

Key words: *AKR MuLV/haemopoietic cells/retrovirus expression/neonatal*

Expression of Ecotropic Murine Leukaemia Virus in Haemopoietic Cells of AKR Mice during the Embryonic and Neonatal Period

By C. M. GODARD,* Y. AUGERY AND C. JASMIN

Institut de Cancérologie et d'Immunogénétique (INSERM U.50), Hôpital Paul-Brousse, 14–16 avenue Paul-Vaillant-Couturier, 94804 Villejuif, France

(Accepted 31 May 1983)

SUMMARY

Cells which produce ecotropic murine leukaemia virus have been detected in the bone marrow and the spleen of weanling AKR mice, using an infectious centre technique based on the XC test. There is a noticeable increase in the number of virus-producing cells between day 3 and day 12 after birth, in both of these organs. Some of the virus-producing cells that appear after day 3 have been identified as haemopoietic precursor cells of the granulocyte–macrophage blood lineage. Such precursor cells do not produce virus during the embryonic period and they progressively become involved in virus production after day 3. By day 12, all of them are active virus producers. Thus, the ecotropic virus is expressed in precursor cells of the haemopoietic system, and the latter represent at least one-third of the virus-producing cells in the bone marrow of young AKR mice.

The N-ecotropic murine leukaemia virus (MuLV) of the AKR inbred strain of mice is an endogenous retrovirus whose expression is induced spontaneously at the end of the embryonic period. Infectious virus can be detected in embryo extracts as soon as day 18 or 20 of gestation (Rowe & Pincus, 1972). However, the number of virus-producing cells present in organs such as the spleen, the liver or the thymus increases dramatically during the period that extends from day 3 to day 10 after birth (Fischinger *et al.*, 1982).

Neutralization of this virus by antiviral antibodies can suppress the development of AKR lymphoma (Huebner *et al.*, 1976; Fischinger *et al.*, 1982). For this purpose, the neonatal period appears to be of paramount importance. Treatment of AKR mice with antibodies to the virus major glycoprotein (gp71) will prevent both the onset of viraemia and the occurrence of the natural disease, provided it is applied within a narrow 'window' period between birth and day 3 (Fischinger *et al.*, 1982).

The identification of the virus-producing cells that appear during the neonatal period is a prerequisite for elucidating the role played by the virus in the leukaemic process and the mechanisms of antiviral immunotherapy (Fischinger *et al.*, 1982).

So far, there have been few attempts to identify the nature of the cells that produce infectious MuLV in the haemopoietic organs of young AKR mice. Recent studies have shown that virus is not expressed in B and T cells from the spleen (Fischinger *et al.*, 1982), whereas it is actively produced by bone marrow fibroblastoid adherent cells which belong to the haemopoietic micro-environment (Zipori & Van Bekkum, 1979).

In recent work, we have analysed in more detail the population of virus-producing cells in the bone marrow of young AKR mice. We have shown that virus production is not restricted to bone marrow stromal cells and that at least some of the haemopoietic precursors present in this organ are active virus producers. Thus, approximately 30% of the virus-producing cells in the bone marrow are immature precursors which belong to the granulocyte–macrophage blood lineage (C. M. Godard, Y. Augery & C. Jasmin, unpublished results).

Table 1. Analysis of the production of XC-positive ecotropic MuLV in the haemopoietic organs of AKR embryos

	Liver			Spleen
	13	16	20	
Age of embryos (days)*	13	16	20	20
No. of embryos tested	9	19	20	10
No. of litters tested	2	2	3	2
Virus-producing cells/10 ⁶ cells	0†	0	0	0
GM-CFC†/10 ⁵ cells	350	NT§	186	56
GM-CFC virus-positive/tested	0/45	NT	0/48	0/72

* Embryonic age was determined by the vaginal plug method (Moore & Metcalf, 1970).

† 3×10^4 cells were seeded in 30 mm plastic dishes containing 1 ml of 0.8% methyl cellulose in α -medium, 20% horse serum and 0.05% GM-CSF. Colonies (aggregates containing more than 50 cells) were counted at day 7.

‡ 2×10^5 cells were seeded in Petri dishes containing 10^5 indicator cells, using five dishes/point.

§ NT, Not tested.

These results have now been extended by analysing virus production in haemopoietic cells during the embryonic and neonatal period, in an attempt to characterize the virus-producing cells whose appearance follows birth.

Foetal haemopoiesis begins in the yolk sac at day 7 of gestation, and becomes apparent in the liver, the spleen and the bone marrow at day 10, day 15 and day 17, respectively (Moore & Metcalf, 1970; Moore & Williams, 1973; Cole, 1975). We have investigated the presence of virus-producing cells in the liver or the spleen of embryos at day 13, day 16 and day 20. Single-cell suspensions were prepared from the liver of individual embryos or from pooled spleens of embryos of the same litter, and virus production was studied using two methods. The cells were plated onto mouse NB-type indicator cells (3T3 F1 clone 5D) (Gisselbrecht *et al.*, 1974) and the number of virus-producing cells was determined using the infectious centre assay developed by Melief *et al.* (1975). In a parallel assay, granulocyte-macrophage progenitor cells (or colony-forming cells: GM-CFC) were stimulated to differentiate *in vitro* by plating the cells in semi-solid medium containing horse serum and granulocyte-macrophage colony-stimulating factor (GM-CSF) from mouse lung-conditioned medium (Sheridan & Metcalf, 1973). Individual colonies were removed from tissue culture dishes 3 days after plating, and co-cultivated with mouse indicator cells. After two passages, the latter were tested for the presence of infectious virus using the XC test (Rowe *et al.*, 1970). This procedure allowed the detection of low virus-yielder cells from individual granulocyte-macrophage colonies that would not score as infectious centres in the assay described by Melief *et al.* (1975).

As shown in Table 1, infectious centres were not detected in the haemopoietic organs of any of the embryos tested, and none of the granulocyte-macrophage colonies assayed for virus production appeared to contain virus-positive cells. This indicates that the spontaneous induction of ecotropic virus which takes place during the late embryonic stage (Rowe & Pincus, 1972) involves cells which differ from the GM-CFC tested as well as from the pluripotent haemopoietic stem cells and erythroid precursors which are known to be present in substantial amounts in the embryonic organs tested (Moore & Metcalf, 1970; Cole, 1975).

Using identical methods, we have studied virus production in the spleen and in the bone marrow of weanling animals, during the period extending from day 4 to day 15 (see Table 2). By day 4, the spleen and the bone marrow still contain very few infectious centres and the granulocyte-macrophage colonies tested appeared to be virus-negative, within the limits of sensitivity of the method. Thereafter, a progressive increase of the infectious centre contents in both of these organs was observed. This increase can be ascribed, at least partially, to the recruitment of virus-producing granulocyte-macrophage precursor cells. Moreover, the virus-producing precursors do not appear preferentially in the spleen or in the bone marrow. Thus, among animals tested at day 8, animal 1 showed the same proportion of virus-positive colonies in both organs. Animal 2, in turn, showed a higher proportion of virus-positive colonies in the spleen, as compared to the marrow, whereas a reverse pattern was observed in animal 3.

Table 2. Production of XC-positive ecotropic MuLV during the neonatal period*

Organ tested	Spleen			Bone marrow		
	Infectious centres /10 ⁵ cells	GM-CFC/10 ⁵ cells	GM-CFC virus- positive/tested	Infectious centres /10 ⁵ cells	GM-CFC/10 ⁵ cells	GM-CFC virus- positive/tested
4 days†	4	27	0/23	29	349	0/23
5 days†	39	150	1/23	65	345	5/23
8 days†						
Animal no. 1	139	213	10/23	126	340	10/23
2	530	294	23/23	562	377	10/23
3	190	287	11/23	462	360	23/23
12 days†						
Animal no. 1	680	100	19/23	660	170	22/23
2	1275	187	20/23	966	220	23/23
3	895	137	23/23	950	190	23/23
15 days†						
Animal no. 1	480	47	23/23	735	253	23/23
2	540	47	23/23	685	339	22/23
3	525	30	23/23	575	323	23/23

* Animals were nursed by their AKR mothers.

† Animals from the same litter were pooled.

‡ Three animals from the same litter (no. 1, 2 and 3) were tested separately.

The infectious centre content reached maximum values by days 10 to 12. By this time, all the granulocyte-macrophage colonies had become virus-positive, and the proportion of virus-producing cells in the spleen and the bone marrow was approx. 0.5% to 1%. The latter values remain unchanged in young adult animals (Fischinger *et al.*, 1982; C. M. Godard, unpublished results).

Control experiments were carried out to check whether the presence of virus-producing cells in granulocyte-macrophage colonies was due to the spread of MuLV infection during the course of colony formation *in vitro*. The infectious MuLV released by bone marrow and spleen cultured cells was neutralized using anti-gp70 serum from Rauscher virus (NIH, Bethesda, Md., U.S.A.). Bone marrow and spleen cells from individual animals, 15 days of age, were cultured in 1 ml methyl cellulose medium containing 40 μ l of a fivefold dilution of the original stock of serum and colonies were assayed for virus production as mentioned above. In each case, the addition of serum did not modify the pattern of virus production. All the spleen and bone marrow colonies obtained in the presence of serum were virus-positive, as were control colonies (12 colonies tested per point).

The neutralizing activity of anti-gp70 serum was checked against Gross MuLV produced by D55-Gross cells, a clone of D55-3T3-F1 chronically infected with Gross virus (Gisselbrecht *et al.*, 1974). Tissue culture supernatant fluid (1 ml) was incubated at 37 °C, for 2 h, with various amounts of serum. The plaque-forming units titre (p.f.u./ml) was determined after plating on D55-3T3-F1 indicator cells and using the XC test. The serum concentration of 40 μ l/ml neutralized 99% of the p.f.u. from tissue culture fluid containing 5×10^3 p.f.u./ml.

The present results clearly show that the haemopoietic precursors that belong to the granulocyte-macrophage lineage are involved in the increase in number of those cells which produce virus which takes place between day 3 and day 10 or 12. Since the infectious centre content is greater than the GM-CFC content at day 15, it must be admitted that cells which differ from the haemopoietic precursors tested are also virus-producers. The nature of these cells is still unknown. Some of them belong to the haemopoietic microenvironment (Zipori & Van Bekkum, 1979); however, pluripotent haemopoietic stem cells, as well as precursors of different blood lineage, and other non-haemopoietic cells might also be involved.

It can be concluded, therefore, that ecotropic AKR MuLV is sequentially expressed in a variety of cells. Haemopoietic cells do not contribute to virus production during the embryonic period (Table 1), and the nature of the embryonic cells in which virus production is initiated has not been identified yet. At this early stage, the highest concentration of infectious virus is observed in bones, and therefore bone-associated cells are potential candidates (Rowe & Pincus, 1972).

Virus-producing haemopoietic precursor cells (GM-CFC) appear at days 3 or 4 after birth, and their number increases rapidly (Table 2). Whether ecotropic virus is spontaneously induced in haemopoietic cells, or whether blood precursors are infected by MuLV which is initially released in the embryo, remains to be determined.

Haemopoietic cells represent over one-third of the virus-producing cells in the bone marrow and must be considered as a major source of infectious ecotropic virus. They represent a substantial proportion of the target cells in the treatment with anti-gp71 antibodies which protect AKR animals against both viraemia and subsequent lymphoma.

This work was supported by a research grant from the Institut National de la Santé et de la Recherche Médicale (CRL 80-20-26).

REFERENCES

- COLE, R. J. (1975). Regulatory functions of microenvironmental and hormonal factors in pre-natal haemopoietic tissues. In *The Early Development of Mammals*, British Society for Developmental Biology Symposia vol. 2, pp. 335-358. Edited by M. Balls & A. E. Wild. Cambridge: Cambridge University Press.
- FISCHINGER, P. J., DUNLOP, N. M., SCHWARZ, H., IHLE, J. N., WEINHOLD, K., BOLOGNESI, D. & SCHÄFER, W. (1982). Properties of mouse leukemia viruses. XVIII. Effective treatment of AKR leukemia with antibody to gp71 eliminates the neonatal burst of ecotropic AKR virus-producing cells. *Virology* **119**, 68-81.

- GISSELBRECHT, S., BASSIN, R. H., GERWIN, B. I. & REIN, A. (1974). Dual susceptibility of a 3T3 mouse cell line to infection by N- and B-tropic murine leukemia virus: apparent lack of expression of the FV-1 gene. *International Journal of Cancer* **14**, 106-113.
- HUEBNER, R. J., GILDEN, R. V., TONI, R., HILL, P. R., TRIMMER, T. W., FISH, D. C. & SASS, B. (1976). Prevention of spontaneous leukemia in AKR mice by type-specific immunosuppression of endogenous virogenes. *Proceedings of the National Academy of Sciences, U.S.A.* **73**, 4633-4635.
- MELIEF, C. J. M., DATTA, S. K., LOUIES, S., JOHNSON, S., MELIEF, M. & SCHWARTZ, R. S. (1975). Splenocyte plaque assay for the detection of murine leukemia virus. *Proceedings of the Society for Experimental Biology and Medicine* **149**, 1015-1018.
- MOORE, M. A. S. & METCALF, D. (1970). Ontogeny of the haemopoietic system: yolk sac origin of *in vivo* and *in vitro* colony-forming cells in the developing mouse embryo. *British Journal of Haematology* **18**, 279-296.
- MOORE, M. A. S. & WILLIAMS, N. (1973). Analysis of proliferation and differentiation of foetal granulocyte-macrophage progenitor cells in haemopoietic tissue. *Cell and Tissue Kinetics* **6**, 461-476.
- ROWE, W. P. & PINCUS, T. (1972). Quantitative studies of naturally occurring murine leukemia virus infection of AKR mice. *Journal of Experimental Medicine* **135**, 429-436.
- ROWE, W. P., PUGH, W. E. & HARTLEY, J. W. (1970). Plaque assay techniques for murine leukemia viruses. *Virology* **42**, 1136-1139.
- SHERIDAN, J. W. & METCALF, D. (1973). A low molecular weight factor in lung-conditioned medium stimulating granulocyte and monocyte colony formation *in vitro*. *Journal of Cellular Physiology* **81**, 11-23.
- ZIPORI, D. & VAN BEKKUM, D. W. (1979). Changes in the fibroblastoid colony forming unit population from mouse bone marrow in early stages of Soule virus-induced murine leukemia. *Experimental Hematology* **7**, 137-144.

(Received 14 January 1983)