Proc. Natl. Acad. Sci. USA
Vol. 78, No. 6, pp. 3600–3604, June 1981
Cell Biology

Differential effects of transforming avian RNA tumor viruses on avian macrophages

(transforming genes/macrophages/acute leukemia viruses/Rous sarcoma virus)

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Communicated by Peter C. Nowell, January 30, 1981

ABSTRACT **Functionally differentiated chicken macrophages were derived by *in vitro* differentiation of embryonic yolk sac cells and were characterized by several macrophage-specific cell markers. Uniform, infected, virus-producing cultures were obtained after exposure of these macrophages to avian myoblastosis virus (AMV), avian myelocytomatosis virus (MC29), myeloblastosis-associated virus (MAV-2), and Prague strain of Rous sarcoma virus (PR-B RSV). Both AMV and MC29 induced morphological transformation typical of the *in vivo* leukemias induced by these virus strains. Analysis of the expression of macrophage-specific markers in these two transformed cell types demonstrated that different markers of the mature macrophage were suppressed by each virus, even though the parental cell immediately preceding the transformation event was a mature macrophage in both cases. Cells infected with PR-B RSV and MAV-2 showed no observable difference from uninfected macrophages in terms of morphological characteristics, growth rate, or expression of the differentiated functions of macrophages. This system provides demonstrations of a cell type that produces infectious, transforming RSV but fails to respond by functional alterations induced by the transforming gene, *src*.**

The transformation of cells by RNA tumor viruses leads to alterations in the phenotypic characteristics of the affected cells. Much effort has been devoted to defining individual changes that occur after transformation of fibroblasts by Rous sarcoma virus (RSV) (1); however, many of the described characteristics of transformed fibroblasts are not appropriate markers for other transformed cell types. A more general feature of the transformed phenotype is the disruption in expression of particular cell type-specific molecules or developmental markers for the cell type that was transformed. Transformation of chondroblasts by Rous sarcoma virus suppresses the synthesis of the chondroblast-specific type of sulfated proteoglycan (2) and type II collagen synthesis (3). Transformed fibroblasts or tendon cells show a reduction of type I collagen synthesis (4, 5). Myoblast fusion and synthesis of muscle-specific products are suppressed by transformation (6–8). Retinal melanoblasts cease pigment synthesis and discard their melanosomes in response to transformation (9). The use of temperature-sensitive, transformation mutants of RSV in these systems has demonstrated that both transformation and disruption of the cell's differentiated state are dependent on continuous function of the transformation gene product, $pp60^{src}$. Recent work from this laboratory and other laboratories has demonstrated that the acute leukemia viruses also affect the state of cell differentiation. The use of functionally differentiated macrophage cultures as target cells allows evaluation of the effects of leukemic transformation on the expression of functions already present in the macrophage and comparison with the effects observed for solid tissue types exposed to RSV. Among the acute leukemia viruses, avian myoblastosis virus (AMV) (10, 11) and avian myelocytomatosis virus (MC29) (12, 13) appear to carry genetically distinct transformation functions and appear to be capable of inducing morphologically distinct transformed cell types (14–17). Earlier experiments had indicated that the functionally mature macrophage could serve as a target for both virus strains (15, 16, 18, 19), which is confirmed in the present work.

The specificity between virus-carried-transformation gene and transformation of defined cell types can also operate on another level. It has been known for some time that fibroblasts are refractory to transformation by AMV (20); in fact, it appears that the target specificity of AMV is probably restricted to macrophages (unpublished data). RSV, on the other hand, induces solid tumors and only rare lymphomas (21), which may be due to transformation defective virus. Hence, it was of interest to determine whether there was a cellular restriction of the transformation function carried by RNA tumor viruses that was independent of virus replication and that could be demonstrated *in vitro*. The results presented here support the existence of such a restriction, which is dependent on the differentiated state of the potential target cell. For the case in point: RSV transforms fibroblasts but not macrophages, whereas AMV transforms macrophages but not fibroblasts.

MATERIALS AND METHODS

Viruses and Cells. SPF-free C/E chicken embryos and AMV plasma were obtained from the Division of Cancer Cause and Prevention, National Cancer Institute. Primary yolk sac cultures were prepared and maintained as described (18, 19).

Virus Assays. The Prague strain (PR-B) of RSV and MC29 were assayed on chicken embryo fibroblasts as described by Temin and Rubin (22) and by Graf (23), respectively. Myeloblastosis-associated virus (MAV-2) was assayed by using the plaque assay (24). AMV was assayed by using yolk sac-derived macrophage cultures (13). Infectious center assays for MC29-, PR-B RSV-, and MAV-2-infected macrophages were performed by dispersing the cells with trypsin/EDTA and seeding them on freshly prepared chicken embryo fibroblast indicator cells; 3 hr later the medium was replaced with agar containing medium, and cultures were processed for focus or plaque assay.

Immunofluorescence. Cells grown on microscope slides were fixed with 2% (vol/vol) formaldehyde in phosphate-buffered saline, washed in saline, treated with 1% Nonidet P-40 in

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Abbreviations: PR-B RSV, Prague strain of Rous sarcoma virus; AMV, avian myoblastosis virus; MAV-2 myeloblastosis-associated virus; MC29, avian myelocytomatosis virus.

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saline, and stained for indirect immunofluorescence with rabbit anti-AMV antiserum (obtained as a gift from W. Mason) and fluorescein isothiocyanate-conjugated goat anti-rabbit antisera (Cappel Laboratories, Cochranville, PA).

Assay for Cell Function. These assays are described in detail elsewhere (17, 18). Briefly, specific phagocytosis was assayed by uptake of sheep erythrocytes opsonized with hyperimmune chicken anti-sheep erythrocyte antisera (obtained from M. Halpern) during a 20-min incubation. Non-specific phagocytosis was assayed by incubation of cells for 3 hr in 1:800 dilution of India ink (Higgins, A. W. Farber–Castell). Acid phosphatase was assayed cytochemically by using the Gomori method as modified by Lake (25) with β-glycerol phosphate (Sigma) as a substrate. Cytoplasmic lipid content was determined by using Sudan Black B stain. Fc fragment receptors were scored by using rosettes formed with sheep erythrocytes coated with hyperimmune chicken anti-sheep erythrocyte antisera. ATPase was determined as described (18). Cell protein was determined with the Bio-Rad protein reagent.

RESULTS

Characteristics of the Cell Population Before Infection. To provide a base line of normal macrophage cell function before transformation and to insure that the infected, transformed cells to be examined arose after the infection of macrophages, it was important to examine both the homogeneity and the expression of cell markers characteristic of functionally mature macrophages in the starting population prior to infection. Yolk sac cells from 13-day chicken embryos were plated in primary culture; the adherent cell population was characterized 6 days later (Table 1). At this time, a significant proportion of the macrophages were still dividing as determined both by mitotic index and by [^{3}H]thymidine incorporation followed by autoradiography. Continued cell proliferation is essential for the establishment of the RNA tumor virus infection (18, 26).

The cultures were examined both as attached cells *in situ* and as cytocentrifuge smears after trypsinization of the cell culture. The cytocentrifuge preparations were used to insure that any loosely attached or floating cells that could represent early cells in the macrophage lineage or other contaminating hematopoietic cells would not be ignored (Table 1). In previous experiments, in which all cell compartments in the macrophage lineage between the blast cells with no recognizable markers and the functionally differentiated macrophage were examined, only cells expressing the full range of functional markers were strongly phagocytic in the carbon-particle test. Either immature cells in the macrophage lineage, contaminating fibroblasts, or other cell types could be easily distinguished. To further confirm that these cells represented a pure macrophage population, acid phosphatase, cytoplasmic lipid accumulation, and cell membrane ATPase were examined. Thus, at the level of detection after examination of more than 5000 individual cells, contamination by cells other than macrophages was <0.02%.

Table 1. Functional characterization of the 6-day-old adherent macrophage-like cells prior to infection

Marker*	Macrophage-like†		Control
	In situ	Trypsinized	
Phagocytosis	+++	+++	—
Acid phosphatase	+++	+++	—
Cytoplasmid lipids	+++	+++	+
ATPase			
Cytochemical			
With ouabain	+++	+	—
No ouabain	+++	+	—
Biochemical‡			
With ouabain	0.030	—	—
No ouabain	0.028	−0.001	0.001
Mitotic index		0.12	0.4

All cells examined in each sample gave the same reaction in the cytochemical tests: +++, very strong reaction; +, weak reaction; —, not detected. Control, chicken embryo fibroblasts.

* Carbon-particle test measured phagocytosis; 5000 cells per sample were scored. In remaining tests, 1000 cells per sample were scored.

† Cells were examined both as attached cells *in situ* and as cytocentrifuge smears after trypsinization.

‡ P_i (μmol/μg of protein) in 30 min; P_i released in absence of ATP was subtracted from P_i released in its presence.

The cell membrane ATPase is not commonly used as a macrophage-specific marker, although a similar enzyme activity has been described on murine macrophages (26). This marker was of particular interest for these studies because it is found on AMV particles isolated from leukemic birds (21) but is not found on AMV particles harvested from fibroblast cultures (20) or on MC29 virus particles produced either *in vivo* or *in vitro* (J. Beard, personal communication). In addition, we have demonstrated that it is expressed only on mature macrophages and not on less differentiated cells in the lineage (15, 16). It is worth noting that this ATPase is very sensitive to trypsin, as has been reported for the virion-associated form (27), and cannot be demonstrated on trypsinized cells (Table 1). The failure of Beug *et al.* (14) to demonstrate the ATPase on macrophages is a result of their use of trypsinized macrophages for the assay.

Infection of Macrophages by Avian RNA Tumor Viruses. Pure cultures of 6-day adherent macrophages (Table 1) were infected with 100-fold concentrates of PR-B RSV, MAV-2, and MC29 or plasma from AMV-infected chickens, and media was changed each day to maximize the level of cell division. The AMV-transformed cells detached from the plate and were morphologically distinct so that pure populations of AMV-transformed macrophages could be easily obtained. Cultures infected with PR-B RSV, MAV-2, and MC29 were assayed as infectious centers on day 5 and day 9 after infection (Table 2). The production of typical fibroblast transformation in the PR-B RSV infectious-center assay demonstrated that these cells carry the complete RSV genome and that no selection for transformation-defective mutants had occurred. For MAV-2, the plaque assay only indicated the presence of a virus with subgroup B or D envelope and did not show that its oncogenic potential had been maintained. The MC29-transformed fibroblasts observed in the infectious-center assay were morphologically distinguishable from the MC29-transformed macrophages.

As an additional control for infection, PR-B RSV and MAV-2 cultures were plated on microscope slides at day 9 after in-

Table 2. Percentage infected cells and proliferative activity

Macrophage sample	Infectious centers,* % infected cells		[^{3}H]dThd incorporation,† cpm per 10^5 cells	
	5 DAI	9 DAI	5 DAI	9 DAI
Uninfected	0	0	1140	763
PR-B RSV-infected	25	70	983	977
MAV-2-infected	30	82	745	757
MC29 (RAV-2)-infected	20	80	1016	4500
AMV-transformed	—	—	3450	6971

DAI, days after infection.

* Macrophages were trypsinized, counted, plated on 2° (secondary) chicken embryo indicator cells, and overlaid with agar at 3 hr.

† Cells were pulsed with [^{3}H]dThd for 4 hr at 37°C.

fection. The cells were fixed and examined by indirect immunofluorescence by using rabbit anti-AMV antiserum to stain for virus group-specific antigen in the cells. All cells exhibited strong cytoplasmic fluorescence indicating the presence of these viral proteins. Although these experiments do not demonstrate conclusively that active viral transforming gene product $pp60^{src}$ is produced in the macrophages, clearly the coding sequences are present and substantial levels of virus products are produced.

Transformation of the Macrophage Population. Fig. 1 shows the morphological characteristics of cells infected with the several avian tumor virus strains and stained *in situ* (or for AMV-infected cells, as a cytocentrifuge smear) with Giemsa stain. The AMV transformed cells were easily distinguished in the stained preparations (Fig. 1*A*) or under phase microscopy *in situ* as smaller loosely adherent cells containing few cytoplasmic vacuoles or granules as compared to the macrophage parent (Fig. 1*C*). The MC29-infected cells (Fig. 1*B*) were similar to the macrophages *in situ* but appeared somewhat smaller and were often loosely attached and, hence, appeared as darkly staining cells. In cytocentrifuge smears they were difficult to distinguish from normal macrophages. Neither the PR-B RSV-infected macrophages (Fig. 1*E*) nor the MAV-2-infected macrophages (Fig. 1*D*) exhibited morphological differences that would distinguish them from the control normal macrophages either in the stained preparations or as living cells under phase microscopy.

The increased proliferative activity of the infected cultures was measured by incorporation of [^{3}H]thymidine for 4 hr on day 5 and on day 9 after infection. Table 2 shows that only the AMV-infected and MC29-infected cultures had a significant increase in labeling rate in comparison to the control uninfected cultures. Again neither the PR-B RSV- nor the MAV-2-infected cells could be distinguished from the uninfected controls.

The Effect of Infection and Transformation on Macrophage Function. One important characteristic of both cells transformed either *in vitro* or *in vivo* by RNA tumor viruses (21) and naturally occurring tumors (28) is a derangement of the expression of the cell products that serve to characterize and define the mature cell phenotype. The expression of several of the macrophage markers in both MC29- and AMV-transformed cells have been reported (14–16), but the particular target cell infected by the virus was not always clear in the earlier studies.

Table 3 shows a more complete comparison of the AMV- and the MC29-transformed macrophages in terms of their ability to continue to express their parental macrophage products after transformation. Although the transformation properties of increased proliferation and altered morphological characteristics of the cell were shared between the two transformed cell populations, most properties of the macrophage parent were not shared. In the AMV-transformed cells, phagocytosis, cytoplasmic lipid accumulation, and acid phosphatase activity were strongly suppressed, and the morphological characteristics of the cell were more similar to those of immature cells in the macrophage lineage; in the MC29-transformed cells, these functions did not appear to be affected, and the morphological properties of the cell were more similar to those of the mature

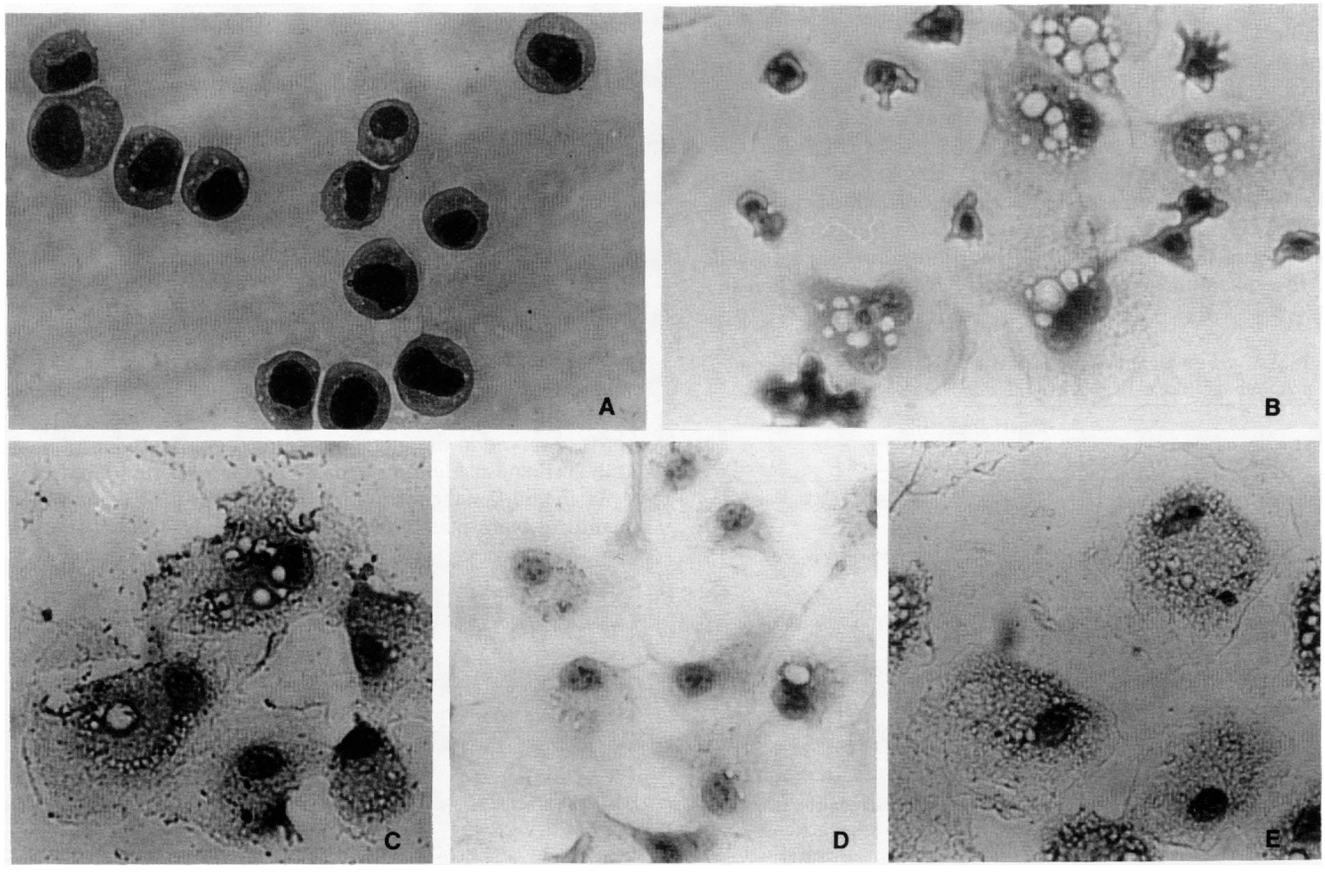

FIG. 1. Morphological appearance of macrophages infected with different strains of avian RNA tumor viruses. Cultures of 6-day yolk sac cells characterized as pure macrophage populations were infected and cultured until the majority of the population registered as infectious centers (Table 2). (*A*) AMV-infected macrophages. (*B*) MC-29-infected macrophages. (*C*) Control macrophages. (*D*) MAV-2-infected macrophages. (*E*) PR-B RSV-infected macrophages. Cultures were stained with Giemsa and photographed *in situ* attached to glass except for AMV-infected cells, which were prepared by cytocentrifuge because the transformed cell population was largely nonadherent.

Table 3. Expression of differentiated functions by the cells 12 days after infection

Cell sample	Phagocytosis		Acid phosphatase	Cytoplasmic lipids	Adherent	Fc receptor, % positive cells	Ouabain resistant ATPase*
	Nonspec.	Spec.					
Uninfected *in situ*	+++	+++	+++	+++	+++	98	0.02
Trypsinized							0.0001
PR-B RSV-infected	+++	+++	+++	+++	+++	95	0.014
MAV-2-infected	+++	+++	+++	+++	+++	90	0.022
MC29-transformed	+++	+++	+++	+++	—/+/+++	89	0.006
AMV-transformed	+	—	+	+	—/+/+++	68	0.022

+, Slightly positive; +++, strongly positive; —, not detectable; spec., specific phagocytosis of opsinized sheep erythrocytes.
* P_i released in the absence of ATP was determined for each sample. This value was subtracted from that obtained in the presence of ATP and reported as μmol of P_i per μg of protein per 30 min.

macrophage. For expression of the cell-surface ATPase, which is characteristic only of the functionally mature macrophage, the opposite pattern of expression was found; ATPase function on AMV-transformed cells was expressed at normal macrophage levels, whereas expression was reduced in MC29-transformed cells. This result is further supported by the presence of this enzyme on virus produced by AMV leukemic cells *in vivo* and its absence on virus produced by MC29 leukemic cells *in vivo* (see above). Thus, individual, distinct transforming genes carried by both AMV and MC29 exert a transforming effect on the same cell type, the macrophage, but each causes a different specific derangement in the expression of normal macrophage functions.

Infection of several differentiated cell types with RSV induces not only morphological alterations but also suppression of specific differentiated functions (2–9). In contrast to the leukemic transformations described above, all specific differentiated cell products thus far examined appear to be suppressed by RSV. Because these examples of effects on expression of differentiation were accompanied by morphological transformation, it was of interest to determine in the macrophage system whether differentiation would be affected in the absence of transformation. Table 3 shows that the PR-B RSV-infected macrophages could not be distinguished from the controls. None of the commonly observed effects of RSV on cells appeared to be operable in this system, even though the cells produced normal transforming virus in infectious-center assays.

MAV-2 is a B-subgroup helper virus associated with the stock of AMV used in these experiments. In addition MC29 was associated with the B-subgroup helper virus, RAV-2, and RSV of B-subgroup was used. The absence of effects of MAV-2 in this system was expected and served as an additional control. However, in *in vivo* experiments, MAV-2 did exhibit some effects on hematopoietic cells and probably on osteoblasts, which are related to the macrophages (29).

DISCUSSION

Functionally differentiated macrophages were used as target cells for infection by several strains of avian RNA tumor viruses. Although it has been demonstrated that macrophage cultures can be transformed by AMV (19) and MC29 (15), Beug *et al.* (14) have questioned whether the macrophages were indeed the target cell and suggested that the cultures were contaminated with "blast-like" cells which were the "true" target cells. We have examined this problem in some detail for the case of AMV (18). In carefully prepared and characterized macrophage cultures, the number of transformed cells generated after exposure to AMV exceeded the possible contamination by blast cells or any other cell type by more than one order of magnitude. The data presented here for MC29 support a similar contention for that virus, although the results are not as definitive as for AMV. These data argue only that functionally differentiated macrophages can serve as targets for transformation by AMV and MC29 and not that they are the only targets.

Gazzolo *et al.* (30) have reported that macrophages infected with several strains of RSV, including the PR-B strain used here, induce giant cell formation in macrophage cultures. The results of Gazzolo *et al.* are difficult to interpret for several reasons: (*i*) the infectious center assays reveal only 1–5% efficiency; (*ii*) giant cell formation was not observed with all strains of RSV that did replicate in the macrophages; and (*iii*) similar giant cell formation will occur in uninfected macrophages and depends on culture conditions. In contrast, in the experiments reported here, no significant changes in cell morphology, growth rate, or differentiated cell function were observed in macrophages producing infectious RSV.

Individual strains of avian RNA tumor virus induce a limited range of tumor types *in vivo* (21). It was possible that the transformation host range, as distinct from a virus-replication host range, may contribute to this restriction. This is demonstrated in the present results. RSV replicates in macrophages but does not transform them. AMV on the other hand replicates and transforms macrophages but only replicates in fibroblasts (20). Thus, cellular products restricted to particular differentiated cell types are important in the susceptibility of the cell to transformation by a particular virus strain.

Transformation genes carried by the various strains of avian tumor virus, also appear to affect the differentiated phenotype of the target cell in specific and characteristic ways. Macrophages transformed by AMV retain the expression of some normal macrophage functions, whereas others are repressed. Transformation of the same cells by MC29 produces a completely different pattern of effects on the normal macrophage functions. This result suggests that different mechanisms of cell transformation are involved and that there is some specificity in the action of transforming gene products on the differentiation program of the target cell.

The situation illustrated by the AMV leukemias and the MC29 leukemias present an interesting dilemma. In both cases the *in vivo* leukemic cells resemble the *in vitro* transformed cells described here; but the AMV leukemia is classified as a stem cell or myeloblastic leukemia, whereas the MC29 leukemia is classed as a myelocytomatosis (31). However, if leukemias are to be classified based on the cell of origin, then both would be monocytic (or perhaps histiocytic) leukemias.‡ Because of the

‡ Langlois *et al.* (31) suggest that both the MC29- and AMV-induced leukemias affect the same cell lineage. However, the case of MC29 is probably more complex because some of the "tumor cells" also appear to be granulated, unlike the *in vitro* transformed macrophages described. MC29 appears to transform a broader range of cell types than AMV, and some of its target cells remain to be defined.

disruption of phenotypic program, which provides the markers allowing the identification of cell types, by the process of cell transformation of tumorigenesis, it may not be possible to precisely identify the normal progenitor to the tumor cell or the original cell that was transformed. Perhaps we have only now discovered in *in vitro* transformation systems some of the complexities that have plagued cancer research for many years.

The authors would like to thank Dr. John Trentin for his help and provision of laboratory space for E.M.D. This work was supported by National Cancer Institute Grant CA16502. E.M.D. was a U.S. Public Health Service Predoctoral Trainee supported by Grant T32 GM07071. D.B. is a Scholar of the Leukemia Society of America.

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