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Peripheral Blood and Lymphatic Organs in BALB/c Mice During the Progressive and Regressive Phases of Moloney Sarcoma Development

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Blood cell counts, differential blood cell counts and weights of the spleen and peripheral lymph nodes draining the area of lesions induced by Moloney sarcoma virus inoculation into the quadriceps shank muscles of inbred BALB/c mice were examined at various stages of tumor development and regression. The blood cell count remained constant through the observation period up to 27 days post tumor development and regression. Differential counts revealed some changes in the cellular composition of the peripheral blood. The most pronounced was an increase of neutrophiles at the stage of tumor development, and their decline with tumour regression. The enlargement of the spleen and of the popliteal lymph nodes draining the tumour site, at the peak of tumor development on day 13 post MSV inoculation, involved at least a doubling of splenic weight, and a much greater weight increase for lymph nodes. This was a long-lasting, although declining event, extending beyond tumor regression.

Key words: Moloney sarcoma virus, peripheral blood analysis, peripheral lymphatic organs.

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Intramuscular inoculation of Moloney sarcoma virus (MSV) into mice or rats results in rapid local development of a tumor (a sarcoma), which in immunocompetent animals spontaneously regresses within a month.

In mice, MSV-induced tumors grew progressively for 12-14 days, then gradually regressed, and disappeared by day 20-24. The regression is mediated mainly by a cellular response (FEFER *et al*. 1967; FEFER *et al*. 1968; FEFER 1969; STUTMAN 1975; RUSEEL & MCINTOSH 1977), although a humoral response also contributes to tumor rejection (FEFER 1969; FRIEDLANDER & MITCHELL 1976).

We examined if this immunologic involvement in MSV tumor regression is mirrored in the peripheral blood composition, and how the lymphatic organs respond to progression and regression of this tumor. To this end the peripheral blood counts, weights of the spleen and lymph nodes draining the lesion site were analyzed in an inbred strain of mice at various stages of MSV tumor development and regression.

Material and Methods

Fifty nine female inbred BALB/c mice, aged 3 months, were used for the experiments. The animals, except for 11 intact mice which were not inoculated and were considered the control group, were inoculated with 0.2 ml of standard MSV suspension into the right femoral muscles. The left, contralateral limb was injected with saline only.

The MSV preparation was a saline homogenate of the tumor, grown in a newborn BALB/c mouse following inoculation of MSV. The tumor was harvested two weeks post inoculation. The virus suspension used in these experiments was prepared according to CHANAILLE *et al.* (1967).

Animals were observed daily, and the time of tumor appearance, growth and disappearance was recorded. Animals which did not develop a tumor were excluded.

Drops of blood were obtained from the tail vein. White cell numbers were counted in a Burcker camera, blood films were stained with Giemsa stain following methanol fixation. The white cell

numbers and morphology were assessed and differential counts were performed. At least 200 nucleated cells were assessed for differential counts at 400x magnification.

A proportion of mice were sacrificed at various times of tumor development in order to evaluate spleen weight and popliteal lymph node weights of both tumor draining and of contralateral, control sites. Spleen weight was measured with an accuracy of 1.0 mg, while lymph nodes with an accuracy of 0.1 mg.

For histological analyses, tumors excised 6, 13, 22 and 27 days post virus inoculation were fixed in Bouin solution, embedded in paraffin and sections were stained with hematoxylin-eosin.

The animals were housed in the animal facility of the Department of Histology, Warsaw Medical University, fed on standard chow and had free access to drinking water. Animals were used in accordance with the Warsaw Medical University Ethics Committee guidelines for the care and use of laboratory animals. Animals were killed by cervical dislocation.

The number of animals used for each time point group evaluation are indicated in the Tables.

The parameters examined were assessed on day 0, 6, 13, 22 and 27 post virus inoculation. The mean values and standard deviations were calculated from the individual counts or measures and the results were analyzed statistically using Student's *t*-test. The differences were considered as statistically significant at $P<0.05$.

Results

The swelling of shanks at the site of MSV inoculation was observed as early as day 4 post inoculation. Tumors grew progressively up to 10-12 days and then gradually declined until day 22. The MSV-induced tumors are pleomorphic and have large elongated cells with a single nucleus and prominent nucleoli, fibroblast-like cells predominating (Fig. 1). Massive destruction of muscles was always observed at the site of virus inoculation during the progressive phase of tumor growth

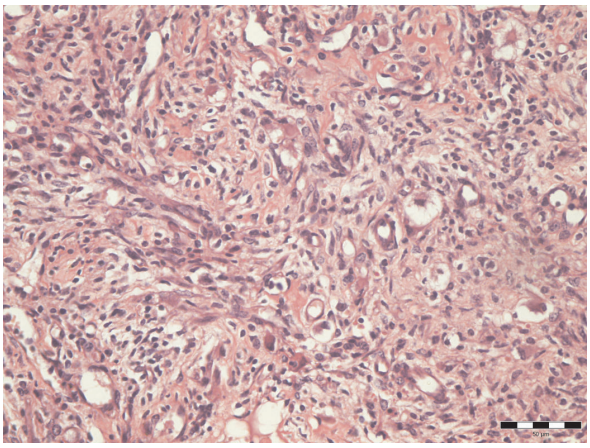

Fig. 1. MSV sarcoma developed on day 12 post MSV inoculation. Polymorphic tumor cells are infiltrated by neutrophiles. Hematoxylin+Eosine (H+E) staining. Bar=50 μm.

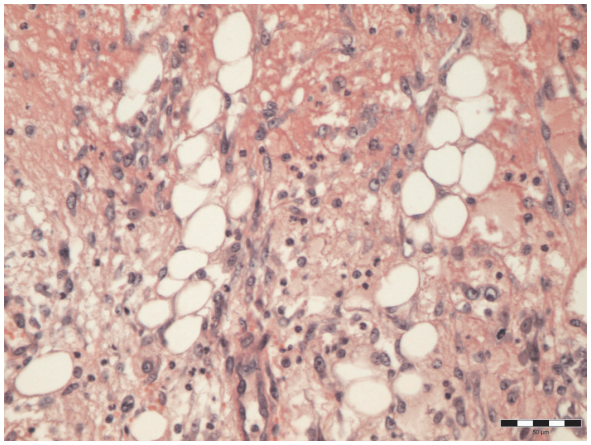

Fig. 2a. Lesions observed 15 days post MSV inoculation. Severe myolysis and impregnation of interstitial with large, mononuclear neoplastic cells. H+E staining. Bar=50 μm.

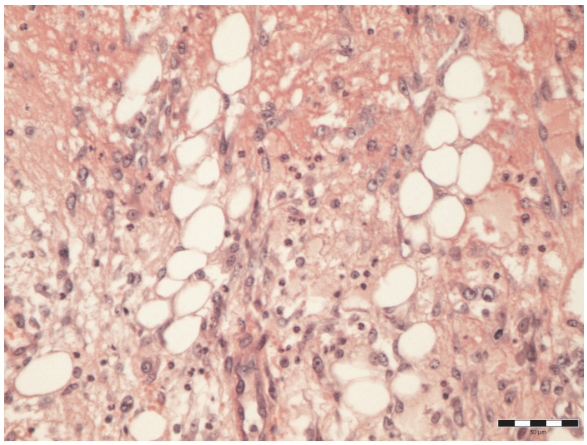

Fig. 2b. Fibrin deposit inside muscles destroyed by MSV on day 15 post inoculation. H+E staining. Bar=50 μm.

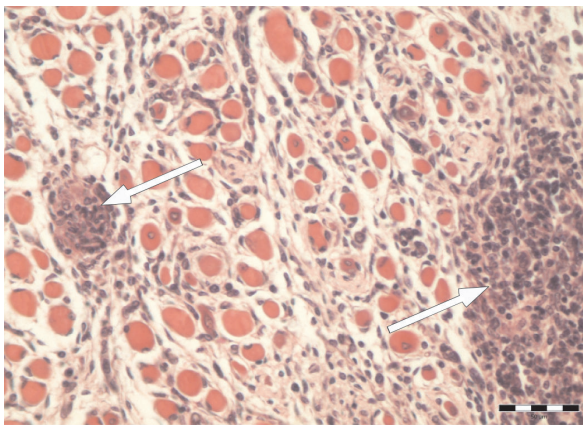

Fig. 3. Regeneration of skeletal muscles in the regressive phase of MSV tumor development. Neoplastic cells vanish and are replaced by connective tissue, although the remnants of sarcoma cells (arrows) are present 27 days post inoculation. H+E staining. Bar=50 μm.

(Fig. 2a, b). The regression of the tumor was associated with the regeneration of skeletal muscles (Fig. 3), disappearance of neoplastic cells and the accumulation in the lesion of macrophages, lymphocytes and fibroblasts.

Between day 22-27 there was no evidence of tumor, and both hind limbs looked similar.

Results of the evaluation of white blood cell count and its cellular composition in the control and tumor-bearing mice at various phases of tumor development are presented in Table 1.

White cell counts did not change during the course of tumor development and its regression, remaining at the level of control values. The differences between groups were not statistically significant, although the highest values were noted among animals with tumor regression (days 22 and 27).

Moderate changes were observed in the differential counts. The fraction of large lymphocytes declined to 16% at an early stage of tumor development (day 6), then gradually returned to the control values (22%), whereas the fraction of small lymphocytes reached the highest value (52%) at the tumor regressive stage (day 22). Monocytes increased to 8% on days 13 and 27 in relation to the control (5%).

The most characteristic feature of the differential count was elevation of polymorphonuclear cells to 35% at an early (day 6) stage of tumor development, followed by a decline below the control (27%) to 17-19% during the regression phase.

The development of MSV tumor was associated with a pronounced increase in splenic and in regional lymph node weights (Table 2). A sharp increase in splenic weight was noted from 114 mg for the control to 240 mg on day 13, i.e. at the peak of tumor growth, then its weight declined during the regressive phase, but was still significantly higher than in the control up to the end of the observation period (day 27).

A similar pattern was observed for popliteal lymph nodes exposed to the tumor. The mean weight of lymph nodes rose from 1.4 mg to 6.1 mg at the peak of tumor development, and the elevation was maintained in the early regression phase (22 day), then slowly declined, but even at day 27 was still above the control.

Histological examination of the tumor

The MSV tumor is highly pleiomorphotic and is characterized by profound destruction of muscles, an appearance of loose tissue with sparse fibrillar and homogenous material. In such areas, large spindle-shaped cells with hypertrophic nuclei, often in mitosis, were found.

During the regressive phase neoplastic cells were infiltrated by lymphocytes, macrophages, and neutrophilic granulocytes. Skeletal muscles were at various stages of regeneration. The shank bones near the tumors were activated and depositing various amounts of periosteal bone.

Table 1

White cell counts and differential counts of peripheral blood in BALB/c mice in the course of M-MSV tumor development

Experimental group (days post MSV inoculation)	No of specimens	Mean white cell count/μl of blood	Differential count (%) ±SD				
			Large lymphocytes	Small lymphocytes	Monocytes	Neutrophiles	Eosinophiles
Control- (0 d)	11	6591 ±1494	22 ±8	45 ±7	5 ±2	27 ±9	1 ±1
MSV- (6 d)	15	6553 ±1611	16 ±7*	42 ±10	5 ±3	35 ±10**	2 ±1**
MSV- (13 d)	26	6319 ±1715	19 ±6	45 ±12	8 ±3*	26 ±12	2 ±1*
MSV- (22 d)	6	6729 ±1807	25 ±6	53 ±10**	4 ±1	17 ±5*	1 ±2
MSV- (27 d)	16	6905 ±1657	26 ±7	44 ±11	8 ±4*	19 ±7*	3 ±1*

* $P<0.05$ against the control value

** $P=0.05$-0.08 against the control value

Table 2

Weight of lymphatic organs in BALB/c mice at various time of exposure to M-MSV sarcoma

Experimental group (days post M-MSV inoculation)	No of specimens	Spleen weight (mg) ±SD	No of specimens	Popliteal lymph nodes (mg) ±SD	
				MSV exposed	Contralateral (not exposed)
Control – (0d)	11	114 ±9	8	–	1.4 ±0.4
MSV – (13d)	16	240 ±94*	14	6.1 ±3.0*	1.4 ±0.4
MSV – (22d)	7	180 ±27*	6	6.2 ±4.0*	2.5 ±1.3
MSV– (27d)	18	166 ±43*	18	3.5 ±1.3*	1.9 ±0.5

Table 3

Comparison of blood cell analysis in mice from various sources

Mean white cell count/μl of blood	Differential count (%)					References
	Lymphocytes	Monocytes	Neutrophiles	Eosinophiles	Basophiles	
8 000	40-60	3-5	20-30	2-3	1	PRZAŁA 1999
10 000	35-90	0-3	10-40	0-7	0-1	KURYSZKO & ZARZYCKI 2000
6 600	67	5	27	1	0	Own results

Discussion

Blood cell counts in female BALB/c mice did not change during MSV tumor development, and in our material was on average 6500/μl.

Blood cell counts and differential counts in our material differ from the values reported for mice by other authors (Table 3), (PRZAŁA 1999; KURYSZKO & ZARZYCKI 2000). These authors have not, however, indicated the strain, sex and age of the mice used. Moreover the values presented were at wide-ranging. They also did not separately count small and large lymphocytes. Thus it was considered important to undertake precise differential white blood cell counts in this study performed on an inbred mouse strain.

Spontaneous regression of the MSV sarcoma is a manifestation of both cellular and humoral immunity (WŁODARSKI *et al*. 1979, 1981; ZANOVELLO *et al*. 1988; ROSATO *et al*. 1995, 1996). In cell mediated immunity, cytotoxic T cells, helper cells, and macrophages are the main participants, but the cytotoxic T cells play a pivotal role in tumor rejection (ZANOVELLO *et al*. 1988). These cells are present in the tumor and in the tumor-draining lymph nodes (SHU *et al*. 2005). One million MSV-sensitized T cells can protect T-cell depleted mice from tumor induction by MSV (MURASKO *et al*. 1983). During the progressive phase of MSV tumor development there is an increase of lymphoblasts (= large lymphocytes) in draining lymph nodes, while during the regression phase their number returns to normal level. During tumor regression large cytolytic T cells are converted into small cells (PLATA *et al*. 1979).

Precursors of cytotoxic lymphocytes (CTL) express phenotype Ly1,2,3+, while cytolysis is executed by cells with phenotype Ly2,3+. Lymphocytes Ly1+ produce anti-viral antibodies (LECLERC & CANTOR 1980).

Humoral immunity against MSV sarcoma is mediated by B cells, and in B-cell depleted animals tumor rejection is impaired, despite a high level of CTL in the spleen (GORDON *et al.* 1982). Macrophages also participate in MSV tumor rejection. They exert cytolytic activity and are activated to this end by T cells (HOLDEN *et al*. 1979; BECKIER & HASKILL 1980).

These cellular changes are not mirrored in the peripheral blood count, whose levels were below 7000/μl, regardless of stage of tumor development.

During the period of rapid tumour growth, the level of large lymphocytes declines and returns to a normal value at the phase of regression. The percentage of circulating small lymphocytes rises on day 22 when tumor regression is completed. A moderate elevation of monocytes is observed on days 13 and 27, i.e. in the regression phase. Polymorphonuclear cells showed a biphasic response, at the early stage of tumor development elevation of neutrophilic granulocytes up to 35% was observed, while during the regression of tumor on days 22 and 27 a significant decline, below the control value, was noted. This suggests the mobili-

zation of these cells at an early stage of tumor growth and that their depletion during tumour regression might be a manifestation of their homing in to the regressing tumour.

An increase of small lymphocytes, observed on day 22, when tumor lysis is advanced, might be considered as a manifestation of the stimulation of lymphopoietic organs. An enlargement of the spleen and of regional lymph nodes indicates antigenic stimulation of these organs. The activation of lymphocytopoiesis in the spleen does not, however, evidently influence the number of circulating lymphocytes.

In general, the peripheral blood analysis does not reflect the dynamic changes observed in the lesion area triggered by MSV inoculation, although the spleen and the local lymph nodes are significantly enlarged, and this state of activation lasts much longer than the duration of MSV lesions.

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