Replicating, Differentiated Macrophages Can Serve as In Vitro Targets for Transformation by Avian Myeloblastosis Virus

ELISA M. DURBAN† AND DAVID BOETTIGER*

Department of Microbiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Pure cultures of chicken macrophages were characterized functionally and transformed by avian myeloblastosis virus. Transformed cells exhibited an altered function. The efficiency of transformation was limited by the mitotic activity of the macrophages.

Avian myeloblastosis virus (AMV) causes an acute leukemia in chickens in which large amounts of immature cells resembling "myeloblasts" (the presumptive, earliest recognizable compartment of the granulocyte lineage) accumulate in the circulating blood (6, 7). In addition, this virus can induce in vitro transformation of avian hemopoietic cells derived either from the bone marrow (3, 8) or from a number of embryonic and adult chicken organs (1, 2). The AMV-transformed cells obtained in vitro are identical to those obtained from the leukemic bone marrow of diseased birds (3, 8). The ability of AMV to induce transformation in vitro correlates with the myelopoietic activity of the various organs at the time of infection (2). More recent studies by Moscovici and collaborators indicated that macrophage-like cells can serve as in vitro targets for transformation by AMV (8, 12, 13). The observation that AMV is capable of transforming macrophages in vitro has been challenged recently by Graf and Beug (10), who noted that macrophage cultures passaged several times are no longer transformable by AMV. Thus, they proposed that, in the macrophage cultures used by Moscovici and co-workers, a small number of immature myeloid cells exist which are lost with passaging and which are the targets for AMV transformation. To date, direct experimentation to show the presence of such cells in the macrophage cultures has not been provided. In this report, we have examined directly the question of whether AMV is capable of transforming mature macrophages in vitro. We show that AMV is indeed capable of infecting and transforming macrophage cultures devoid of immature cells of the granulocyte-macrophage lineages. We further show that the efficiency with which the transforming event occurs correlates with the proliferative activity of the macrophage cultures at the time of infection.

† Present address: Department of Virology and Epidemiology, Baylor College of Medicine, Houston, TX 77030.

In the first series of experiments, 9-day-old adherent macrophage cultures derived from the yolk sac were carefully characterized both morphologically and functionally and then examined for their capacity to generate AMV-transformed cells in liquid culture as a function of time. Macrophage cultures were obtained by trypsinization of the adherent cell population on day 6 after isolation from the yolk sac. Trypsinized cells were seeded at a density of 5×10^6 cells per 100-mm culture dish. Three days later, the cells were characterized both in situ and in cytocentrifuged smears of trypsinized cells. Parallel 9-day-old adherent cultures were infected with 10^4 focus-forming units of AMV standard after a medium change. The appearance of AMV-transformed cells in these cultures was monitored by differential cell counting at 2-day intervals.

These day 9 macrophage cultures were characterized at the time of infection to determine the level of contamination by immature cells in the macrophage lineage or other cell types which could serve as potential targets for AMV transformation. Since the immature cells are less adherent, the culture supernatants, including any loosely attached cells which could be removed by pipetting, were collected by centrifugation, and cytocentrifuge preparations were made. Both the adherent cell population and the loosely adherent cytocentrifuge preparations were examined for morphology, acid phosphatase, membrane ATPase, cytoplasmic lipids, and phagocytosis (Table 1). The loosely adherent population contained about 60% nonviable cells as determined by trypan blue exclusion. All (100%) of the cells examined in both populations were strongly positive for all of the macrophage markers; 96% had a typical macrophage morphology (Fig. 1A) and 4% were large polykaryons (Fig. 1B). The initial yolk sac cell population used to derive these macrophage cultures contains predominantly blast cells which differentiate in vitro. All of the intermediate cell types

TABLE 1. *Functional characterization of day 9 adherent macrophage-like cells^a*

Marker	Macrophages		Controls	
	In situ	Trypsinized and cytocentrifuged	CEF	AMV-transformed cells
Phagocytosis	+++	+++	—	+
Acid phosphatase	+++	+++	—	+
Cytoplasmic lipids	+++	+++	+	+
ATPase	+++	+	—	+++

^a Third-passage chicken embryo fibroblasts (CEF) were examined in situ, whereas AMV-transformed cells were examined after cytocentrifugation. Phagocytosis was tested by ingestion of carbon particles, using 0.5 ml of a 1:50 dilution of India ink per 100-mm dish. Test cells were incubated for 3 h at 41°C and then washed three times with phosphate-buffered saline. Nonadherent cells were cytocentrifuged. A total of 5,000 cells per sample were scored in this assay. Acid phosphatase activity was measured by the lead acetate method of Lake (11). Cellular lipid components were stained with 0.3% Sudan Black B in 70% ethanol for 5 min after fixation in Formalin-gum-sucrose (11). ATPase activity was determined by the method of Gordon and Cohn (9). A total of 1,000 cells per sample were scored in the last three assays. —, Not detectable; +, slightly positive, +++, strongly positive.

present between the blast cells and the functionally mature macrophage have been extensively characterized and quantitated. The immature cells can be distinguished by absence of membrane ATPase and cytoplasmic lipids and greatly reduced acid phosphatase and phagocytic activity (E. M. Durban, Ph.D. thesis, University of Pennsylvania, Philadelphia, 1980; Durban and Boettiger, manuscript in preparation). The carbon particle test for phagocytosis is particularly easy to score since the immature cells are negatively or weakly positive, whereas the mature cells are all strongly positive (i.e., almost completely black). A total of 5,000 cells were scored with these tests, and 100% were functionally mature macrophages.

Infection of these 9-day-old cultures with AMV resulted in the appearance of AMV-transformed cells (Fig. 1C), which could be detected by day 4. Approximately 0.2% of the cells were transformed (Fig. 2), a 10-fold-higher amount than the maximum level of contamination by immature cell types. It is unlikely that the majority of these transformed cells arise from division of originally transformed cells since the doubling time of the transformed cells in the culture is about 4 days (Fig. 2). Also, it would be expected that some of the later contributions to the transformed cell pool were derived from later

transformation events related to the slow rate of macrophage proliferation in vitro. Thus, 0.2% represents a reasonable estimate of the number of primary transformation events to the culture. These results are in agreement with those of Gazzolo et al. (8), who reported a similar percentage of adherent peripheral blood macrophages to be sensitive to AMV transformation.

Successful infection and transformation by Rous sarcoma virus requires cellular DNA synthesis and mitosis; however, virus infection does not induce a round of DNA synthesis in cells that have ceased to divide (5, 14, 15). Likewise, AMV does not exert a mitogenic effect on the infected cells before transformation (E. M. Durban, Ph.D. thesis, University of Pennsylvania, Philadelphia, 1980). Hence, in the second series of experiments the transformation potential of adherent macrophages of different ages was examined in relation to their proliferative activity at the time of infection. The focus assay system developed by Moscovici et al. (13) was used for these experiments.

Six-, 9-, 13-, and 16-day-old adherent yolk sac cells were trypsinized and seeded at a density of 3×10^5 cells per 60-mm culture dish. Triplicate samples were infected 3 h after seeding with 10^3 focus-forming units of AMV-B and overlaid with agar 5 h later, and foci were scored 2 weeks later. DNA synthesis by the adherent cells was examined by autoradiography after pulsing parallel cultures at the time of infection with 10 μ Ci of [3 H]thymidine for 3 h. The mitotic index of the adherent populations was determined by cytocentrifugation of the trypsinized cells. At least 1,000 cells per time point were scored (Table 2). A near-perfect correlation was observed between the proliferative activity of the cells at the time of infection and the number of transformed foci obtained. Namely, the higher the proliferative activity of the adherent cells, the higher the efficiency of transformation by AMV under the conditions of the focus assay. Control uninfected cultures were used for all experiments. In both the liquid culture system and the focus assay under agar, spontaneous transformation yielding a population of dividing or morphologically transformed cells has not been observed. Generally, spontaneous transformation of chicken embryo cells does not occur with significant frequency in culture.

In the macrophage culture system described here, the maximum level of contamination by immature cells in the macrophage lineage or by other cell types was less than 1 in 5,000. Hence, the level of immature cells is not sufficient to account for the level of transformation events in the liquid assay or the number of foci in the focus assay. Therefore, the AMV-transformed

FIG. 1. Morphological appearance of normal and transformed cells. (A) Macrophage-like cells; (B) multi-nucleated cells; (C) AMV-transformed cells. Bars, 20 μm (A, B) and 10 μm (C).

FIG. 2. Generation of AMV-transformed cells from the adherent day 9 macrophage-like population. Nine-day-old macrophage cultures were infected with 10^4 focus-forming units of AMV after a medium change. The appearance of AMV-transformed cells in these cultures was monitored by differential cell counting at 2-day intervals from the time of infection. The transformed cells were not detectable on day 2 after infection.

TABLE 2. *Correlation between transformation efficiency and proliferative activity at the time of infection^a*

Age of adherent cells at time of infection (days)	Foci per 3 × 10 ⁵ adherent cells	Labeling index	Mitotic index
6	TMTC	5.8	0.12
9	127	3.8	0.05
13	8.3	0.5	0.001
16	1	—	—

^a Adherent cells were infected at each time point with 10³ focus-forming units of AMV-B. Foci were counted 2 weeks after infection; the number represents the average of triplicate samples. The labeling index represents the percentage of labeled cells and is the average of triplicate samples; 1,000 cells per sample were scored. The mitotic index represents the percentage of mitotic cells, and 1,000 adherent cells per time point were scored after cytocentrifugation. TMTC, Too many to count. —, Not detectable. AMV-B was assayed on yolk sac macrophages, using the method of Moscovici et al. (13).

cells must be able to arise by transformation of the functionally mature macrophage. This result implies that models for AMV-induced cell transformation which depend on a simple block in the normal differentiation of hematopoietic cells (10) are insufficient to account for the facts.

In spite of the homogeneity of these cultures with respect to morphology and several macrophage cytochemical markers, the proportion of effective targets for AMV-induced transformation appears to be limited. Part of this limitation appears to be imposed by the decreased proliferative activity of the macrophage cultures. We suggest that this decrease in proliferation, rather than dilution of progenitor cells, is responsible for the observations of Graf and Beug (10) that macrophage cultures lose susceptibility to transformation as they are passaged *in vitro*. Rough calculations of the rate of cell division from the data in Table 2 suggest that the low frequency of transformation is not due exclusively to limitation on cell division but also may depend on other variations in the physiological state of the macrophages. However, in the absence of an independent measure of the efficiency of the *in vitro* transformation systems, other experimental approaches will be required to resolve this issue.

This work was supported by Public Health Service grant CA16502 from the National Cancer Institute. E.M.D. was a recipient of Public Health Service predoctoral trainee grant T32 GM07071 from the National Institute of General Medical Sciences. D.B. is a scholar of the Leukemia Society of America.

LITERATURE CITED

1. Baluda, M. A. 1962. Properties of cells infected with avian myeloblastosis virus. Cold Spring Harbor Symp. Quant. Biol. 27:415-425.
2. Baluda, M. A., and I. E. Goetz. 1961. Morphological conversion of cell cultures by avian myeloblastosis virus. Virology 15:185-197.
3. Beaudreau, G. S., C. Becker, R. A. Bonar, A. M. Wallbank, D. Beard, and J. W. Beard. 1960. Virus of avian myeloblastosis. XIV. Neoplastic response of normal chicken bone marrow treated with virus in tissue culture. J. Natl. Cancer Inst. 24:395-415.
4. Beug, H., A. vonKirchbach, D. Dodevlein, J. F. Conscience, and T. Graf. 1979. Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. Cell 18:375-390.
5. Boettiger, D., and H. M. Temin. 1970. Light inactivation of focus formation by chick embryo fibroblasts infected with avian sarcoma virus in the presence of 5-bromo-deoxyuridine. Nature (London) 228:622-624.
6. Eckert, E. A., D. Beard, and J. W. Beard. 1951. Dose-response relations in experimental transmission of avian erythromyeloblastic leukemia. I. Host response to the virus. J. Natl. Cancer Inst. 12:447-463.
7. Eckert, E. A., D. Beard, and J. Beard. 1953. Dose-response relations in experimental transmission of avian erythromyeloblastosis. II. Host response to whole blood and to washed primitive cells. J. Natl. Cancer Inst. 13:1167-1184.
8. Gazzolo, L., C. Moscovici, M. G. Moscovici, and J. Samarut. 1979. Response of hematopoietic cells to avian acute leukemia viruses: effects on the differentiation of the target cells. Cell 16:627-638.
9. Gordon, S., and Z. Cohn. 1970. Macrophage-melanocyte heterokaryons. I. Preparation and properties. J. Exp. Med. 131:981-1003.
10. Graf, T., and H. Beug. 1978. Avian leukemia viruses: interaction with their target cells *in vivo* and *in vitro*. Biochim. Biophys. Acta 516:269-299.
11. Lake, B. D. 1965. The histochemistry of phosphatases: the use of lead acetate instead of lead nitrate. J. R. Microsc. Soc. 85:73-75.
12. Moscovici, C. 1967. A quantitative assay for avian myeloblastosis virus. Proc. Soc. Exp. Biol. Med. 125:1213-1215.
13. Moscovici, C., L. Gazzolo, and M. G. Moscovici. 1975. Focus assay and defectiveness of avian myeloblastosis virus. Virology 68:173-181.
14. Temin, H. M. 1967. Studies on carcinogenesis by avian sarcoma viruses. V. Requirement for new DNA synthesis and cell division. J. Cell Physiol. 69:53-63.
15. Weiss, R. A. 1971. Cell transformation induced by Rous sarcoma virus: analysis of density dependence. Virology 46:209-220.