Europ. J. Cancer Vol. 16, pp. 43–51

0014-2964/80/0101–0043 $02.00/0

Study of a Virus-Induced Myeloproliferative Syndrome Associated with Tumor Formation in Mice*

M. C. LE BOUSSE-KERDILES,†‡ F. SMADJA-JOFFE,† B. KLEIN,† B. CAILLOU§ and C. JASMIN†

†*Département de Virologie, Institut de Cancérologie et d'Immunogénétique*, 14 *avenue Paul-Vaillant Couturier*, 94800 *Villejuif, France*

§*Laboratoire d'Anatomopathologie, Institut Gustave Roussy*, 16 *bis avenue Paul-Vaillant Couturier*, 94800 *Villejuif, France*

Abstract—*In* 1968, *Chirigos* et al. *showed that the PP-SV strain (plasma passage of Moloney sarcoma virus) induced the appearance of tumor nodules in the spleen and then in other hemopoietic and non-hemopoietic organs; these nodules were constituted mainly of large fusiform cells, undifferentiated mesenchyme cells and fibrosarcoma-like cells and destroyed the normal architecture of the affected organs.*

In this paper, we show that this tumoral phase is preceded and accompanied by important alterations of all hemopoietic organs such as spleen, thymus, liver, bone marrow as well as peripheral blood. The granulocytic and erythroid lines are the most involved by the pathological process, which is characterized by a hyperplasia of the splenic red pulp, a hematopoietic metaplasia in the liver, invasion of the blood by young cells and fibrosis of the bone marrow. All this suggests that the neoplasia is a malignant hemopathy, or a mesenchymal sarcoma characterized by its association with significant hemopoietic alterations. As the original strain of Chirigos' virus has been passaged in vivo *for* 2 yr *before its use in this study, we have called our strain MPSV (myeloproliferative sarcoma virus). MPSV may constitute a unique murine virus which can transform* in vivo *hemopoietic and non-hemopoietic cells. It may be used to study the relationship between fibroblastic cells and hemopoiesis. It appears also to induce a myeloproliferative sarcoma.*

INTRODUCTION

THE CHIRIGOS [1] plasma passage murine sarcoma virus (PP-SV) was defined by these authors as an undifferentiated sarcoma characterized by the invasion of the spleen, muscles and lymph nodes by tumoral nodules composed of: (1) tumoral cells less differentiated than cells observed in the rhabdomyosarcoma induced by MSV (original murine sarcoma virus); (2) undifferentiated mesenchyme cells; (3) fibroblast-like cells. The study did not provide any hematological data.

We obtained in 1974 from Chirigos the PP-SV virus and maintained it by *in vivo* acellular passages on adult DBA/2 mice. We report here new properties of the virus, which we found capable of inducing a myeloproliferative syndrome with myelofibrosis associated with the tumoral changes described by Chirigos.

Accepted 29 May 1979.

*This work was supported by Contrat I.N.S.E.R.M. No. 77.5.050.2.

‡Research Fellow of the L.N.F.C. (Ligue Nationale Française contre le Cancer).

MATERIALS AND METHODS

Virus

The viral suspension used in our experiments was obtained from a pool of leukemic spleens homogenized with an ultraturax in a solution of PBS (phosphate buffered saline) and saccharose 0.5 M. After two centrifugations (5000 ***g***) of 10 min at 4°C, the final supernatant was collected and frozen at −70°C. The titer of the virus expressed in spleen-dose 50% (SD_{50}) was determined by the method of Rowe and Brodsky [2]; one SD_{50} corresponds to the viral concentration inducing splenomegaly (spleen weighing at least 250 mg) in 50% of the injected mice, 3 weeks after the virus injection.

In our experiments, 0.2 ml per mouse of

viral suspension (containing 50 SD_{50}) was inoculated into the retro-orbital sinus. The PP-SV virus suspension used was found free of the following viruses: pneumonia virus of mice, reovirus 3, sendai, polyoma, mouse adenovirus and lymphocytic choriomeningitis.

Mice

DBA/2 MrcCbilco (from Iffa-Credo, France), male, 2-month old mice (weighing approximately 25–30 g) were used.

Histological study

Groups of 5 infected mice and non-infected mice were killed every 7 days after virus inoculation till the spontaneous death of the leukemic mice, which occurred around day 35,

At autopsy, the following organs were removed for study: femur, spleen, liver, thymus, axillary lymph-nodes, heart, lungs, pectoral and femoral muscles, and kidneys. Spleen, liver and thymus were weighed. All organs were fixed with buffered formalin. Histological sections (thickness: 3 μm) were performed and stained by hematoxylin–eosin. Histochemical reactions to detect reticulin and NASD cholesterases were made, as well as Mallory and methylgreen–pyromine reactions. A trichromatic stain (hematein, eosin, saffran) was also performed.

Cytological study

In order to follow the quantitative evolution of the different hemopoietic series in the spleen, smears were obtained from a cellular suspension with a cytocentrifuge (cytospin–Shandwood) and stained with orthodianizidin–Giemsa.

At the terminal stage of the disease, the following cytochemical stains were used on these smears: P.A.S. (periodic acid–Schiff), acid and alcalin phosphatases, sudan black, peroxydases, non-specific esterases, inhibition of non-specific esterases by NaF (sodium fluoride), chloroesterases.

Hematological study

Blood. The blood was collected with heparin by heart puncture. The number of nucleated cells per mm^3 was determined by double numeration on a Malassez Chamber. The hematocrit was measured in heparinized capillary tubes. The percentage of reticulocytes was calculated on 1000 erytrocytes.

Bone marrow. Nucleated cells of femoral bone marrow were counted by the following method: one femur was taken and cleaned, a needle was inserted through each epiphysis (diameter: 0.45×13 mm). Then, the femur was washed twice with 5 ml of MEM medium (minimum essential medium from Gibco, U.S.A.) containing 30% of fetal calf serum. The number of nucleated cells was determined by double counting in a Malassez Chamber. Differential cytological counts on blood and bone marrow were made on smears stained with orthodianizidin–Giemsa after 10 min fixation in absolute methanol.

RESULTS

Growth of the spleen and the liver

The relationship between the spleen weight and the time after viral injection (Fig. 1) shows 3 distinct kinetic phases:

(1) *An exponential growth phase.* This lasted till day 20. During this phase, the spleen weight increased exponentially and was multiplied 10-fold, reaching 1 g. This very steep growth curve was characterized by a doubling time of 4.5 days.

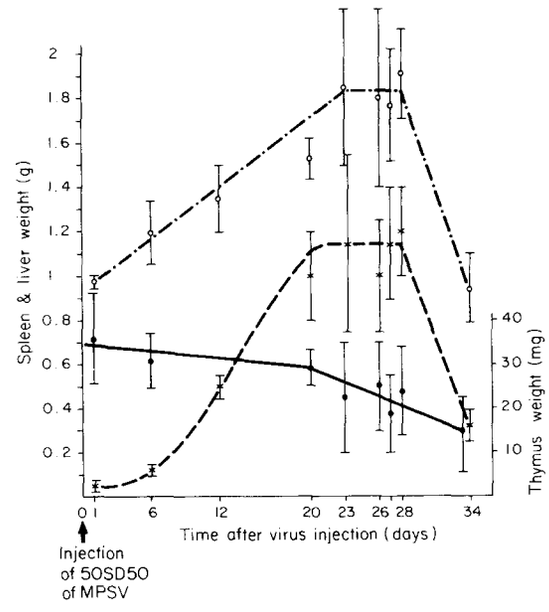

Fig. 1. Changes in the weights of spleen (----), *liver* (-·-·-) *and thymus* (— — —) *after MPSV injection. The points represent 1 standard deviation* (S.D.) (*the mean weight was* 80 ± 12 mg, 1 ± 0.049 g, 36 ± 0.3 mg *for control spleen, liver and thymus respectively*).

(2) *A plateau phase.* This phase lasted from day 20 to day 28 after virus injection. The spleen weight did not exceed 1.2 g.

(3) *A regression phase.* During this phase an important and rapid decrease from 1.2 g to 300 mg of the spleen weight was observed from day 28 to day 34. The death of mice

Table 1. Histological features of some tissues after MPSV injection

Day after MPSV injection / Organ	D6	D12	D20 to D28	D34
Spleen	Structure is normal	Disappearance of normal structure	Complete disappearance of normal structure	Structures are non-existant
	Hyperplasia of hemopoietic cells in red pulp	Appearance of clear, whitish areas around centro-lobular arteriose of lymphoid follicles	Parenchyma entirely replaced by cellular proliferation forming clear areas	Clear areas decreased in size and number
	Hyperplasia of some lymphoid follicles		Increase of the reticulum	Cells are well differentiated The reticulum became very dense and tangled
	Weight: 120 ± 20 mg	Weight: 500 ± 30 mg	Weight: 1–1.2 g	Weight: 300 ± 75 mg
Liver	Apparently normal	Erythroid and granulocytic metaplasia in veinous sinuses and around portal tract	Erythroid and granulocytic metaplasia	Erythroid and granulocytic metaplasia
			Presence of some clear nodules (30% of cases) Increase of the reticulum	Presence of clear nodules (50% of cases) Increase of the reticulum
Thymus	*idem.*	Appearance of clear nodular areas in the cortex (25% of cases)	Irregularly affected by proliferation (50% of cases) forming clear nodules	Irregularly affected (50% of cases)
Muscles	*idem.*	Apparently normal	Irregularly invaded by distinct nodules	Numerous nodules in all femoral and pectoral muscles
Lymph nodes	*idem.*	*idem.*	Medullar and paracortical diffuse infiltration by eosinophilic polymorphonuclear cells	Infiltration by eosinophilic and polymorphonuclear cells
Other organs	*idem.*	*idem.*	Genitals affected (70%) Hemorrhage, pulmonary foci (30%) Rare nodules in kidneys and heart	Genitals affected (60%) Peritoneal membrane affected
Bone marrow	*idem.*	*idem.*	Progressive increase of the reticulum	Significant increase of the reticulum

Histological study was carried out weekly after the injection of 50 SD_{50} of MPSV (4 mice per point).

occurred at day 36. The growth of the liver followed the same pattern as that of the spleen (Fig. 1).

Histological study

The histological modifications observed after virus injection are summarized in Table 1. From this table, it appears that the spleen was the first affected organ since, as soon as day 6 after virus injection, its weight increased slightly but significantly (120 instead of 80 mg for normal mouse); this increase was not only due to a hyperplasia of the red pulp but also of the lymphoid follicles, the size of which doubled. Two other important modifications occurred later in this organ: the first one, also described by Chirigos, consists in the apparition of whitish nodules, areas which extended around the centrolobular arterioles of the lymphoid follicles, that entirely replaced the splenic parenchyma which was destroyed. These nodules were composed of large blast-like cells (about 25–30%) negative for all cytochemical staining, granulocytic cells (about 30%) and fibroblast-like cells (about 20%) (Fig. 2). The second modification was the late but very rapid decrease of spleen weight, accompanied by a decrease in the number and the size of the clear nodules and a spectacular modification of the reticulum, which became very dense and tangled (Fig. 3). Some giant multinucleated cells were also observed in the spleen (Fig. 4). Similar tumoral nodules were also observed in the thymus (Fig. 5), the lymph nodes and some non-hemopoietic organs (muscles, lungs, kidneys, genitals) (Table 1) as described by Chirigos. In the liver, which has not been previously described, we observed, from day 20 after virus injection, a hemopoietic metaplasia in venous sinuses and around the portal spaces, then the presence of 'whitish nodular areas' around the portal spaces, both associated with an increase of the reticulum.

Hematological studies

(1) *Blood.* Table 2 shows that this disease appears to be essentially characterized by a sudden but irregular increase of the nucleated cell number at day 26 in the peripheral blood which was concomitant with the spleen regression. At the terminal phase of the disease, the nucleated cell count reached 4.2×10^4 cells/mm^3 as compared to 1.7×10^3 cells/mm^3 in non-injected control mice.

This increase was due to 2 events: (a) the percentage of metamyelocytes and polymorphonuclears reached 55% (2.2×10^4 cells/mm^3) at the last stage of disease as against 30% (5.4×10^2 cells/mm^3) in control mice. The percen-

Table 2. Changes in the hematological features after MPSV injection

Day after MPSV injection	D0	D6	D20	D26	D34
Total mean number of nucleated cells per mm^3	1726 ± 365	1193 ± 113	2002 ± 840	16,232 ± 9419	42,250 ± 28,889
Myeloblasts Promyelocytes (%)	0	0	0.6 ± 0.7	0.2 ± 0.2	1.5 ± 1.8
"Tissue" cells (%)	0	0	4.2 ± 2.6	2.3 ± 2.1	1.8 ± 3.1
Myelocytes (%)	0	0	0	0.5 ± 0.1	4 ± 2.8
Metamyelocytes Polymorphonuclears (%)	32.5 ± 4	26.4 ± 7.5	39.2 ± 15	39.7 ± 16	52.4 ± 1.8
Lymphocytes (%)	65.5 ± 4	70 ± 7	45 ± 13	30 ± 8	12 ± 4
Monocytes (%)	2.3 ± 0.6	4 ± 0.7	4.2 ± 2	1.6 ± 0.6	1.7 ± 0.3
Proerythroblasts Basophilic erythroblasts (%)	0	0	1.6 ± 1.7	2 ± 2	5.2 ± 3
Polychromatophilic acidophilic erythroblasts (%)	0	0	5.2 ± 2	24.5 ± 4	22.2 ± 5
Reticulocytes (%) (calculated on 1000 erythrocytes)	2.7 ± 1.5	6.4 ± 1.5	9.2 ± 1.3	17.7 ± 8	26.2 ± 0.8
Number of platelets per mm^3 in the peripheral blood	2.3 ± 0.3	2.6 ± 0.3	2.2 ± 0.3	1.1 ± 0.1	0.6 ± 0.1

Injection of 50 SD_{50} of MPSV.
Mean ± 1 S.E.M. (4 mice per point).
Differential cell count was made on 200 cells per mouse (4 mice per point).

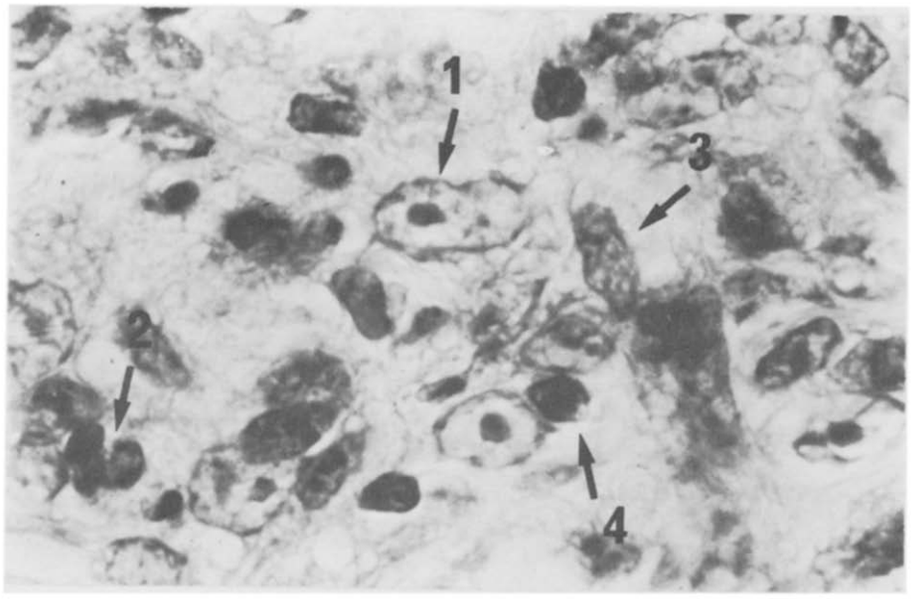

Fig. 2. *Cellular composition of the 'clear nodules'. Regardless of their localization, the nodules are morphologically identical and essentially composed as follows:* (1) *large 'blastic-like' cells;* (2) *granulocytic cells;* (3) *'fibroblastic-like' cells;* (4) *differentiated cells. Hematoxylin and eosin.* × 1400.

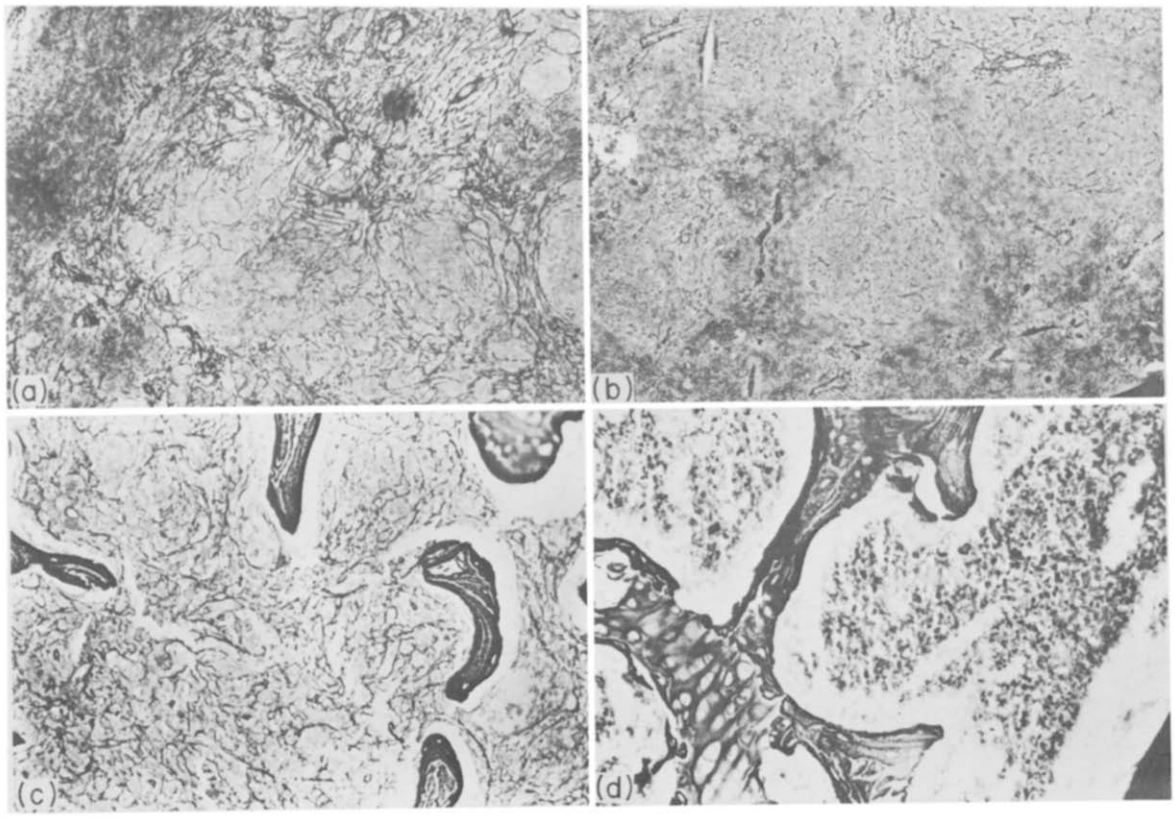

Fig. 3. (*a*) day 28 *after MPSV injection. Note the dense and tangled reticulum in the clear nodules.* (*b*) *normal aspect of the reticulum around lymphoid follicles in the spleen of non-injected control mice. Gordon and Sweets.* × 87. (*c*) day 34 *after MPSV injection—increased reticulum in the bone marrow, resulting in a myclosclerosis in MPSV injected mice.* (*d*) *Normal reticulum in the bone marrow of control mice. Gordon and Sweets.* × 220.

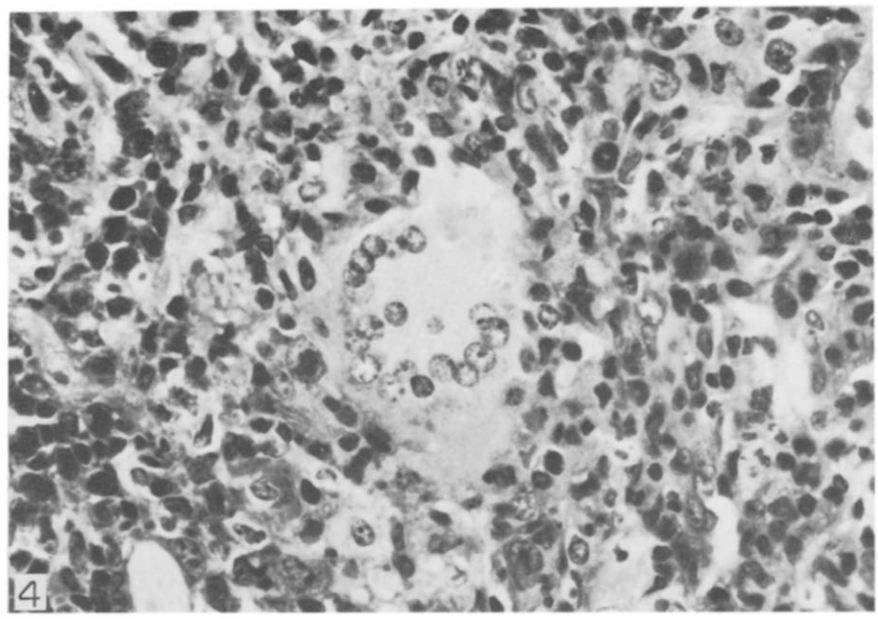

Fig. 4. Giant multinucleated cell found in spleen. Hematoxylin and eosin. × 560.

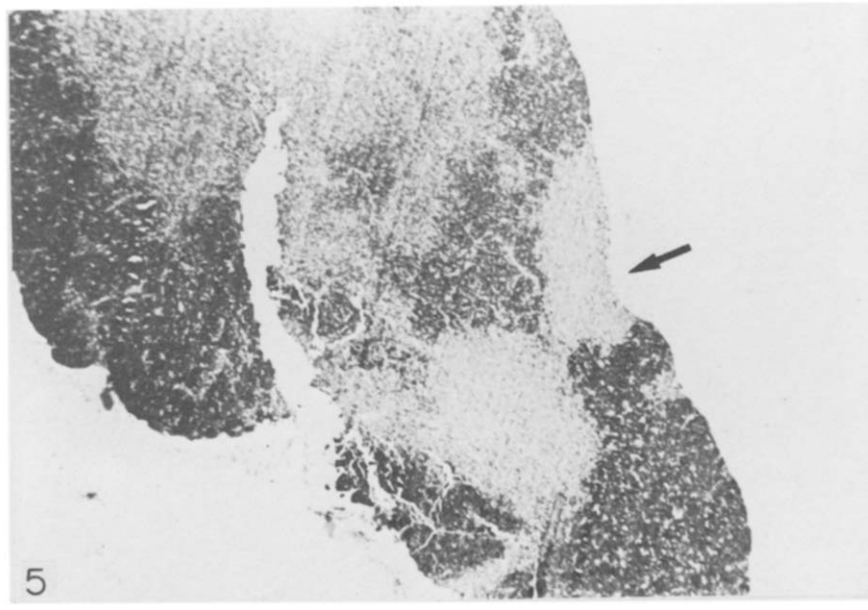

Fig. 5. Day 28: *thymus is invaded by nodules in the cortical zone* (→). *Hematoxylin and eosin.* × 35.

tage of myelocytic cells increased from 0% in the control mice to 6% (2.8 ± 10^3 cells/mm^3) in the leukemic mice at day 35, just before death. (b) An erythroblastemia: this erythroblastemia was observed at day 20 and the percentage of erythroblasts (mainly polychromatophilic and acidophilic erythroblasts) reached up to 25% (8.5×10^3 cells/mm^3).

The number of lymphocytes remained constant whereas their percentage, in relation to the other nucleated cells per mm^3, of blood had clearly decreased (range: 60% in non-injected control mice to 5% in infected mice).

Finally, we note the evolution of other important hematological parameters in injected mice (Table 2): the hematocrit decreased from 45% in control mice to 28% in infected mice at the last stage of the disease. The platelet number clearly decreased from 2.3 $\times 10^5$ cells/mm^3 at day 0, to 0.6 10^5 cells/mm^3 at day 34. The percentage of reticulocytes greatly increased, from 2.7% at day 0 to 26% at the end of the course.

(2) *Bone marrow.* The nucleated cell number per femur remained quite unchanged during the course of the disease: yet, the study of the medullogram showed two important points (Table 3): (a) an increase of the young granulocyte percentage (myeloblast and promyelocyte percentage increased from 4% in control mice to 11% in the infected mice at day 34) and of the eosinophilic polymorphonuclear percentage from 1 to 10% at day 28. (b) a decrease of the percentage of erythroid cells from 32% to 15–20% and of the percentage of lymphocytes. These variations, expressed as percentage, reflect the variation in absolute number, considering that cell number per femur remained constant.

Finally, and principally, from day 20 to day 34, a very important increase of the medullary reticulum with myelosclerosis (Fig. 3) was observed.

DISCUSSION

The murine virus induced leukemias provide examples of specific alterations of the main hemopoietic cell lines, such as the erythroid cell line, by the Friend and Rauscher viruses, and the myeloid cell line by the Graffi virus, perhaps also by the Rauscher virus at a late phase of its action, and by the MyLV virus recently isolated by M. P. McGarry [3].

Unfortunately, virus-induced myeloid leukemias have been difficult to reproduce regularly and, therefore, the blood in alteration, which involved mainly the granulocyte cell line, explains our interest for this disease. Since the original study of Chirigos [1] did not mention this hematological alteration, and since it has not been possible to obtain the original early passages of the PP-SV strain, it is possible that viral recombinations occurred during the multiple *in vivo* passages. We propose, therefore, to call our strain of virus: myeloproliferative sarcoma virus (MPSV). The MPSV is defective, it induces foci of transformation in murine embryonic fibroblasts and it has been cloned *in vitro* and separated from a helper virus. (Ostertag *et al.*, manuscript in preparation). The cloned transforming virus has kept the capacity to induce hemopoietic perturbations. This would mean

Table 3. *Percentage of nucleated cells in the femoral bone-marrow after MPSV injection*

Cells	Day after virus injection	D0	D6	D28	D34
Blasts					
Myeloblasts	(%)	4.2±1.5	10.5±5.4	11.6±1.2	11.5±2
Promyelocytes					
Myelocytes					
Metamyelocytes	(%)	36±2.4	43.4±6.8	37.3±3	43±10
Polymorphonuclears					
Eosinophils	(%)	0	1.3±1.9	10.2±2.1	4.8±2
Lymphocytes	(%)	26.7±1.6	22.2±4	22.7±0.6	19±5
Monocytes	(%)	0.1±0.3	2.3±1.8	3.3±1.8	1.3±0.5
Plasmocytes	(%)	0.06±0.1	0.16±0.2	0	0.14±0.12
Erythroid cells	(%)	32.6±4.6	20.3±3.4	15.6±2	20.4±4.2
Number of nucleated cells per femur ($\times 10^7$)		1±0.1	1.3±0.2	0.7±0.2	1.1±0.2

Injection of 50 SD_{50} of MPSV virus.
Mean±S.E.M. (4 mice per point).
Differential cell count was made on 4000 cells per point (4 mice per point).

that MPSV is a unique murine virus which transforms hemopoietic and non-hemopoietic cells.

We observed a hyperplasia of the splenic red pulp, a hemopoietic metaplasia in the liver, a myelemia and a medullary fibrosis, which suggests that the neoplasia could be a hematosarcoma.

The splenic hyperplasia is not due to a bacterial or a viral contamination of our virus stock (see Materials and Methods). However it can be supposed that it is just a leukemoid reaction to a tumoral transformation of non-hemopoietic cells (fibroblast-like cells and perhaps undifferentiated blast cells in this case).

Indeed, numerous authors have shown the importance of such leukemoid reactions in tumor-bearing mice [4, 5]. However, the splenic hyperplasia appears to be virus-specific, since we have shown (unpublished experiments) a direct relationship between the MPSV virus dose and the onset of the hyperplasia. The hyperplasia could be due to a direct transformation of the proliferative hematopoietic compartment, as in the case of the Friend leukemia [6], or to a cytolytic effect of the virus on mature hemopoietic cells, provoking a hyperplastic reaction of the precursor cells [7].

The second phase of the disease characterized by the spread of the nodular structures in hemopoietic and non-hemopoietic organs, is obviously the most interesting. Indeed the invading structure is a cellular association of three types of cells: undifferentiated blast-cells, fibroblast and granulocytic cells. Such an association, which is independent of its anatomical localization, raises, strikingly, the problem of the relationship between these 3 types of cells. To explain the proliferation of fibroblastic cells in these nodules, the most simple hypothesis is that it is provoked by the action of the MSV genome contained in the MPSV genome. Indeed Chirigos obtained 100% of tumours when the virus was injected into the leg of BALB/c new-born mice [1], and we confirmed this finding. The hypothesis is strengthened by the fact that MPSV can transform *in vitro* murine embryonic fibroblasts [8]. However, the role of fibroblasts as a component of the hemopoietic microenvironment has been strongly suggested [9] and we must study in this experimental model, the possibility that the proliferation of the fibroblastic cells may be closely related to the pathological proliferation of the other hemopoietic cells observed in the same nodules, particularly the granulocytic cells.

It has not been possible, in this morphological study to elucidate the cytological nature of the undifferentiated blast cells: all the cytochemical staining which was performed was negative but as no muscular striation was observed after the Mallory staining they are probably not mesenchyme cells. Our histological observations strongly suggest that they are more probably hemopoietic cells (or cells closely related to hemopoiesis). Indeed, we have shown that these cells appear firstly in hemopoietic organs; they are always associated with granulocytic cells, and they always appear around the central arterioles of the splenic lymphoid follicles, which is the site where myeloid colonies appear in lethally irradiated recipients [10].

The constant association, in the nodules, of granulopoiesis with fibroblast-like and undifferentiated blastic cells may represent a good model for the study *in vivo* and *in vitro* of the respective interaction of these cell populations.

Finally, MPSV-induced disease is another example of an oncornavirus-induced model for multistep carcinogenesis as in the Friend leukemia. Our work suggests that such a multistep carcinogenesis is probably the case for MPSV induced disease where, firstly, a hyperplasic phase appears, followed by the apparition of nodules, which represent histologically tumoral characteristics. If this is confirmed by physiological studies, then multistep carcinogenesis would not be restricted to chemically-induced tumors [11], but would also be demonstrable in virus-induced malignancies.

Acknowledgements—The authors thank Professor H. Rappaport for his helpful discussions, Mrs. M. Bennoun and Mrs. A. Girot for their excellent technical assistance, Mrs. G. Martin for her excellent photographs, and Mrs. R. Dauvilliers for secretarial assistance.

REFERENCES

1. M. A. Chirigos, D. Scott, W. Turner and K. Perk, Biological, pathological and physical characterization of a possible variant of a murine sarcoma virus (Moloney). *Int. J. Cancer* **3,** 223 (1968).
2. W. P. Rowe and I. Brosky, A graded response assay for Friend leukemia virus. *J. nat. Cancer Inst. (Wash.)* **23,** 1239 (1959).

3. M. P. McGarry, R. A. Steeves, R. Jeckner, E. A. Mirand and P. J. Trudel., Isolation of a myelogenous leukemia inducing virus from mice infected with the Friend virus complex. *Int. J. Cancer* **13,** 867 (1974).
4. R. L. Degowin and D. P. Gibson, Suppression of erythropoiesis in the marrow by extramedullary tumour. *Exp. Hematol.* **5,** 49 (1977).
5. P. E. Tambourin, Haematopoietic stem cells and murine viral leukaemogenesis. In *Stem cells and tissue homeostasis.* (Edited by Lord Pohen and Cole) p. 259. Cambridge University Press (1978).
6. P. E. Tambourin and F. Wendling, Malignant transformation and erythroid differentiation by polycythemia-inducing Friend virus. *Nature New Biology* **234,** 230 (1971).
7. M. H. Umar, Aspects of the pathogenesis of the Raucher murine leukemia infection. Thesis 1977, Rotterdam.
8. J. C. Chermann, Personal communication.
9. A. J. Friedenstein, U. F. Deriglasova, N. N. Kulagina, A. P. Panasuk, S. F. Rudakowa, E. A. Luria and I. A. Rudakow, Precursors for fibroblasts in different populations of haematopoietic cells as detected by the *in vitro* colony assay method. *Exp. Hematol.* **2,** 83 (1974).
10. R. V. Petrov, R. M. Khaitov, N. V. Aleinikova and L. V. Gulak, Factors controlling stem cell recirculation. III. Effect of the thymus on the migration and differentiation of hemopoietic stem cells. *Blood* **49,** 865 (1977).
11. I. Berenblum, Sequential aspects of chemical carcinogenesis: skin. In *Cancer: a Comprehensive Treatise*, (Edited by F. F. Becker) Vol. 1 p. 323. Plenum Press, New York (1975).