Mechanism of Leukemogenesis and of Target Cell Specificity by Defective Avian Leukemia Viruses: A Hypothesis

Graf, T., v. Kirchbach, A., Beug, H.

Biologisch-Medizinische Abteilung, Max-Planck-Institut für Virusforschung, Spemannstr. 35. 7400 Tübingen, Federal Republic of Germany. Present adress: Institut f. Virusforschung. Deutsches Krebsforschungszentrum, D-6900 Heidelberg, Federal Republic of Germany

Introduction

Avian leukemia viruses comprise a class of retroviruses isolated in their majority from field cases of the domestic chicken. A group of these agents, which are defective for replication and are therefore referred to as "defective leukemia viruses" (DLV), cause a variety of types of acute leukemia and other neoplasms of nonhematopoietic origin within weeks or months after infection. All DLV-strains isolated so far are capable of in vitro transformation of hematopoietic tissues (for review see Graf and Beug, 1978). In our recent studies, we have mainly concentrated on two model virus strains: avian erythroblastosis virus (AEV) and myelocytomatosis virus strain MC29. Clone-purified AEV causes an acute erythroleukemia but also sarcomas (Graf et al., 1977). MC29 causes myelocytomatosis and tumors of predominantly epitheloid origin (for references, see Graf and Beug, 1978).

The aim of this article is to summarize the present state of our research on AEV and MC29 with regard to their interaction with hematopoietic cells and to present a new hypothesis about their target cell specificity and mechanism of leukemogenesis.

Results and Discussion

Characterization of Hematopoietic Cells Transformed by AEV and MC29

The first question we asked was: do hematopoietic cells transformed by AEV and MC29 in vitro express erythroid and myeloid differentiation parameters, respectively, as do leukemic cells from virus-infected birds on the basis of morphological and staining criteria?

To study this question, chick bone marrow cells were infected with the respective viruses and colonies of transformed cells isolated and grown into mass cultures as described before (Graf, 1973; Graf, 1975; Graf et al., 1978b). These cultures were then examined with regard to the expression of a series of erythroid and myeloid differentiation parameters. The results obtained, some of which have been published in a preliminary form (Graf et al., 1976a, b; Graf et al., 1977; Graf et al., 1978b) are summarized in Table 1. As can be seen, AEV-transformed cells are erythroid in nature, whereas MC29-transformed cells have myeloid properties and in several respects resemble

Table 1. Differentiation parameters in AEV- and MC29-transformed chick bone marrow cells

Differentiation parameters		Virus used for infection	
		AEV	MC29
Erythroid	Hemoglobin	+	–
	Histone H5	+	–
	Erythrocyte cell surface antigen	+	–
Myeloid	Phagocytic capacity	–	+
	Fc receptors	–	+
	Macrophage/granulocyte cell surface antigen	–	+

macrophages. With both viruses, in particular with AEV, the transformed cells express differentiated functions but at a lesser degree than terminally differentiated cells of the corresponding lineage. For example, AEV-transformed cells exhibit levels of hemoglobin which are about 100 times lower than those of adult erythrocytes (Graf et al., 1978b).

Leukemia cells from AEV-infected birds, when brought into culture, were indistinguishable from the in vitro transformed cells for all differentiation parameters mentioned in Table 1.

Characterization of the Hematopoietic Target Cells for Transformation by AEV and MC29

The second question asked was: do both our model leukemia viruses affect pluripotent hematopoietic stem cells, inducing them to differentiate exclusively into one or the other differentiation lineages, or do AEV and MC29 transform erythroid and myeloid progenitor cells, respectively?

That the latter alternative is correct could be shown by the separation of the target cells prior to infection and by the demonstration that they already express lineage specific membrane antigens. The corresponding body of evidence (Graf et al., 1978b; Graf and Beug, 1978, and unpublished results of Beug, v. Kirchbach, Meyer-Glauner, Royer-Pokora and Graf) cannot be described here in detail and is only briefly summarized in Table 2.

Taken together, our results suggest that the hematopoietic cells transformed by AEV and MC29 as well as the normal hematopoietic target cells for transformation by these viruses represent immature erythroid and myeloid cells, respectively.

Table 2. Characterization of the hematopoietic target cells of AEV and MC29

Properties of the target cells of	
AEV	MC29
Nonadherent	Adherent + nonadherent
Not phagocytic	A fraction is phagocytic
Erythroid cell surface antigens	Macrophage/granulocyte cell surface antigen(s)

A Viral Gene Product is Required for the Maintenance of the Undifferentiated Stage of DLV-transformed Hematopoietic Cells

The above results, in conjunction with the finding that AEV transformed erythroblasts could be induced to differentiate by addition of butyric acid (Graf et al., 1978b), raised the possibility that AEV and MC29 transform hematopoietic cells by blocking their capacity to differentiate.

The third question, therefore, was: is a viral gene product required for the maintenance of the "transformed" state of the leukemic cells? And if so, can the leukemic cells be induced to differentiate further by inactivating this gene product?

If both of the above assumptions were true, it should be possible to isolate mutants of AEV or MC29 temperature sensitive (ts) for the maintenance of the undifferentiated state in the respective transformed cells. Attempts to isolate such mutants were made in the AEV system, mainly because simple assays were available for the qualitative and quantitative detection of hemoglobin in chicken hematopoietic cells.

It was indeed possible to isolate a mutant of the desired type (Graf et al., 1978a). At 35°, cells transformed by this mutant (designated *ts* 34 AEV) exhibited low amounts of hemoglobin as detected by benzidine staining or by radioimmunoassay. After 3 days of shift to 41°, with both tests, a drastic increase in hemoglobin levels was observed (Fig. 1 and 2; and Graf et al., 1978a). Control experiments showed that the ts-lesion was located in the defective, transforming ts34 AEV virus and not in its helper virus (Graf et al., 1978a). As shown in Fig. 2, the shift-induced increase in hemoglobin was reversible

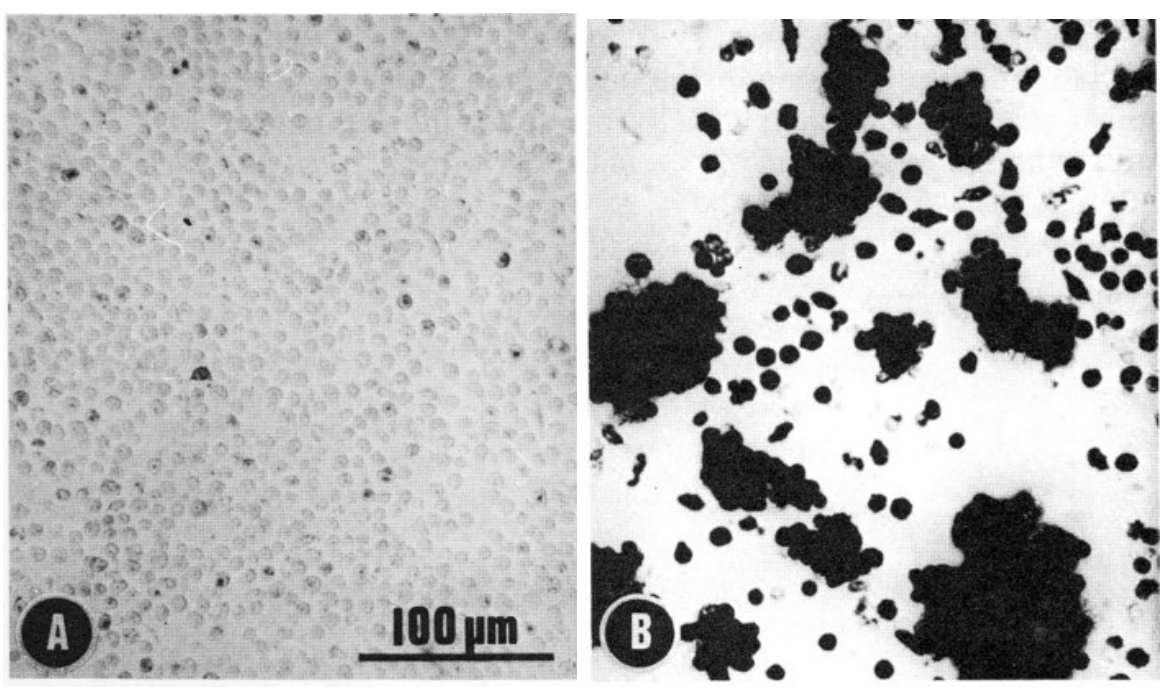

Fig. 1. Effect of temperature on the expression of hemoglobin in ts 34 AEV-infected chick bone marrow cells. Erythroblasts from a single transformed colony were grown up at 35° and aliquots were seeded at 35° (A) and 41° (B). Three days later the cells were stained with benzidine by the method of Orkin et al. (1975)

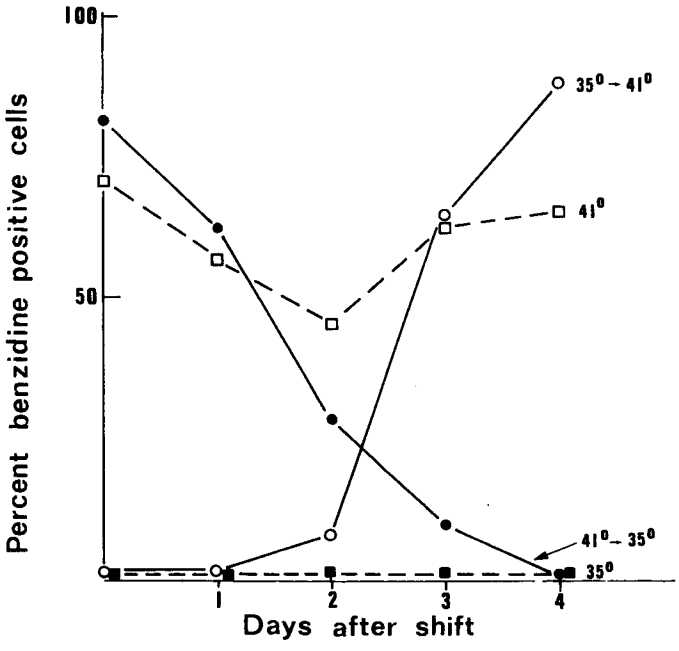

Fig. 2. Kinetics of hemoglobin expression in ts34 AEV-erythroblasts as determined by benzidine staining

after shift back of the cells to 35°. Because of the high body temperature of the chicken (41,8°), the mutant could also be tested for its leukemogenicity under "non-permissive" conditions. As shown in Fig. 3, *ts* 34 AEV is in fact significantly less leukemogenic than the parental wild type AEV.

The data discussed so far support the notion that AEV and MC29 specifically transform immature hematopoietic cells already exhibiting lineage specific differentiation markers, and that these cells are blocked in their ability to terminally differentiate by the action of a viral gene product. This concept is schematically depicted in Fig. 4. Similar ideas have been proposed in earlier communications (Graf et al., 1976a, b; Graf and Beug, 1978b).

The model shown, contains no information about the molecular basis of the observed target cell specificity nor about the mechanism of leukemic transformation by AEV and MC29. In the following, a few hypotheses relevant to these topics will be discussed.

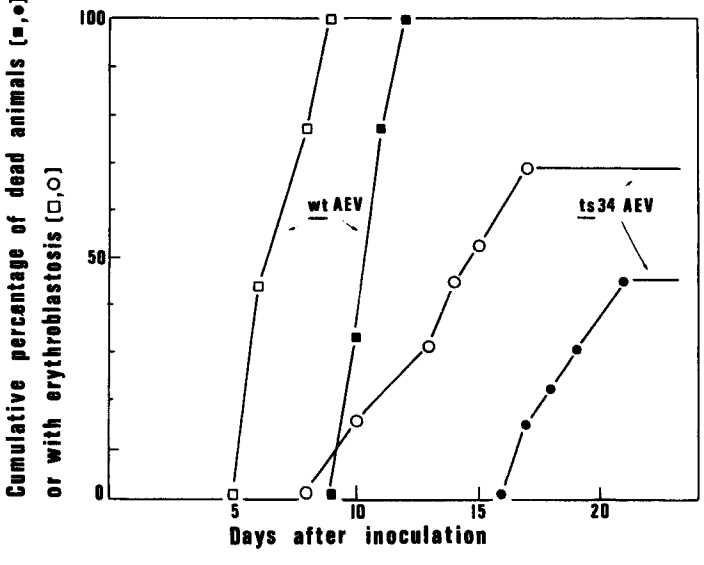

Fig. 3. Leukemogenicity of ts34 AEV. Three groups of 8-day old chicks were injected i.v. with 0,1 ml of growth medium containing either 1000 colony forming units of cell-free ts34 AEV (13 chicks) or 10 colony forming units of cell-free wild type AEV (9 chicks). All animals were observed at 2–3 day intervals for the appearance of erythroblastosis by preparing blood smears and for their death over a period of 3 months. Chicks with ts34 AEV, circles; with AEV, squares

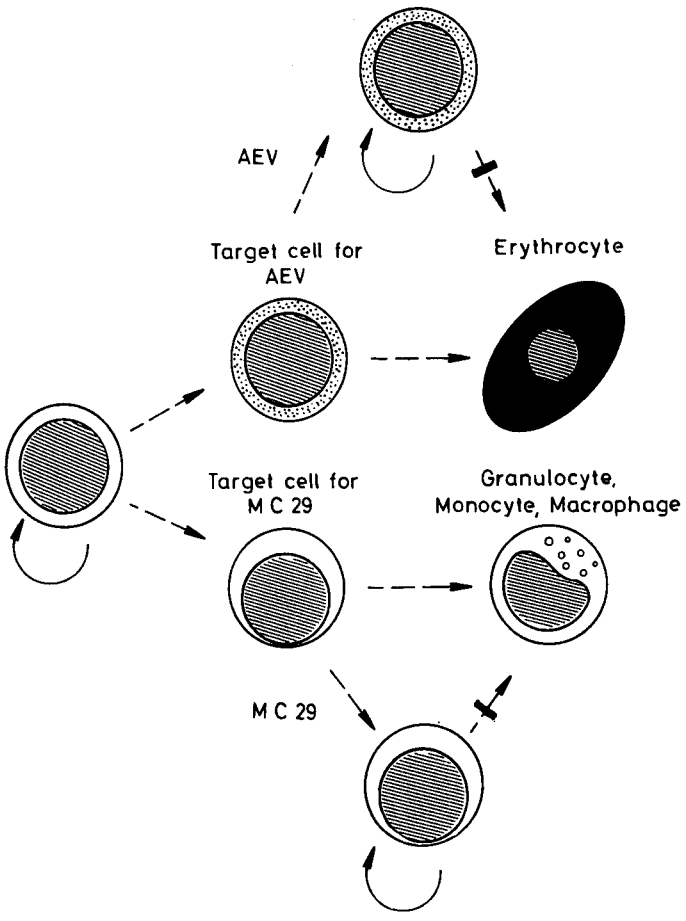

Fig. 4. Diagram illustrating the lineage specific leukemic transformation of hematopoietic cells by AEV and MC29. Curved arrows: proliferation; broken arrows: differentiation, bars: block of differentiation

Basic Possibilities to Explain the Target Cell Specificity of AEV and MC29

The simplest explanation for the transformation target cell specificity of AEV and MC29 would be that these viruses are unable to replicate in cells which they cannot transform. We have tested this model using chick macrophage cultures free of erythroid cells and fibroblasts. They were shown earlier to be resistant to transformation by AEV but not to MC29 (Graf et al., 1976a, b). Macrophages were infected with AEV and tested for the amount and type of virus produced at various days after infection. The results in Fig. 5 show that after an eclipse period of about two days both transforming AEV and its helper virus are synthesized by the infected macrophages. In a similar experiment, MC29 was found to be synthesized by AEV-transformed erythroblasts after superinfection with MC29 virus (unpublished results).

That DLV's can replicate (in the presence of a helper virus) in hematopoietic cells not susceptible to transformation rules out the possibility that they are restricted in nontarget cells at the level of penetration. In addition, if it is assumed that integration is a prerequisite for replication (Bishop, 1978) these results, together with those obtained with the ts mutant of AEV discussed above, indicate that integration of the viral genome alone cannot be sufficient to induce a leukemic cell transformation. They are neither compatible with any simple model in which an integration specificity accounts for the target cell specificity of DLV's. Instead, it appears as if the continuous

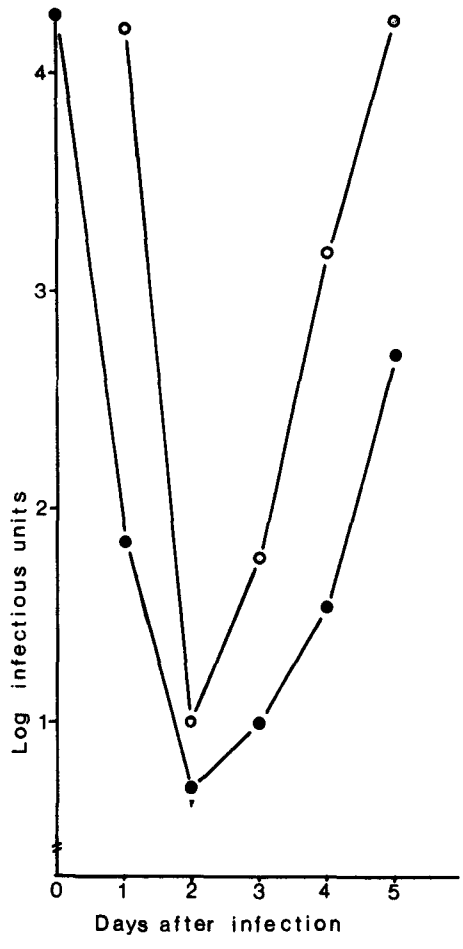

Fig. 5. Replication of AEV in macrophages. Tertiary macrophage cultures derived from the bone marrow of a 3 week old chick were infected with a RAV-2 pseudotype of AEV. Virus infectivity was determined at daily intervals thereafter and the medium was changed after each harvest. Closed circles: focus forming units/ml; open circles: plaque forming units per ml (the number of plaques is a measure of the infectivity of the helper virus)

expression of a viral gene product (probably a protein) is required for the maintenance of leukemic transformation and the restriction is therefore likely to occur either at the level of protein expression or protein recognition. In the first case, viral transformation protein would be synthesized in target cells only. In the second case it would be synthesized in hematopoietic cells from all lineages but since it would not be recognized in nontarget cells, it would induce a transformation in specific target cells only.

We favor the latter possibility and have constructed a new hypothesis which will be outlined in the following.

A Hypothesis Explaining Leukemogenesis and Target Cell Specificity of DLV's (Fig. 6)

Recent findings have shown that the *src* gene of avian sarcoma viruses is present in the genome of normal cells (Stéhelin et al., 1976). Similar data are emerging for AEV and MC29-specific sequences (the putative transforming sequences or *"onc"* genes).

We now propose that certain genes involved in hematopoietic differentiation, arbitrarily termed b, have been picked up (and modified) by DLV's during evolution, now constituting the transforming sequences of the virus (b*). Consequently, transforming proteins of DLV's (B*) represent modified hematopoietic differentiation proteins which differ for different virus strains.

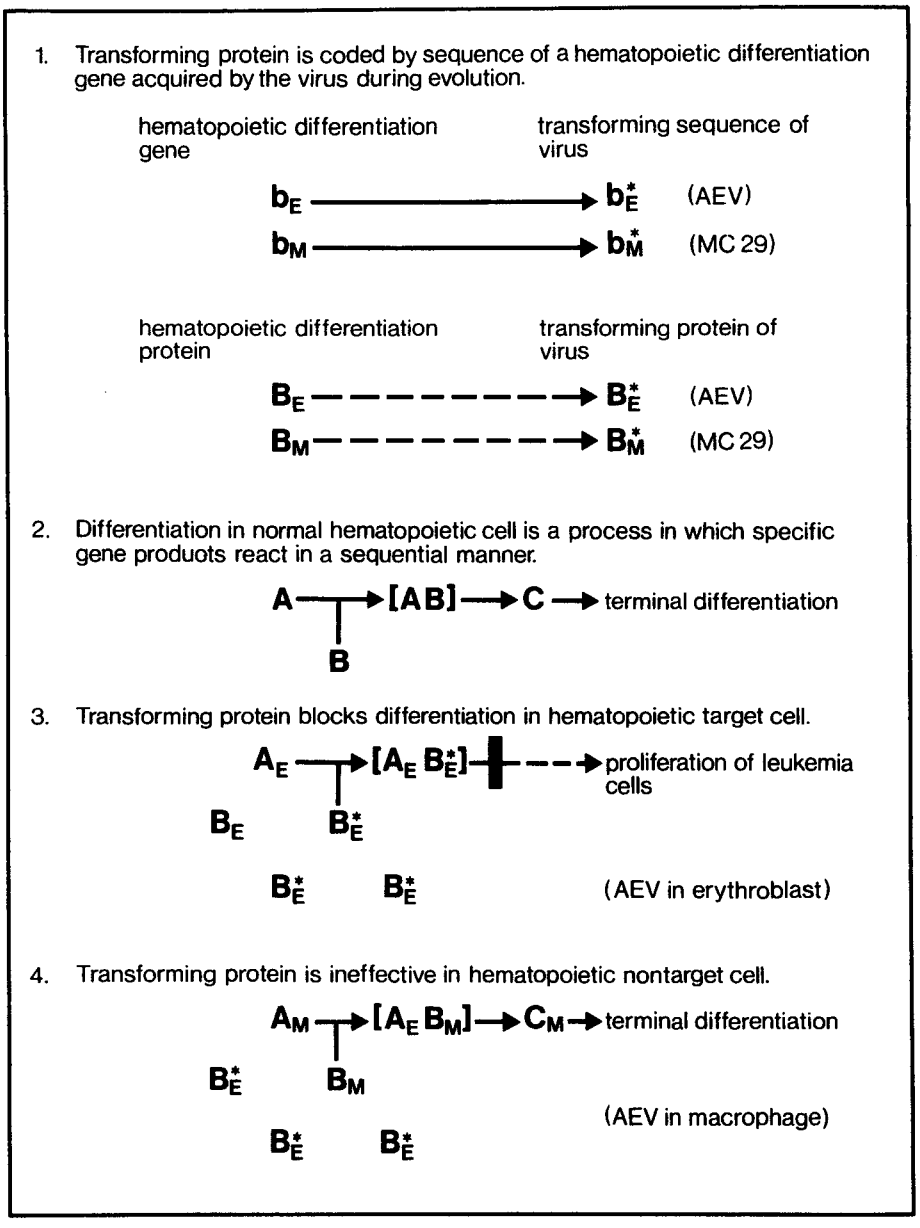

Fig. 6. Schematic representation of a hypothesis explaining the target cell specificity of DLV's and the mechanism of leukemogenesis as consequences of a differentiation block

Thus, AEV would code for a modified erythroid differentiation protein (B^*_E) and MC29 for a modified myeloid protein (B^*_M). It should be emphasized here that the designations used serve only formal purposes and cellular proteins such as B_E and B_M are not meant to be homologous in a functional way.

The second assumption is that a necessary step in hematopoietic differentiation is a process in which specific gene products react in a sequential manner. For example, a differentiation protein B interacts with a receptor A forming a complex [AB] which in turn can be converted to or induces another differentiation factor C etc., finally leading to terminal differentiation.

In the DLV-infected hematopoietic target cell (exemplified in Fig. 6, point 3 by an erythroblast infected with AEV), viral transforming protein (B^*_E) is synthesized in large amounts, thus displacing by competition its normal cel-

lular counterpart (B_E) from its corresponding receptor (A_E). We postulate that because of the modification in B^*_E the complex formed is nonfunctional and therefore causes a block in differentiation. In the infected hematopoietic nontarget cell (exemplified in Fig. 6, point 4 by an immature macrophage infected with AEV), there is no receptor of the A_E-type for B_E to react with and terminal differentiation can therefore proceed undisturbed.

Our hypothesis allows several predictions to be made which are experimentally testable.

First, sequences related to viral *onc* sequences should be present in the DNA of normal cells. This seems to be indeed the case for both AEV (Saule, Roussell and Stéhelin, personal communication), and for MC29 (Sheiness and Bishop, personal communication). In addition, these sequences appear to be highly conserved during evolution as might be expected from genes which play a role during differentiation.

Second, RNA homologous to the *onc* sequences of AEV should be expressed in normal erythroid target cells; and those of MC29 should be expressed in normal myeloid cells. This possibility is currently being explored in collaboration with the group of D. Stéhelin.

Third, DLV's which differ in their target cell specificity should have different *onc* genes and those with a similar specificity should have related ones. Preliminary experiments have shown that MC29-specific cDNA (probably homologous to the onc_{MC29} sequences) does not hybridize to AEV RNA but to MH2 RNA (Sheiness and Bishop, personal communication). MH2 is a virus which has similar biological properties as MC29 (Vogt et al., 1977; for discussion, see also Graf and Beug, 1978). Conversely, AEV-specific cDNA does not hybridize with RNA from MC29 (Saule, Roussell and Stéhelin, personal communication).

Fourth, viral transforming proteins should not only be expressed in target cells but also in virus-infected nontarget cells.

Fifth, transforming protein of AEV should crossreact with a cellular protein synthesized in erythroid cells but not in other hematopoietic cells while that of MC29 should crossreact with a protein present in normal myeloid cells. Candidates for such transforming proteins are a 75000 d protein synthesized in AEV-transformed cells (Hayman et al., 1978) and a 110000 d protein found in MC29-transformed cells (Bister et al., 1977). We are currently trying to develop antibodies against these polypeptides which would be of much help in testing predictions 4 and 5, the perhaps most crucial predictions of our hypothesis.

Our hypothesis can also be extended to explain leukemogenesis by nondefective leukemia viruses with a long period of latency. In this case we postulate that copies of their DNA provirus may integrate in or adjacent to genes of the b-type, thus causing a modification of the corresponding gene product. The production of aberrant B-type proteins would then lead to an arrest of differentiation and to the proliferation of immature "leukemic" cells. The long period of latency of nondefective leukemia viruses would be explained by the assumption that this process occurs only rarely.

Concluding Remarks

What is the significance of the studies described with defective avian leukemia virus for the problem of human leukemia?

Earlier hopes that infectious leukemia viruses play a role in the etiology of the human disease could not be substantiated. In addition, most if not all human leukemias are monoclonal disorders (for review, see Fialkow, 1976). Despite these obvious differences a basic similarity to virus-induced avian leukemias remains: it is becoming increasingly evident that cells from different types of human lymphoid and myeloid leukemia express differentiation parameters similar to those of normal hematopoietic cells at different stages of differentiation (for review, see Greaves and Janossy, 1978; Greaves et al., this volume). These cells can be induced in tissue culture to further differentiate by the addition of chemicals such as dimethylsulfoxide (Collins et al., 1978), in a similar way as Friend leukemia virus- or AEV-transformed erythroleukemia cells (Friend et al., 1971; Graf et al., 1978b) or myeloid cells transformed by Rauscher leukemia virus (Fibach et al., 1972). This suggests that in both virus-induced leukemias and spontaneous leukemias, the differentiation of early hematopoietic cells is blocked during leukemogenesis. In the light of our hypothesis for the mechanism of transformation by DLV's, that specific transformation proteins representing modified hematopoietic differentiation proteins of the cell cause a differentiation block in the leukemia cells, analogous proteins might be altered in human leukemia cells such as by somatic mutations in the respective genes.

Elucidation of the mechanism of leukemic transformation by defective avian leukemia viruses may thus lead to a better understanding of human leukemia as a disease of differentiation.

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