Hormone Independent in Vitro Erythroid Colony Formation by Mouse Bone Marrow Cells

K. Nooter (1, 2), R. Ghio (3), K. J. v. d. Berg (1) and P. A. J. Bentvelzen (1).

Radiological Institute TNA Rigwigh (ZH), The Netherlands

Introduction

In vitro transformation of normal cells by oncogenic viruses, inducing growth patterns comparable to those of neoplastic cells, has become a powerful tool in the study of these viruses (1, 2). The most used targets for oncogenic viruses are embryonic fibroblasts, but murine leukemia viruses can rarely transform this kind of cell. The obvious targets for this group of viruses seem to be hemopoietic cells. To develop a suitable transformation assay, possibilities to discriminate between normal and leukemic cells on the basis of differences in growth patterns *in vitro* are requied.

Murine erythroid precursor cells (CFU-E) produce within a few days many colonies of hemoglobin-synthesizing cells in vitro in the presence of the hormone erythropoietin (EP) (3, 4). With a longer culture period in the presence of larger amounts of EP, so-called bursts consisting of large, dispersed colonies of erythroid cells appear (3, 5). The burst forming unit (BFU-E) is thought to be a more primitive member of the erythroid series than is the CFU-E.

I. Growth differences between normal and virus-infected B. M. cells

Bone marrow cells from RLV-infected BALB/c mice were compared with cells from uninfected mice with regard to their dependency on EP for the *in vitro* development of erythroid colonies.

BALB/c mice were injected i.p. with RLV and examined twice a week thereafter for the development of the disease. A continuing increase in spleen weight begins at day 5 postinfection; a maximum weight of three grams is reached in the terminal stage at 4–5 weeks after inoculation. The production of *in vitro* erythroid colonies

1. Radiobiological Institute TNO,
Lange Kleiweg 151,
Rijswijk (ZH),
The Netherlands.
2. Part of this investigation was supported by a grant from the Netherlands Organization for Fundamental Medical Research.
3. Riccardo Ghio was on leave of absence from the I.S.M.I., Università Cattedra di Ematologia, Genoa, Italy. He received a fellowship from the Netherlands Organization for the Advancement of Science under the auspices of a treaty between this organization and the Centro Nazionale di Richerche, Italy.

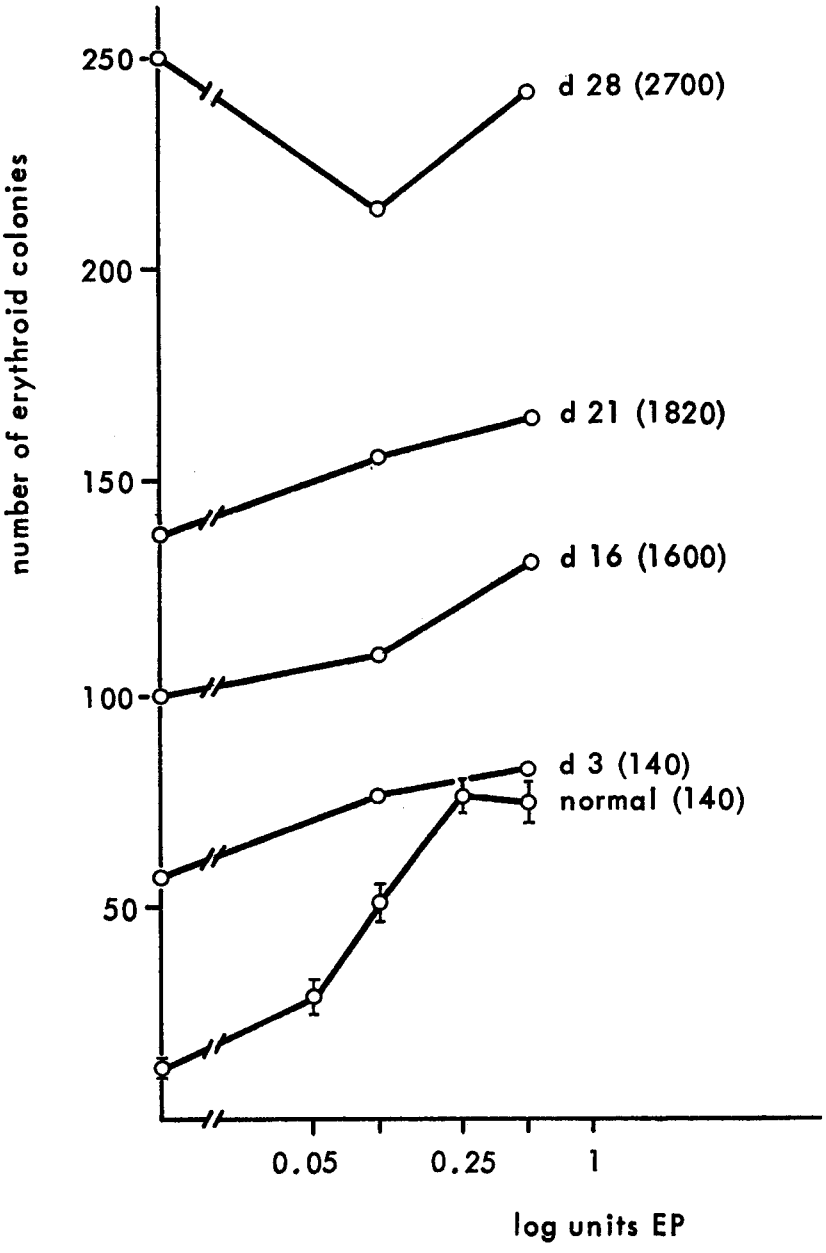

Fig. 1: Erythropoietin dose-response curves of normal and RLV-infected BALB/c mice bone marrow.
Normal curve: mean ± SE of 7 separate experiments. RLV-infected animals: three (d3). sixteen (d16), twenty-one (d21) and twenty-eight (d28) days after infection.
Numbers in parentheses indicate spleen weights.

at several concentrations of EP by normal bone marrow (BM) cells and cells taken from mice at several days after infection with RLV is presented in Fig. 1. A few erythroid colonies are produced by the controls in the absence of exogenous EP (Connaught, Step III), but the addition of the hormone leads to a marked elevation with an optimum at 0.25 I.U. Three days after infection with the virus when clinical signs of the disease are not yet present, a five-fold increase in the number of colonies formed in the absence of exogenous EP is found. During the progression of the disease, a remarkable increase in the number of EP-independent colonies can be observed.

Evidence for the specificity of this spontaneous colony formation is demonstrated by the following experiments: Plating different cell concentrations (1, 2, 5, 10, and 20 x 10^4) from RLV-infected BM reveals a linear relationship with the number of erythroid colonies formed, both with and without EP in the culture medium. To complete rule out the possibility that the BM cells of RLV-infected mice produce endogenous EP feeder-layer experiments were done with normal and infected BM cells. No significant erythropoietic activity by either normal or RLV-infected BM could be detected which would give rise to erythroid colony formation by normal BM cells.

To exclude the possibility that the observed hormone-independency of BM cells from RLV-infected mice is due only to a high erythropoietic stimulation *in vivo*, we performed the following experiments. Anaemia was induced in BALB/c mice by phenylhydrazine treatment. Two days later the haematocrit and EP responsiveness *in vitro* were determined. The haematocrit values were decreased from 48–50 % to 31–40 %. The maximum number of erythroid colonies per 2 x 10^5 BM cells increased 3–5 times, but the colony formation was still hormone-dependent. Even after a prolonged phenylhydrazine treatment of 14 days, the erythroid-colony-forming units (CFU-E) were dependent on the addition of EP *in vitro*.

In antoher experiment, RLV-infected mice were hypertransfused with packed red cells at various time intervals after infection. Bone marrow was cultured with 0.25 I.U. EP and in medium containing no EP. Both infected and uninfected mice showed a drastic decrease in the number of colonies in the presence of EP. However, the colony formation was still completely EP-independent in the infected animals, while the BM cells of the controls needed the hormone.

Not only the CFU-E but also the BFU-E seems to have become hormone independent as large bursts of erythroid cells after 10 days of cultures are found by BM cells from mice which have been infected with RLV 15 days earlier (Table 1).

Table I: Number of EP-independent BFU-E in bone of marrow of RLV-infected BALB/c mice 15 days after injection

	number of bursts after 10 days of culture	
Treatment animals	+ EP*	– EP
–	17	2
RLV	21	8

* The amount of EP added to the cultures is 1 I. U.

II. Enhancement of erythroblasts by CFA

Complete Freund's adjuvant (CFA) strongly enhances the erythroblastosis induced by Rauscher murine leukemia virus (RLV) (6, 7). On the basis of this fact, it was concluded that the pluripotent hemopoietic stem cell would be involved in the development of splenomegaly (6). On the other hand, the course of the disease can be influenced by manipulation of the erythroid compartment (8, 11) suggesting

that an erythroipoietic-sensitive cell would be the target for the transforming activity of the virus.

The *in vitro* techniques mentioned above were used to determine the mechanism by which CFA enhances RLV-induced erythroblastosis.

BALB/c mice were injected with crude cell-free RLV preparation and the same day with CFA(0.2 ml. i.p.).

The number of CFU-E per 10^5 normal bone marrow cells as determined in methylcellulose cultures with erythropoietin greatly increases after administration of CFA (fig. 2). A peak is reached at 5 days after inoculation; after which a plateau level is maintained.

The number of CFU-E producing erythroid colonies in the absence of erythropoietin increases steadily after RLV-infection (fig. 3). The administration of CFA also has an enhancing effect on the number of EP-independent CFU-E, but this effect becomes noticeable only when bone marrow is taken 10 days or later after infection. The most likely explanation for the strong increase induced by the treatment with CFA is the recruitment of new target cells for virus released by previously infected cells.

Rather unexpected was the marked increase in CFU-E after CFA administration. This is certainly not due to the slight anemia induced by CFA, because that is preceded by the rise in CFU-E. Furthermore, a drop of less than 10 % in the hematocrit does not induce a 6-fold increase in CFU-E (12). The adjuvant, like other antigens, is known to stimulate the proliferation of pluripotent hemopoietic stem cells as has been described for a variety of other antigen (133). There is probably no competition at the stem cell level for differentiation into a a specific

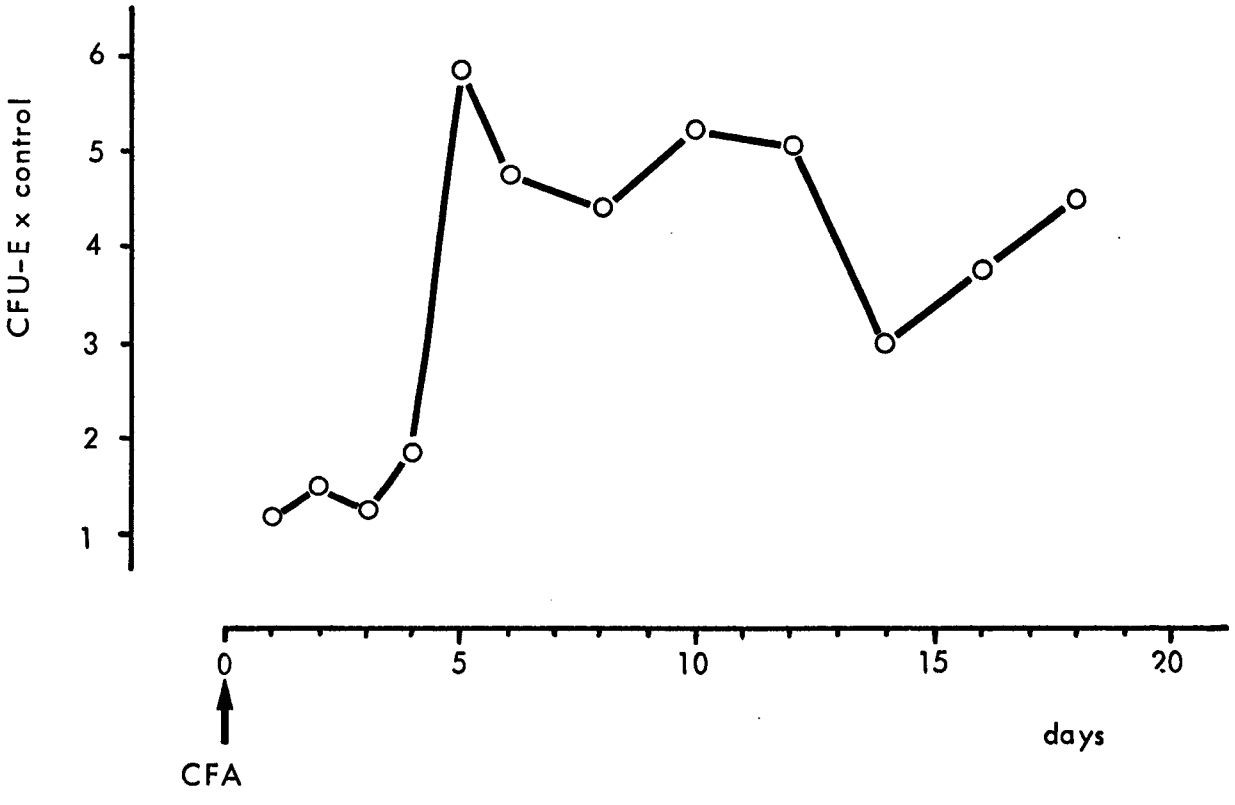

Fig. 2: Increase in the number of CFU-E in bone marrow of BALB-c mice induced by CFA, expressed proportional to control bone marrow of untreated animals.

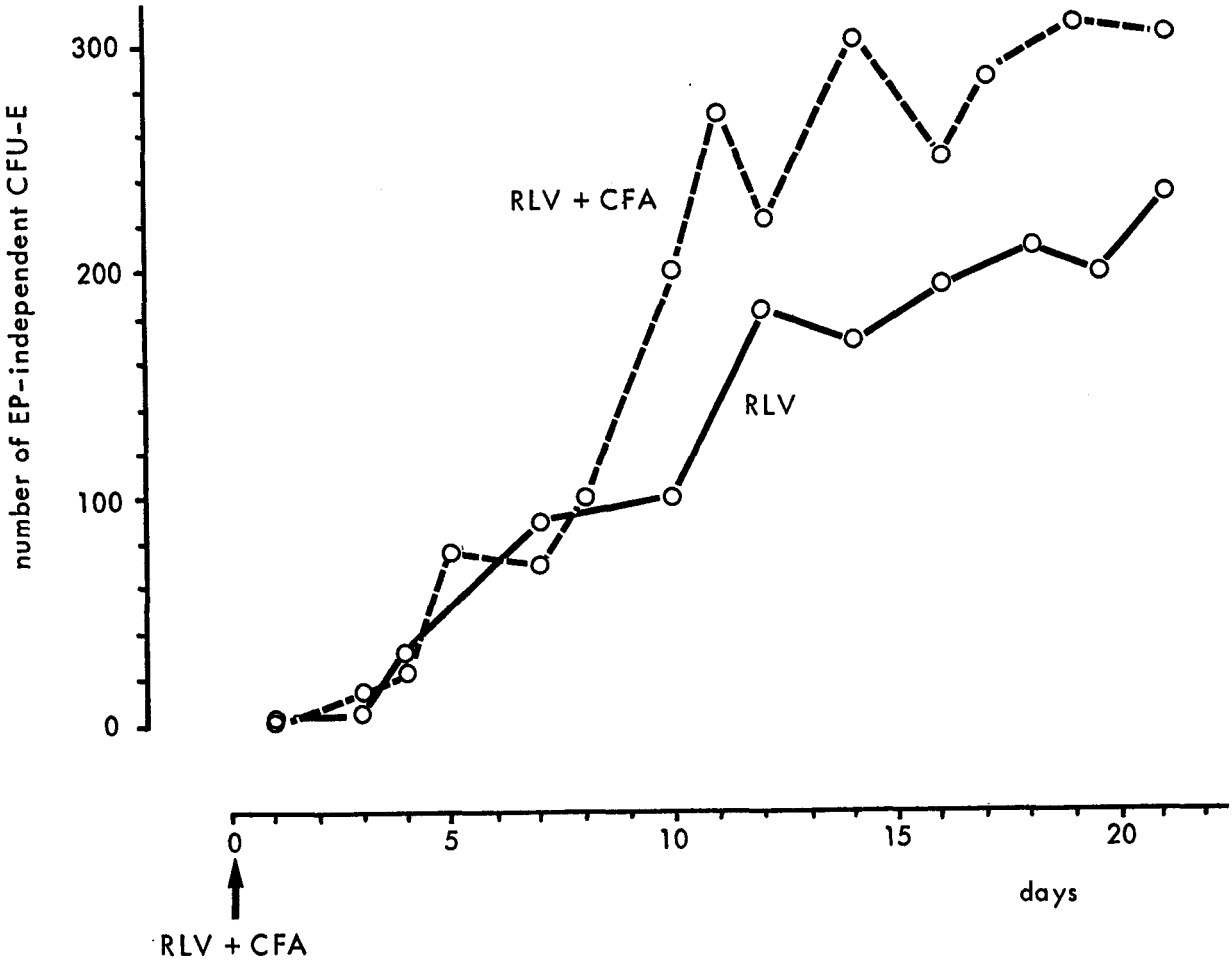

Fig. 3: Number of EP-independent CFU-E in bone marrow of BALB/c mice treated with only RLV or RLV + CFA.

hematologic series which would promote proliferation of myeloid progenitor cells over that of erythropoietic-sensitive cells. The very marked increase in CFU-E does not lead to polycythemia. There is even a slight decrease in the hematocrit. The number of erythroblasts in the bone marrow also declines after administration of CFA (14). There seems to be an maturation block *in vivo* beyond the stage of CFU-E.

III. Transformation in vitro

A subsequent step in our study was the induction of such hormone-indepency by incubation *in vitro* of normal BM cells with RLV. In table 2 is presented the number of erythroid colonies produced at various intervals after plating for bone marrow incubated with 10^7 XC-PFU of purified RLV isolated from leukemic spleen. As controls, the results of bone marrow kept for 4 hours under the same conditions in the absence of RLV are given. An additional control is the addition of EP to the culture medium. Already 2 days after plating no sign of EP-dependency can be observed in the RLV-infected cells, while the addition of EP to the normal bone marrow cultures leads to a marked elevation in colony production. A very high number of EP-independent colonies is noticed at day 5 after plating

of RLV-incubated cells. The numbers of erythroid colonies produced 5 days after plating in the absence of EP and after incubation with various dilutions of RLV are presented in table 3. The results suggest a linear dose-response relationship. An endpoint seems to be reached at the dilution of 3200x, which corresponds with 10^4 PFU in the reverse XC test. Transformation could be prevented by incubation with specific antiserum. Mammary tumor virus or a myeloid leukemia virus did not induce this physiologic transformation.

Table II: In vitro induction by RLV of E.P.-independency of erythroid colony formation

Days after plating	Incubated with RLV number of erythroid colonies[1]		Incubated without RLV number of erythroid colonies[1]	
	presence of EP[2]	absence of EP	presence of EP[2]	absence of EP
2	166	207	290	82
5	1404	1342	174	54
7	1306	1211	201	43

[1] per 2 x 10^5 B. M. cells.
[2] 0.5 I. U. per dish.

Table III: Dose-response relationship of EP-independent colony-formation after incubation with R.L.V.

Dilution of RLV stock suspension	Number of EP-independent colonies 5 days after plating[1]
10	538
100	481
200	374
400	220
800	96
1600	58
3200	24
6400	15
12800	19

[1] per 2 x 10^5 B. M. cells.

The influence of EP on *in vitro* transformation was investigated by incubating bone marrow with RLV in the presence of EP for 16 hours. Thereafter, the cells were plated in the same way as described above. A slight inhibitory effect of the hormone on *in vitro* transformation can be observed in both experiments (table 4).

Possibly during the 16 hour incubation period the hormone induces maturation

of the target cells beyond a stage where transformation can still take place. The hormone cannot compensate for this by the production of new target cells from more primitive hemopoietic cells under the employed *in vitro* conditions.

Table IV: Influence of incubation with EP on in vitro transformation by RLV

Incubation		Number of EP-independent colonies at day 5[1]	
1 mg RLV	1 IU EP	Experiment 1	Experiment 2
–	–	32	76
–	+	15	53
+	–	686	939
+	+	483	632

[1] per 2 x 10^5 B. M. cells.

IV. Transfection

Hill and Hillova (15) were the first to carry out "transfection" experiments using proviral DNA of Rous sarcoma virus. Their results gave clear evidence that DNA from RNA virus-infected cells can carry virus-specific information responsible for the transformation.

In the present study, we used spontaneous erythroid colony formation by mouse bone marrow cells as a criterion for transformation. DNA was isolated by standard procedures from spleen cells of BALB/c mice which had been infected with RLV. The DNA solution was buffered with Hepesphosphate and treated with a high calcium concentration according to Graham and Van der Eb (16). A 0.5 ml sample of DNA suspension was then incubated with BALB/c mouse bone marrow cells for 4 hr and after washing the cells were plated in methyl-cellulose in the absence of erythropoietin.

After 5 days the number of erythroid colonies were counted. From table 5 it can be seen that DNA prepared from RLV-infected spleen cells has a transforming capacity. The control experiments show that the observed phenomenon is specific. Experiments with the reversed XC-test (17) showed that virus reproduction also

Table V: Transfection of mouse bone marrow cells with proviral DNA

Additions	Number of EP-independent colonies at day 5[1]
RLV-spleen-DNA 50 ug	744
rat-spleen-DNA 50 ug	22
RLV-spleen-DNA[2] 50 ug + DNA-se	16
High calcium buffer only	20

[1] per 2 x 10^5 B. M. cells.
[2] incubated for 60 min. at 37 °C with 50 ug/ml of DNA-se before addition of calcium.

takes place after incubation of bone marrow cells with DNA from RLV-infected cells. The procedure utilized here to isolate DNA may give a range of molecular weights between 20 x 10^6 and 200 x 10^6 daltons. More detailed studies will be undertaken to determine the minimal size of the DNA fragments which can lead to virus reproduction and to transformation of haemopoietic cells.

V. Concluding remarks

B.M. cells of RLV-infected BALB/c mice can proliferate in methylcellulose in the absence of E.P., while normal B.M. cells cannot (12). Not only the CFU-E but also the more primitive BFU-E shows hormone-independency (18). This phenomenon is in favour of the view that the Rauscher virus induced erythroblastosis is a true neoplasia although transplantation experiments failed so far.

The experiments in which transformation *in vitro* of B.M. cells by RLV is established (19) show that the CFU-E can serve as a target for the virus.

Treatment of normal mice with CFA leads to a rapid increase in CFU-E in the bone marrow (18). Splenomegaly of RLV-infected mice is enhanced by CFA-treatment probably due to an increase in targets. Transfection with proviral DNA also can transform the CFU-E of BALB-c mice. This approach allows *in vitro* studies on the resistence of mouse strains to RLV *in vitro*.

The studies are of interest for the human disease in two aspects. *In vitro* transformation assays are needed to study the oncogenic potential of putative human leukemia viruses. Furthermore the studies have yielded some new insight in the pathogenesis of virally induced erythroblastosis. This might serve as a model for e.g. acute myeloid leukemia in man.

References

1. Macpherson, I. The characteristics of animal cells transformed in vitro. Adv. Cancer Res. *13*, 169–209 (1970).
2. Pontén, J. Spontaneous and virus induced transformation in cell culture. Virology Monogr. *8*, Springer Verlag, New York (1971).
3. Iscove, F. N. and Sieber, F. Erythroid progenitor in mouse bone marrow detected by macroscopic colony formation in culture. Exp. Hematol. *3*, 32–43 (1975).
4. Stephenson, J. R., Axelrad, A. A., McLeod, D. L. et al. Induction of colonies of hemoglobin-synthesizing cells by erythropoietin in vitro. Proc. Nat. Acad. Sci. USA *68*, 1542–1546 (1971).
5. Axelrad, A. A., McLeod, D. L., Shreeve, M. M. et al. Properties of cells that produce erythrocytic colonies in vitro. In hemopoiesis in culture. (Robinson, W. A., ed.). DHEW publication No (NIH) 74–205 (1974), pp. 226–234.
6. Siegel, B. V., and Morton, J. I. Immunologic stimuli in relation to leukemogenesis. I. Potentiation of Rauscher viral leukemia by Freund's adjuvants. J. Nat. Cancer Inst. *48*, 1681–1686 (1972).
7. Brommer, E. J. and Bentvelzen, P. A. J. Interactions between differentiation and leukaemogenesis in Rauscher murine leukaemia. Bibl. Haemat. *39*, 929–934 (1973).

8. Dunn, T. B., Malmgren, R. A. and Carney, P. C. et al. PTU and transfusion modification of the effects of Rauscher virus in BALB/c mice. J. Nat. Cancer Inst. *36*, 1003–1025 (1966).
9. Pluznik, D. H., Sachs, L. and Resnitzky, P. The mechanism of leukemogenesis by the Rauscher leukemia virus. Nat. Cancer Inst. Monogr. *22*, 3–14 (1966).
10. Brommer, E. J. The role of the stem cell in Rauscher murine leukaemia TNO, The Hague, (1973).
11. Seidel, H. J. Die Blutzellbildung bei der Rauscher-Leukämie (Mäusestamm BALB/c) und ihre Beeinflussung durch Hypertransfusion. Z.Schr. Krebsforsch. *77*, 155–165 (1972).
12. Nooter, K. and Ghio, R. Hormone-independent in vitro erythroid colony formation by bone marrow cells from Rauscher virus-infected mice. J. Nat. Cancer Inst. *55*, 59–64 (1975).
13. Boggs, D. R., Marsh, J. C. and Chervenick, P. A. et al. Factors influencing hematopoietic spleen colony formation in irradiated mice. J. Exp. Med. *126*, 851–870 (1967).
14. McNeill, T. A. Antigenic stimulation of bone marrow colony forming cells. Immunology *18*, 61–72 (1970).
15. Hill, M. and Hillowa, J. Virus recovering in chicken cells tested with Rous sarcoma cell DNA. Nature New Biology *237*, 35–39 (1972).
16. Graham, F. L. and Van der Eb, A. J. A new technique for the assay of infectivity of human adeno virus 5 DNA. Virology *52*, 456–467 (1973).
17. Niwa, O., Declève, A. and Liberman, M. et al. Adaptation of plague assay methods to the in vitro quantitation of radiation leukemia virus. J. Virol. *12*, 68–73 (1973).
18. Nooter, K. and Bentvelzen, P. A. J. In vitro studies on the enhancement of Rauscher virus-induced erythroblastosis by complete Freund's adjuvant in BALB/c mice. J. Nat. Cancer Inst. In press. (1976).
19. Nooter, K. and Bentvelzen, P. A. J. In vitro transformation of murine erythroid cells by Rauscher leukemia virus. Cancer letters, 155–160 (1976).