^7$ cells in 1.0 ml of complete BT88 medium and infected with 0.1 ml of diluted

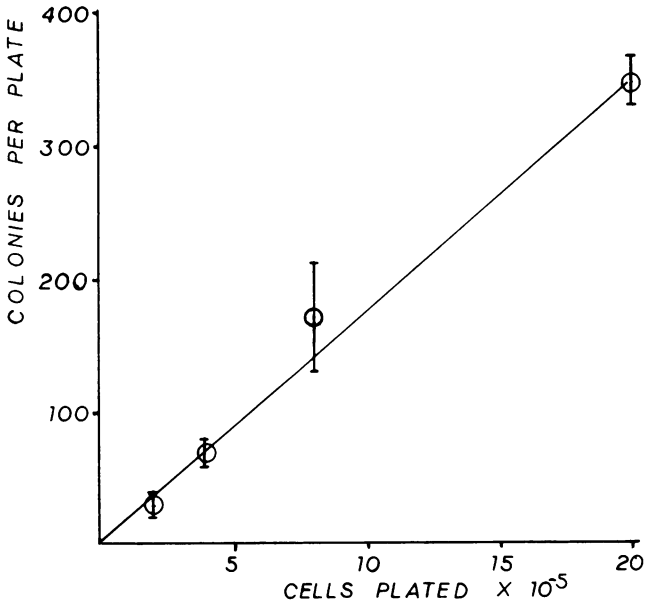


FIG. 1. Linearity of the agar colony assay. Fresh cultures of yolk sac cells were infected 4 h after plating with 0.1 ml of a $10^{-3}$ dilution of AMV stock no. 3 per $2 \times 10^7$ cells. On day 1, agar colonies were prepared in duplicate containing $2 \times 10^5$ to $2 \times 10^6$ cells per dish. Error bars indicate ±1 standard deviation.

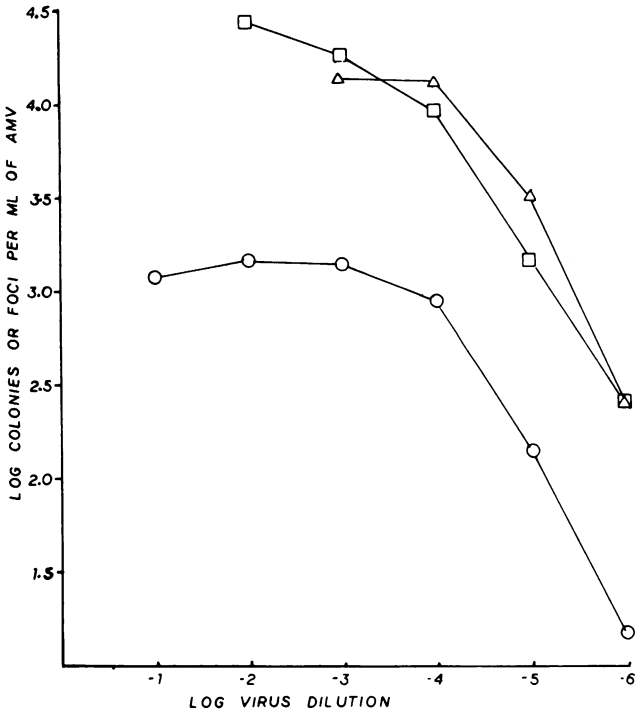


FIG. 2. Assay curves for AMV. Stocks of AMV were assayed by using either the focus assay (○ [stock no. 3]) or the agar colony assay (□ [stock no. 3] and △ [stock no. 4]). The virus dilution is plotted against the number of agar colonies or foci obtained per milliliter of AMV used.

AMV stock at 2 h after plating. In the experiment shown here (Fig. 2), the cells were seeded in agar 22 h after infection, but similar results were obtained at 1 h to at least 30 h after infection. At low virus inputs, the assay was linear with a slope of ca. 1, as expected. This linear region of the titration was used to calculate the virus titer. Stock 3 gave $2.3 \times 10^8$ CFU/ml and stock 4 gave $2.5 \times 10^8$ CFU/ml. These two stocks were obtained from separate AMV pellets produced by Life Sciences Inc. Each pellet was suspended as a 5% homogenate which served as our undiluted stock. The virus concentration was about 20-fold higher than that obtained from leukemic plasma. With the calculations from Smith and Berstein (25), the undiluted stock was estimated to contain $4 \times 10^{13}$ virus particles per ml. For use, a $10^{-1}$ dilution was filtered through a filter (0.22-μm pore size) and frozen at −80°C as the stock virus.

This assay system was compared with the macrophage focus assay of Moscovici et al. (21) with stock 3 in parallel to the yolk sac cell assay (Fig. 2). The shape of the titration curve was similar to that obtained for yolk sac cells, and the calculated titer was $1.7 \times 10^7$ focus-forming units per ml or about 17-fold less than that obtained with yolk sac cells. More recently, Graf et al. (16) have presented a similar assay for AMV which uses bone marrow. Their plasma stocks gave titers of $2.5 \times 10^4$ CFU/ml which, after an allowance of an additional 20-fold dilution of our stock to reach normal plasma levels, still left a 500-fold difference. This could be due to a difference in titer in their plasma stock or to a difference in assay sensitivity or both.

In addition to producing a linear portion for titration at low virus inputs, all titrations produced a plateau region at high virus inputs (Fig. 2). A similar result has also been obtained for bone marrow (16). This plateau could result either from saturation of potential target cells (resulting either from state of cell differentiation or from physiological state) or from a limitation in the virus stock. Since the virus stock contained both AMV and MAV-1 and MAV-2 helper viruses as well as noninfectious virus particles, an unfavorable ratio of AMV to the remaining particles could, at a high virus input, saturate the virus receptors and interfere with infection by AMV. This phenomenon of early interference has been described by Steck and Rubin (26).

**Test for viral interference and estimate of receptors.** To provide a source of noninfectious virus particles which could still occupy receptor sites, a $10^{-1}$ dilution of stock 3 was UV irradiated. Of this irradiated virus stock, 0.1 ml was used to infect $2 \times 10^7$ yolk sac cells, and no transformed colonies were produced. This proved to be the limit of the assay, since the undiluted virus stock was too thick to be irradiated efficiently with UV. The stock of UV-irradiated virus was serially diluted, and each dilution was mixed with a constant amount of infectious virus, corresponding to a final dilution of $2 \times 10^{-3}$. Figure 3 shows the titration curves for stock 3 and the mixture in the same experiment. Stock 3 gave a

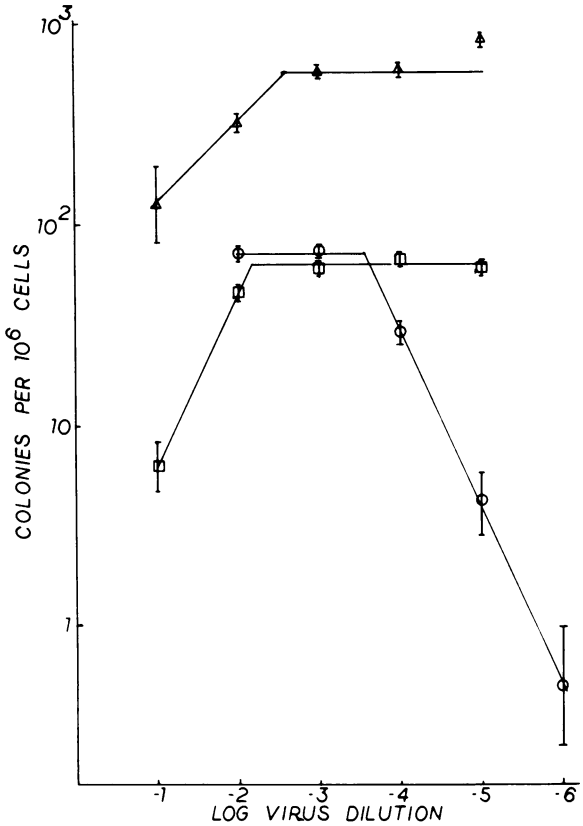


FIG. 3. Interference assay for AMV. Fresh yolk sac cultures were infected with 10-fold serial dilutions of stock no. 3 AMV or UV-irradiated stock no. 3 AMV (a $10^{-1}$ dilution of AMV stock irradiated for 7 min in a UV hood) or a mixture of serial dilutions of the UV-irradiated virus plus a constant $10^{-3}$ dilution of normal AMV. Unirradiated AMV, ○; UV-irradiated AMV mixed with unirradiated AMV, □. (Both of these assays were scored for transformed colonies only.) UV-irradiated AMV only, △. (No transformed colonies were produced, normal macrophage colonies were scored.) Error bars indicate ±1 standard deviation.

similar result as that graphed in Fig. 2. The mixture produced a plateau at low levels of irradiated virus, but a sharp decrease was observed starting near the $10^{-2}$ dilution. Since it was possible that the virus stock itself was toxic and hence this decrease was nonspecific, serial dilutions of the irradiated virus only were used to mock infect yolk sac cells, and the effect on normal macrophage colony formation was determined (Fig. 3). These results suggested that the high virus concentrations were indeed toxic and inhibited normal macrophage colonies; however, the slope of the inhibition was less than that observed in the interference assay. It appears that both early interference and toxic factors contributed to the inhibition of transformed colony formation with virus inputs of greater than the $10^{-2}$ dilution. Thus, the plateau region from $10^{-2}$ to $10^{-4}$ appears to be due to the limitation in the number of virus target cells and not to a property of the virus stock used.

These interference values were combined with the estimated concentration of virus particles in the virus stock to estimate the average number of virus receptors per cell. If early interference occurs at the $10^{-2}$ dilution, this level of virus must saturate the receptors. One-tenth milliliter of the $10^{-2}$ dilution on $2 \times 10^7$ cells represents a multiplicity of ca. 2,000 particles per cell. Hence, the average number of virus receptors was about 2,000 per cell. The beginning of the plateau occurs at the $10^{-4}$ dilution, which represents ca. 20 particles per cell. This suggests that at least 1 in 20 of the particles in the stock represents infectious AMV. This estimate is quite high in comparison to earlier estimates.

**Cell multiplication in liquid culture.** Since cell division is a requirement for infection and expression of viral products, the dynamics of cell proliferation in primary yolk sac cell cultures was examined. Cell proliferation was examined by: (i) [$^3$H]thymidine incorporation during a 3-h pulse followed by autoradiography and (ii) mitotic index. Both procedures gave qualitatively similar results, but the autoradiographic emulsion interfered with the MacNeal staining, making unequivocal identification of each stage of cell differentiation difficult. Hence, only the results obtained from determining the mitotic index are presented. If one assumes an average mitotic transit time of 0.5 h, then 48 times the mitotic index gives the percentage of cells expected to divide during 24 h. Table 1 displays the percentage of cells expected to divide in 24 h and the measured mitotic index of samples taken on successive days of culture. Since only the macrophage lineage cells were affected by AMV, only these data are included. The blast cell compartment certainly contains many committed granulocyte progenitors and some erythroid progenitors; these cannot be distinguished at this stage. The majority of the initial cell population divided in the first 24 h. These conditions are excellent for the establishment of infection. On subsequent days, there was a rapid decrease in proliferation rate as the cells progressed to more mature stages of differentiation. During this period there was also considerable cell death, as expected for primary cultures, so there was a general decrease in total cell number.

**Kinetics of AMV-induced cell transformation.** The previous experiments have established an optimal, saturating AMV input level to affect all the susceptible target cells available in the fresh yolk sac cell cultures. No transformed cells were identified at 24 h after infection, but 5 to 10% of the cells present at 48 h could be morphologically identified as AMV-transformed cells. To determine the proportion of transformants observed in liquid culture which was due to primary infection and the proportion which was due to secondary infection, a reconstruction experiment was performed. A series of primary yolk sac cultures were prepared; one-half of the cultures were infected with AMV at 2 h as described above. On each successive day of incubation, a parallel uninfected culture was infected by using the total cell-free supernatant from a culture infected on day 0 to replace the medium in the uninfected culture. At 3 days after infection in each case, the cells present were examined for the presence of transformed cells. At least 96 h were required before secondary transformants could be detected, and the level of these events was less than 2% of the total transformants (Table 2). The originally infected cells produced ca. $4 \times 10^6$ transformants by day 3 (Table 3), whereas the secondary infections produced only $8 \times 10^4$ by day 8. The decreased rate of cell proliferation did contribute to a loss of sensitivity for detection of virus production; however, this demonstrated that secondary infection did not make an important contribution to the total number of transformants produced in this system. Given the relatively high efficiency of the

TABLE 1. Multiplication of yolk sac macrophage lineage cells in vitro

| Cell type | Mitotic index ± SEM on culture day[a]: | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 |
| Blast | | | | | | |
| Control | 1.7 ± 0.45 (85) | 0.62 ± 0.12 (30) | 0[b] | 0 | 0 | 0 |
| AMV-infected cultures | 1.7 ± 0.45 (85) | 0.95 ± 0.06 (46) | 0 | 0 | 0 | 0 |
| Promonocyte | | | | | | |
| Control | 0 | 0.35 ± 0.20 (17) | 0.30 ± 0.09 (14) | 0.06 ± 0.06 (3) | 0.04 ± 0.13 (2) | 0 |
| AMV-infected cultures | 0 | 0.53 ± 0.13 (25) | 0.32 ± 0.07 (15) | 0.04 ± 0.06 (2) | 0.06 ± 0.08 (3) | 0 |
| Monocyte | | | | | | |
| Control | 0 | 0 | 0.04 ± 0.05 (2) | 0.11 ± 0.06 (5) | 0.13 ± 0.08 (6) | 0.12 ± 0.07 (6) |
| AMV-infected cultures | 0 | 0 | 0.02 ± 0.04 (1) | 0.17 ± 0.07 (8) | 0.04 ± 0.05 (2) | 0.11 ± 0.08 (5) |
| Macrophage | | | | | | |
| Control | 0 | 0 | 0.07 ± 0.04 (3) | 0.17 ± 0.03 (8) | 0.10 ± 0.02 (5) | 0.13 ± 0.82 (6) |
| AMV-infected cultures | 0 | 0 | 0.02 ± 0.02 (3) | 0 | 0.07 ± 0.13 (3) | 0 |

[a] Values are for several fields in three separate experiments. Numbers in parentheses represent the corresponding percentage of cells which would divide in 24 h, with an assumed mitotic transit time of 0.5 h. All numbers refer to normal cells only in either control or AMV-infected cultures.

[b] 0, Too few to score, i.e., less than 2% total cell population.

TABLE 2. Extent of secondary infection in AMV-infected yolk sac cultures[a]

| Virus inoculum | Total transformed cells 72 h after infection |
|---|---|
| 0-h standard AMV stock | $9 \times 10^5$ |
| 24-h supernatant | $<10^3$ |
| 48-h supernatant | $<10^3$ |
| 72-h supernatant | $<10^3$ |
| 96-h supernatant | $<10^3$ |
| 120-h supernatant | $8 \times 10^4$ |

[a] Total supernatants collected from cultures infected with the standard AMV dose were transferred to parallel cultures of the same age at successive days of culture. Cultures were assayed at 72 h post-infection for total number of AMV-transformed cells. Values represent averages of duplicate cultures.

assay, this implied that the blast cell stage of macrophage progenitors was susceptible to infection by AMV.

The kinetics of cell transformation could be followed in the liquid culture system by evaluating morphologically the number of transformed cells present on each successive day after infection by AMV. Since cell multiplication may also contribute to the total cell number, the rate of cell multiplication of the transformed cells was determined by evaluating the mitotic index for transformed cells on each day. Initially $2 \times 10^7$ cells were infected; this produced $1.5 \times 10^6$ transformed cells at 2 days. This amount corresponds to 7.5% of the original cell number or about 4% of the cells from day 0 accounting for cell proliferation (Table 3). Measurement of the mitotic index revealed that there was an increase from 0.2 at 2 days to 0.75 at 5 days. The 0.2 level was similar to that of normal promonocytes and monocytes at the same time of culture (Table 1); the 0.75 indicated a cell doubling time of about 3 days, which was the same as that we measured for established transformed cells and similar to that observed by others for AMV-transformed cells in vitro (3, 17). Thus, the initial morphological transformation preceded the increase in cell growth rate.

To evaluate the synchrony of cell transformation, the mitotic index was used to approximate the growth rate of the transformants and hence, to determine the proportion of new transformation events which would be required to generate the number of transformants actually observed on the next day. Of the transformation events, 60% occurred on day 2, with the remainder split between days 3 and 4. After day 4, all subsequent increases could be accounted for by the multiplication of already transformed cells. This time course correlates with the disappearance of the blast cells (which were reduced to 40% by day 1 and were essentially gone by day 2) and the appearance of promonocytes and monocytes which made their first appearance on day 1 and continued to mature and accumulate, mostly as adherent macrophages, on days 4 and 5.

TABLE 3. Kinetics of liquid culture transformation by AMV

| Time (days) | No. of transformants | Mitotic index | New transformants (% of total)[b] |
|---|---|---|---|
| 0 | 0 | | |
| 1 | 0 | | |
| 2 | $1.5 \times 10^6$ | 0.20 | 60 |
| 3 | $2.2 \times 10^6$ | 0.32 | 20 |
| 4 | $3.2 \times 10^6$ | 0.50 | 20 |
| 5 | $4.1 \times 10^6$ | 0.75 | 0 |

[a] Number of morphologically transformed cells per dish initiated with $2 \times 10^7$ initially infected yolk sac cells.

[b] Proportion of transformation events detected on that day required to account for total number of transformants observed. The mitotic index and the assumption of a mitotic transit time of 0.5 h were used to calculate growth of cells already present. Cell death is not accounted for in the transformed cell population.

**Purification of yolk sac target cells.** The above experiments demonstrated that the virus inputs which we used saturated the available target cells present in the 12- to 14-day chicken embryo yolk sac. Further, it appears that the target cell population represented only a minority component of ca. 4% of the starting cell population. To achieve a better definition of the target cell subpopulations, the freshly prepared yolk sac cells were separated on a preformed Percoll equilibrium density gradient. Cells ($8 \times 10^8$) were layered on a 30-ml gradient. Total cell recovery was 78%. The majority of the cell population was concentrated in the denser region of the gradient (Fig. 4). These cells consisted primarily of early stages of erythroid cell differentiation. The target cells for AMV were found in a peak at the lighter end of the gradient. This peak corresponded exactly to the peak for normal macrophage colony-forming cells in yolk sacs (data not shown). Based on the total cell population, this gradient produced a ca. 20-fold enrichment for AMV target cells. The use of the 14-day yolk sacs in this experiment biased the results slightly in favor of the erythroid cells which are present at higher proportion then than in the 12- to 13-day yolk sac cells. The enrichment for 12- to 13-day yolk sac cells was about 10-fold. If, as the liquid cultures imply, 4% of the total yolk sac cells could be transformed by AMV, then 40 to 50% of the enriched population should be target cells. In all experiments examined by the agar colony assay with saturating doses of AMV, a reasonable proportion of normal

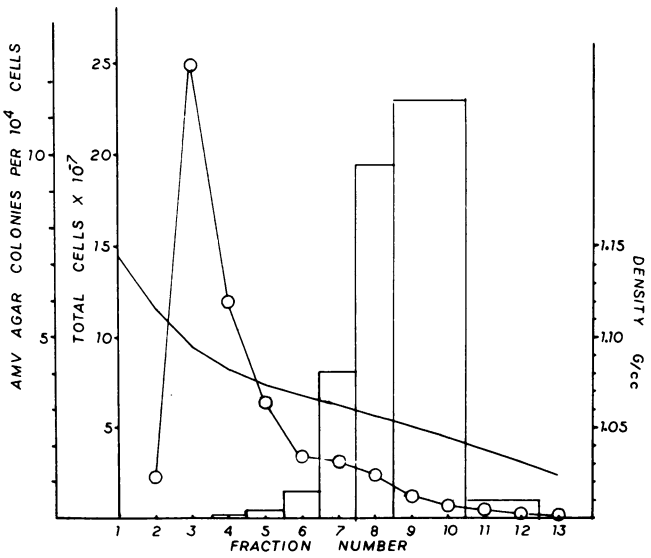


FIG. 4. Percoll gradient separation of AMV target cells. Fresh yolk sac cells ($8 \times 10^8$) were separated on a 50% Percoll self-generated gradient. Fractions were scored for number of cells (○) and AMV target cells (bar graph; the wider bars for fractions 9 and 10 and fractions 11 and 12 indicate that fractions were pooled for the assay, owing to the limited number of cells in single fractions). Density was determined by using density marker beads (solid line graph).

macrophage colonies were found. Thus, it would appear that only a subset of the macrophage lineage(s) may serve as targets for AMV. A similar conclusion has been suggested in our experiments involving the transformation of adherent macrophages by AMV (12).

## DISCUSSION

The embryonic avian yolk sac is a primary site of hemopoiesis in embryos (10). We have characterized the markers present in the myeloid cell subpopulation and examined the progressive appearance of myeloid markers for both the granulocyte and macrophage lineages during their synchronous in vitro differentiation (5; Durban, Ph.D. thesis). These studies have demonstrated that the vast majority of myeloid cells present in the yolk sac contain no characteristic myeloid markers and hence are classed as blast-stage myeloid progenitors. The data presented here demonstrate that these cells were susceptible to infection by AMV since: (i) they could be cloned in agar directly after infection and they produced transformed colonies and (ii) they transformed efficiently in liquid culture under conditions in which secondary infection plays an insignificant role (quantitatively). Earlier experiments have established that the functionally mature adherent macrophages can serve as targets (12). Additional experiments with both yolk sac and bone marrow (13, 15, 16) have provided evidence for AMV targets being less mature than adherent macrophages. With the present data, it appears likely that all stages of macrophage differentiation, from the committed progenitor to the mature macrophage, may serve as targets for infection and give rise to transformed progeny cells.

An agar colony assay for AMV in primary yolk sac cells was presented which compared favorably with the previous macrophage focus assay (21) and bone marrow colony assays (13, 16). Even this assay appears to be rather inefficient in comparison to the liquid culture transformation. To achieve saturation levels capable of transforming about 5% of the total population required $2 \times 10^4$ CFU for $2 \times 10^7$ cells or a multiplicity of infection of 0.001 CFU per cell. This finding suggests that the efficiency of the agar assay may be 0.1 to 1% of potential transformants producing a colony. For the gradient-purified population, the estimated efficiency based on target cell calculations described above was about 0.5%. That potential AMV transformants exhibited such a low plating efficiency strengthens the conclusion that the committed macrophage progenitors, and not the low level contamination of promonocytes, were serving as the principle target for infection in these assays.

The initial target cell in the yolk sac copurified with the normal macrophage colony-forming cell on the Percoll gradients. Since even the more mature macrophages score in this assay (11), this result merely indicated that target cells were restricted to the macrophage lineage. This conclusion was strengthened by recent results which demonstrate that both granulocyte and erythrocyte precursors are separated from the macrophage precursors by this gradient (M. Olsen and D. Boettiger, manuscript in preparation). The major target cell in yolk sac and the major target cell in bone marrow represent different cell populations and can be distinguished on the basis of their requirements for CSA. The yolk sac targets require three- to fivefold more CSA for colony formation (4). Since CSA is required for differentiation (19) as well as for survival and growth (27), and the bone marrow contains a substantial number of cells which express some macrophage markers (13), it appears that the target cell in yolk sac is, on the average, more primitive than that in bone marrow. The kinetics of transformation of yolk sac cells in liquid culture required a minimum of 2 days; whereas the transformation of fibroblasts by Rous sarcoma virus at saturating levels of virus input has been reported to occur in 28 h (18). Given the high mitotic index of the primary yolk sac cells and their susceptibility to infection, it is unlikely that the cell cycle-dependent events in virus replication were responsible for the delay. Both this and the normal development of the macrophage progenitors to promonocytes and monocytes during this period argue for differentiation steps being involved in the delay (7; Durban, Ph.D. thesis; Durban and Boettiger, in preparation).

Transformation which is dependent on the process of cell differentiation has also been implicated in other systems. Samarut and Gazzolo (24) have described the process for avian erythroblastosis virus in which the target cell for infection was the erythroid burst-forming unit (BFUe), but the transformed cell was more similar to the more mature erythroid colony-forming unit (CFUe). They postulate that the transition from BFUe to CFUe was essential for cell transformation. Dexter et al. (9) have demonstrated that in long-term murine bone marrow cultures infected with Friend erythroleukemia virus, the addition of anemic mouse serum was required for transformation. Since the primary effect of the anemic mouse serum was to induce erythropoiesis in the cultures, it appeared that some additional cell differentiation steps were required for cell transformation. This phenomenon is not limited to leukemia viruses. When early chicken embryonic limb bud cells are infected with Rous sarcoma virus the chondrogenic progenitors present do not appear to be affected until they undergo a differentiation transition and initiate the synthesis of the characteristic chondroblast products (5, 6).

The process of cell transformation generally encompasses both a change in cell growth properties and a change in cell morphology. It is generally assumed that these events occur simultaneously; however, there are few systems available which can allow the simultaneous quantitative evaluation of both parameters. In the transformation of yolk sac cells by AMV in liquid cultures, these two parameters were measured. The initially transformed cell could be identified morphologically but retained a mitotic index similar to its immediate parent (the promonocytes and monocytes). The mitotic index gradually increased over the next 3 days to reach a level characteristic of long-term-transformed cells. Thus, the morphological changes preceded the altered growth rate.

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