Original Articles

Immune Characterization of the Lewis Rats Inoculated with K2 Sarcoma Cell Line and Newly Derived R5-28 Malignant Cells

(rat sarcoma / malignancy / myeloid cells / splenomegaly / natural killer cells / cytotoxic cells)

A. MORÁVKOVÁ[1], O. MÁLEK[1, 3], E. POKORNÁ[1, 2], J. STRNÁDEL[1, 3], J. HRADECKÝ[1], V. HORÁK[1]

[1]Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Liběchov, Czech Republic
[2]Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic
[3]Czech University of Agriculture, Prague, Czech Republic

Abstract. Sarcoma is a relatively rare malignant disease with high mortality, bad prognosis and response to conventional therapy. Two possible models of this disease were tested: the K2 rat sarcoma cell line, which was described previously, and the new rat R5-28 cell line derived from a spontaneously growing rat neoplasm with sarcoma morphology. While all rats inoculated with K2 cells developed tumours at 22^{th}–25^{th} day after inoculation (D = 22–25), only 60%–75% of R5-28-inoculated rats were affected by tumours. The frequency and progress of the disease depended on the number of inoculated cells. No metastases were detected in both cases. All affected animals showed large splenomegaly. A possible response of some immune system components to tumours was tested. No tumour-infiltrating lymphocytes were revealed in the tumour tissue. Anti-tumour antibodies were not found in tumour-bearing animal sera. Appropriate changes in peripheral blood lymphocyte subsets were explored. While the relative numbers of both NK cells and Tc were impaired, no changes were noted in numbers of $CD4^+CD8^-$ T helper cells. Leukocytosis with highly increased numbers of $CD11b^+$ myeloid cells displaying variable expression of CD4 was detected in terminal stages of the disease.

Sarcomas are rare but aggressive tumours of mesenchymal origin. They consist of poorly differentiated or undifferentiated tumour cells. On that account sarcomas compose several heterogeneous histological groups with different clinical markers. Prognosis is usually bad and standard therapies remain limited.

Several animal models of sarcoma are known, one of them was described by Pokorna et al. (1994). K2, T15 and A8 Lewis rat sarcoma cell lines were established. Their shape, cytoskeletal structure and mobility were determined *in vitro*. Different metastatic potential was shown for the three sarcoma cell lines. Rats inoculated subcutaneously with K2 cells manifested pulmonary metastases only occasionally (low malignancy), T15 cells gave rise to metastases in 40% (intermediate malignancy) and A8 cells developed metastases in 80% of animals (high malignancy).

Contrary to other tumours such as melanoma or colon carcinoma, sarcomas are not very well defined from the immunological point of view, particularly due to the variety of differentiation stages of tumour cells and therefore wide spectra of expressed antigens. It was shown recently that more than 70% of human sarcomas bear tumour testicular antigens NY-ESO-1 and SP110 (Ayyoub et al., 2004). Two possible routes of immune reaction against these antigens were described. Tumour-specific $CD8^+$ T lymphocytes were found in human synovial sarcoma (Ayyoub et al., 2004). This observation accords well with the generally accepted theory that the main anti-tumour immune response is of Th1 (inflammatory) type, which involves mainly activation of cytotoxic immune cells. However, specific anti-tumour antibodies were found in sera of sarcoma patients recently (Segal et al., 2005).

To identify host-tumour interactions and immune system changes in this cancer disease we utilized the Lewis rat sarcoma model. We selected the low-malignant K2 cells to develop subcutaneous tumours in experimental animals. Tumour growth, metastatic potential and abundance of immune cells in peripheral blood were monitored. We also included in this study

Received October 6, 2005. Accepted October 17, 2005.

This work was supported by grants IAPG/05/16, GACR 524/04/0102 and by IRP IAPG No. AVOZ50450515.

Corresponding author: Alena Morávková, Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Rumburská 89, 277 21 Liběchov. Tel.: (+420) 315 639 573; Fax: (+420) 315 639 510; e-mail: Moravkova.Alena@seznam.cz.

Abbreviations: D – day, DC – dendritic cells, iMC – immature myeloid cells, NK – natural killer, PCNA – proliferating cell nuclear antigen, Tc – cytotoxic T lymphocyte.

new R5-28 cells originating from a tumour with sarcoma histology (spontaneously growing in a female Lewis rat) that was isolated recently in our laboratory to characterize them *in vivo*.

Material and Methods

Animals

Rats of the Lewis strain used in this study were obtained from the Institute of Physiology in Prague (Academy of Sciences of the Czech Republic). Twenty-six male rats were used in total. Two groups of rats (18 animals) received R5-28 cells, four rats were inoculated with K2 cells, and four animals used as a negative control were injected with PBS. Peripheral blood was obtained from vena caudalis of each experimental and control animal twice a week starting from day (D) = 0 until the end of the study, D = 45, D = 35, or D = 70 (K2- inoculated, R5-28-inoculated group B, group A, and control rats, respectively).

Cells

The K2 cells (Pokorná et al., 2003) were cultivated *in vitro* using DMEM medium (Sigma-Aldrich, Steinheim, Germany) supplemented with 10% foetal calf serum (FCS) (Sigma-Aldrich).

We isolated the R5-28 cells recently from a spontaneously growing Lewis rat tumour with sarcoma histology. These cells were expanded *in vitro* in DMEM medium with 10% FCS, frozen in aliquots and stored in liquid nitrogen for following experiments.

Both the K2 and R5-28 cells growing *in vitro* were washed with PBS, trypsinized and gently washed twice in PBS before injection. Cell suspensions were applied subcutaneously into two places on the back at a dose of 3×10^5 or 5×10^5 cells in 0.1 ml PBS for group A or group B, respectively. The control rats were injected with 0.1 PBS per place only.

Antibodies

Monoclonal mouse anti-rat FITC or RPE-conjugated primary CD3, CD4, CD8 (Becton Dickinson, Franklin Lakes, NJ) and CD11b (Serotec, Kidlington, UK) antibodies were used for FACS analyses.

For immunohistochemical staining, anti-vimentin (Boehringer Mannheim, Mannheim, Germany), anti-cytokeratin 18 (Labsystems, Helsinki, Finland) and anti-proliferating cell nuclear antigen (PCNA) (Dako Cytomation, Glostrup, Denmark) antibodies were used.

Staining of blood cells and FACS analysis

For FACS analysis, we followed the staining protocol for Easy Lyse solution (Dako Cytomation) with minute modifications. Briefly, 50 µl of whole heparinized blood were mixed with 1 µl of each antibody per sample and incubated for 30 min at room temperature in the dark. Thereafter, 1 ml of Easy Lyse working solution was added and incubated for 10 min. After incubation, samples were washed in PBS with 0.2% gelatin from cold water fish skin (Sigma) and 0.1% sodium azide, centrifuged (200 *g*, 5 min, 4ºC) and immediately measured in the FACS Calibur (Becton Dickinson). Data from the cytometer were evaluated using Summit software (Dako Cytomation).

Blood smears were stained for microscopic analysis using standard May-Grünwald and Giemsa-Romanowski staining.

Immunocytochemical staining

Cells were grown *in vitro* on glass coverslips in DMEM medium with FCS until they almost reached confluence. Then, the medium was removed, cells were washed shortly in PBS and fixed with cold ethanol for 10 min. After washing in PBS with 0.2% bovine serum albumin (BSA) (3 times, 5 min each), unspecific staining was blocked with 10% goat serum and primary antibodies were applied overnight in 4°C. Thorough washing in PBS with 0.2% BSA followed and cells were incubated in the dark with appropriate Cy3-conjugated secondary antibody for 1 h at room temperature. Cell nuclei were counterstained with DAPI. Finally, stained cells were embedded in Mowiol with propyl gallate and evaluated with a Provis AX70 fluorescent microscope (Olympus, Prague, Czech Republic).

Non-fixed cryosections of tumour tissue were used to determine the presence of anti-tumour antibodies (Alexander et al., 1996). Samples were blocked with 2% BSA in PBS and incubated 1 h at room temperature with plasma collected from experimental animals and diluted 1 : 100 in PBS. FITC-conjugated rabbit anti-rat Ig was used as secondary antibody. Cell nuclei were counterstained with DAPI.

Results

K2 sarcoma cells form highly progressive sarcoma tumours in the Lewis rat

Four Lewis male rats were inoculated with K2 cells as described above (day D = 0). No significant changes in the health status were seen in first two weeks after K2 cell application. The first macroscopic tumours were observed on D = 22–25 in all animals. Once the tumours appeared, extensive tumour growth and disease progression started. All animals became apathetic and cachectic with large (71 x 43 x 32 mm) tumour lesions within 3 weeks. Rats were sacrified shortly before death due to tumour progression in advanced stage of the disease (D = 45). No spontaneous regression of sarcoma tumours was observed.

Subcutaneous tumours were freely movable and capsulated in connective tissue. Tumour vascularization was weak and mainly superficial. The central part of all tumours was necrotic, but no cysts, haemorrhagic areas or cavities filled with fluid were found there. In two

animals, initially subcutaneous tumours penetrated into abdominal and thoracic cavity, respectively.

Splenomegaly (51 x 15 mm vs. 34 x 9 mm in control rats) was found at autopsy in tumour-bearing animals. No other organs were pathologically changed and metastases of the sarcoma cells affected no inner organs.

R5-28 cells form tumours in 60% of inoculated Lewis rats

The cells named R5-28 were derived from a spontaneous neoplasm that developed in one Lewis rat kept in our laboratory. These cells were expanded *in vitro* and partially characterized. The cells have a spherical shape with short dendritic protuberances (Fig. 1). Immunocytochemical analysis showed that these cells are positive for vimentin and negative for cytokeratin 18. Staining performed for PCNA showed positivity in almost all cells. It corresponds well with good proliferation of R5-28 cells *in vitro*. Particular morphological and immunocytochemical characterization of the R5-28 cells is a matter of running experiments *in vitro*.

The malignancy and tumorigenicity of the R5-28 cells was determined in two groups of male Lewis rats inoculated subcutaneously with different quantities of R5-28 cells (day D = 0). To study the relation between the tumorigenic potential and quantity of inoculated R5-28 cells, we divided rats into two groups. Group A (10 animals) obtained the same dose as K2-inoculated rats (3 x 10^5 cells/ml). Group B (8 animals) was injected with a higher dose (5 x 10^5 cells/ml). No significant changes in the health status of animals were seen during two weeks after R5-28 cell administration.

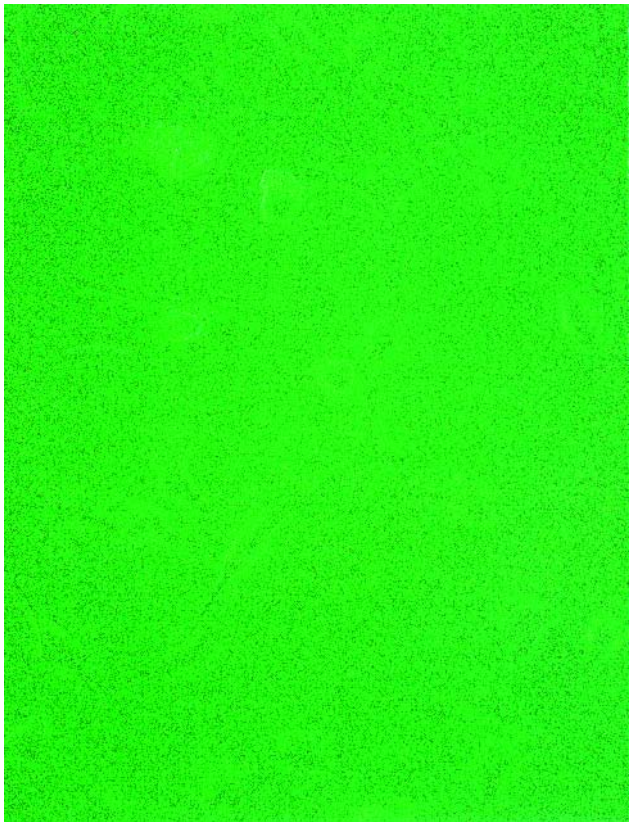

Fig. 1. R5-28 cells in vitro. Cells have spherical shape with dendritic protuberances and are easily maintained *in vitro*.

Tumours in group A appeared subsequently in the 4th week after cell inoculation (3 animals on D = 25; 2 animals on D = 27; 1 animal on D = 31). The other four animals developed no tumour over the whole experiment (D = 70). Relatively slow, but constant tumour progression was obvious in all tumour-bearing animals without any signs of spontaneous regression. Their health status after five to six weeks (D = 36–45) was practically the same as in K2 sarcoma-bearing rats in terminal stage of the disease, including tumour size (74 x 52 x 36 mm) (Fig. 2). All tumour-bearing as well as non-affected and control (PBS injected) animals were sacrificed on D = 70.

The situation in group B was slightly different. The first tumours appeared already on D = 14 in 75% of rats (six animals). However, there was a significant variation among individuals. Whereas three rats quickly developed larger tumours (14 x 30 mm on average), another three animals bore only small tumours (10 x 12.5 mm on average) on D = 14. The two remaining animals never developed any visible tumours over the whole experiment. The next progression of tumours clearly depended on the state of the disease on D = 14. All severely affected rats showed rapid tumour growth with fast general pathogenetic changes and were sacrificed on D = 35 due to bad health condition. By contrast, rats that showed only small tumours in early stages of the disease stopped tumour growth on D = 18 and subsequently regressed spontaneously. Finally, no tumours were detected on D = 27 and all three animals were without any pathological findings until D = 50. We can therefore conclude that a high dose of R5-28 cells induced tumours in 75% of animals (vs. 60% in group A). On the other hand, no spontaneous tumour regression was detected in group A, while in group B 50% of mildly affected animals rejected tumours in a short time.

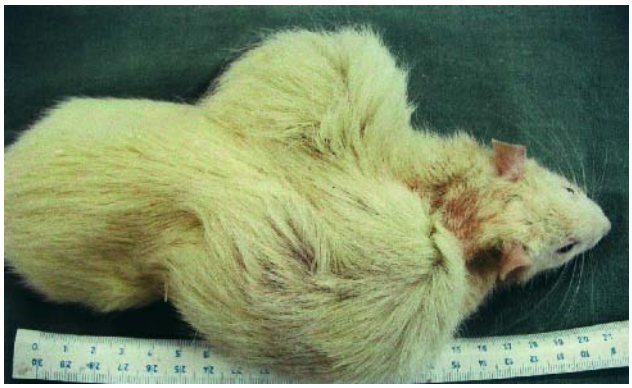

Fig. 2. Tumour-bearing rat 45 days after R5-28 cell application.

To determine the immunogenicity of R5-28 cells and to decide whether or not there is some immunologic memory, we re-injected three animals recovered by spontaneous regression with 5 x 10^5 R5-28 cells. No tumours were detected even on D = 80.

The histological features of growing R5-28 tumours were the same for both groups of tumour-bearing animals. All tumours were freely movable, capsulated in connective tissue, similarly to K2 tumours. The inner part of R5-28 tumours was heavily necrotic, with large haemorrhagic cysts filled by clear red fluid. Tissue samples taken from all tumours at autopsy showed sarcoma-like morphology on histologically stained sections. Similarly as in the K2 tumour-bearing rats, the spleens of R5-28 tumour-bearing rats were enlarged in size (49 x 13 mm), in contrast to non-affected rats (34.5 x 9 mm) and control animals (34 x 9 mm). No other organs were pathologically changed or affected by metastases.

Interaction of immune cells with tumours

Interactions of some components of the immune system with both K2 and R5-28 tumour cells were monitored. No leukocyte infiltration was observed either by histology or by flow cytometry (not shown) in samples taken from K2 and R5-28 subcutaneous tumours at autopsy. No anti-tumour antibodies were detected in the plasma of both K2- and R5-28-inoculated rats by immunohistochemical methods.

FACS analysis of peripheral blood showed that the relative number of NK cells and Tc slightly increased after inoculation with both K2 and R5-28 cells as compared to control animals, where no significant changes were noted. A decrease of relative NK and Tc cell numbers was observed after this elevation during following weeks. This decrease was noted in K2 tumour-bearing animals as well as in R5-28-inoculated (both tumour-bearing and non-affected) rats. However, these changes were not found in control animals. No significant changes occurred in $CD4^+CD8^-$ helper cells during the study in experimental and control animals.

As tumours progressed, myeloid cell populations strongly increased and nearly surpassed lymphocytes in terminal stages of the disease. All these cells showed CD11b on the cell surface. The majority of these cells fell into the $FSC^{hi}SSC^{hi}$ cell compartment in FACS analysis. Besides that, a new population of FSC^{hi} SSC^{med} cells appeared in flow cytometry around D = 20, D = 30 or D = 14 for K2 tumour and group A or group B of R5-28 tumour-bearing rats, respectively. These cells were classified as $CD45^+CD3^-CD4^-CD8^-$ $CD11b^+$ (Fig. 3) and their number increased as the tumours progressed. It was in contrast to control animals, where most of $SSC^{med}CD11b^+$ cells co-expressed CD4, so they could be classified as monocytes. In tumour-bearing animals of all groups, $CD11b^+CD4^-$ cells surpassed the $CD11b^+CD4^+$ population as the disease progressed. Histologically, a significantly increased number of myeloid cells (20% in controls vs. 90% of myeloid cells in tumour-bearing rats) with highly segmented nuclei was confirmed in blood smears (Fig. 4). Moreover, myeloid cells in blood of control animals had 9.3 μm in diameter, whereas in tumour-bearing animals it was 10.5 μm. However, this difference was not statistically significant.

Discussion

Mouse and rat inbred strains with inoculated tumour cells are often used as animal models of various cancers. Interactions of tumour and immune cells, cytokine expression and effect of new therapeutic procedures or anti-tumour drugs can be easily monitored using such models. We utilized in our study Lewis rats with inoculated K2 and R5-28 rat tumorigenic cells to reveal a potential reaction of the immune system to growing subcutaneous tumours since application of the sarcoma cells to final stages of the cancer disease. All animals that obtained K2 sarcoma cells showed tumour growth, in agreement with our previous results (unpublished). Tumour progression was very rapid and animals were dying during 7 weeks after inoculation. Contrary to Veselý et al. (1987) we did not find lung metastases in tissue sections. However, Veselý and colleagues described only low metastatic potential of K2 cells. No more than one or two metastases per 10% of inoculated animals were found. This low metastatic potential is compatible with our previous observation. Although we used four animals in this experiment only, more than 10 other experiments with K2 cells were done before and approximately 10% of animals showed metastases generally.

The R5-28 cells were derived from a spontaneously growing rat neoplasm. These cells growing *in vitro* show mainly spherical shape with small dendritic-like protuberances. Pokorná et al. (2003) compared three rat sarcoma cell lines, and the R5-28 cells are similar in shape to K2 cells. This morphotype is characteristic for slow-moving cells with low malignancy, which R5-28 cells are. Immunocytochemical staining determined these cells as vimentin-positive and cytokeratin 18-negative. These findings correlate well with the observed sarcoma-like histology of subcutaneous R5-28 tumours and confirm the mesenchymal origin of these cells. Almost all R5-28 cells also show positivity for PCNA, which is involved in cell division. This suggests high proliferative capacity of R5-28 cells.

Two groups of Lewis rats were inoculated with R5-28 cells. Group A obtained 3 x 10^5 cells/ml, group B was inoculated with 5 x 10^6 cells/ml. In group A (N = 10) tumours developed in six animals after 25–31 days. The remaining four animals did not show any symptoms during all the experiment (70 days) and no pathological changes were observed in tissue sections. In group B (N = 8) tumours developed in 75% of animals

(N = 6). One half (N = 3) of affected rats showed larger tumours with rapid progression. However, the second half of affected rats (N = 3) developed only small tumours and these neoplasms were quickly rejected. The remaining two animals did not develop any tumour during the whole experiment (D = 70). The R5-28 cells were derived from a Lewis rat spontaneous neoplasm, so it was supposed to be synergic. Nevertheless, some genetic or expression pattern abnormalities may have occurred by neoplasm developing *in vivo*. Moreover, there could be some variability in genes participating in immunosurveillance among animals of the Lewis inbred line. R5-28 may thus not be absolutely synergic for all animals, and/or the immune system balance and responsiveness among rats differs. In any case, rejection of the R5-28 tumour cells has to occur shortly after inoculation, because no tumours were observed in 40% of rats in group A. On the contrary, three animals of group B showed tumour rejection shortly after appearance. This surprising finding could be paradoxically explained by the lower dose of R5-28 cells in group A. A lower number of tumour cells may be rejected very rapidly after application, so that no macroscopic marks of tumour growth were observed. Contrary to that, a higher number of R5-28 cells can form small tumours that are subsequently destroyed by the immune system. The experiment with repeated administration of R5-28 cells into rats of group B that underwent spontaneous

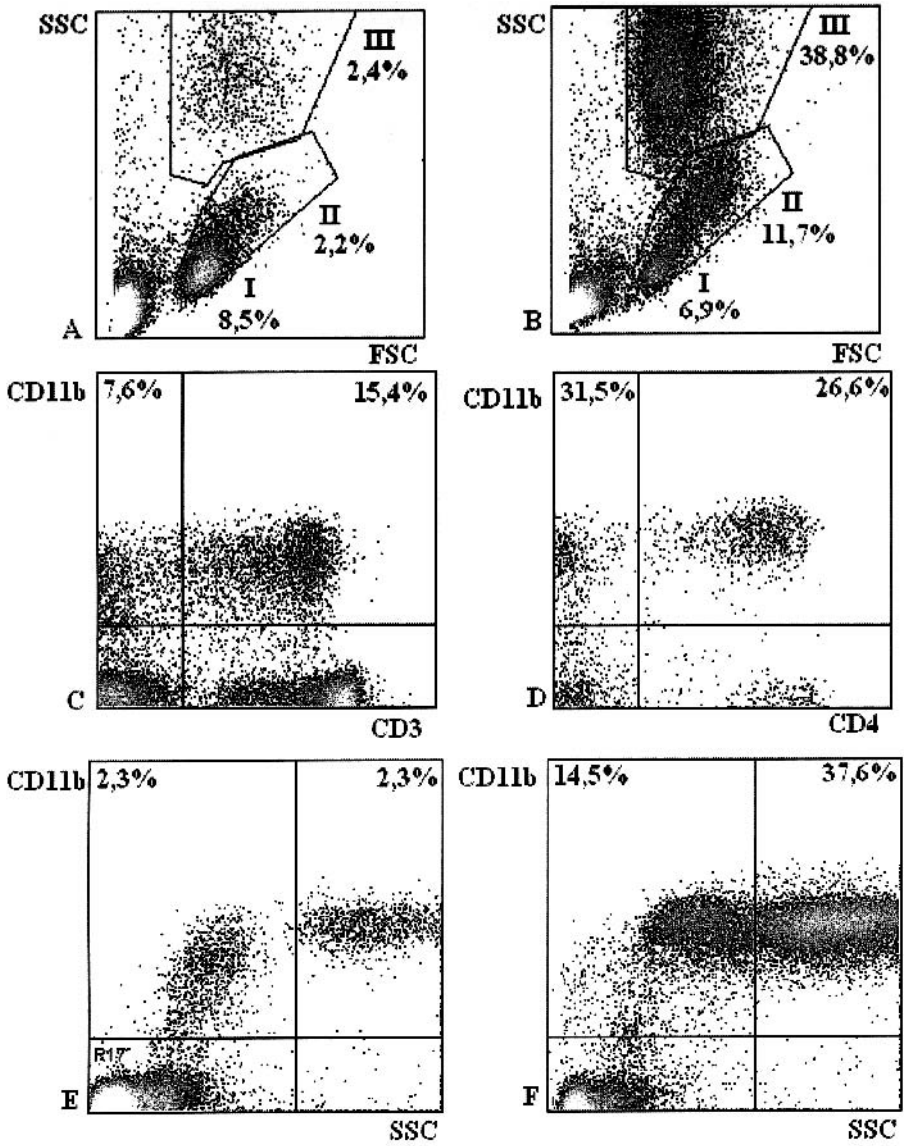

Fig. 3. Phenotyping of peripheral blood cells in healthy and R5-28 tumour-bearing rats in advanced stage of the disease. A highly increased number of granulocytes (III) and decreased amount of lymphocytes (I) is clearly visible in R5-28 tumour-bearing rat sample (B) compared to a healthy animal (A) at FSC/SSC parameter analyses. Moreover, the SSC^{med} cell population (II) is increased in R5-28 tumour-bearing rat. All these cells maintain the CD11b molecule on their surface. Approximately half to 2/3 of them are $CD4^+$ in control rats (C). Contrary to that, $CD4^-$ cells increase in number in tumour-bearing rats (D). CD11b-bearing cells differ between healthy (E) and R5-28 tumour-bearing rat (F). The number of CD11b-positive cells both SSC^{med} and SSC^{hi} are increased more than 10 times in R5-28 tumour-bearing rat.

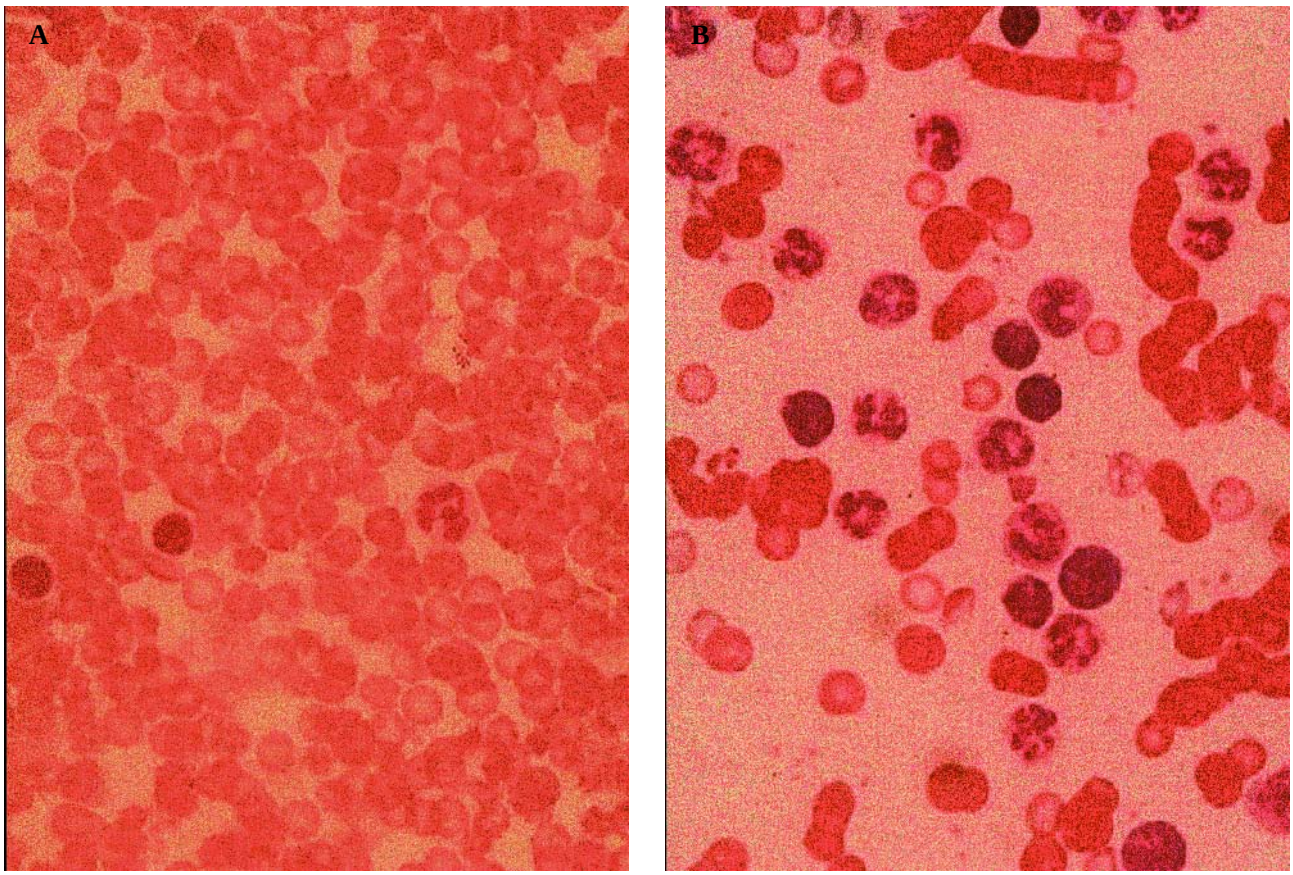

Fig. 4. A considerable increase of myeloid cells is clearly seen in blood smears of a tumour-bearing animal (B) in comparison with a control rat (A).

regression suggests there may be some immunological memory. Although the rats developed small tumours after the first tumour cell application, absolutely no changes in the health status were observed after the second one. No R5-28 cell metastases were observed in experimental animals in all groups. This may signify that R5-28 cells have no or only low metastatic potential similar to that of K2 cells.

The inner part of both K2-derived and R5-28-derived tumours was necrotic. In the case of R5-28 tumours there were large cavities filled by fluid. Surprisingly, leukocytes were detected neither in tumours nor in the cavity fluid. Both K2 and R5-28 tumours form blood vessels mainly at their surface. The inner part of the tumour therefore has not enough nutrition and dies either by necrosis or apoptosis. On the other hand, this anatomic organization together with a compact character of tumour tissue may implicate worse accessibility of inner parts of the tumour for leukocytes. In any case, the same situation is generally known for other rapidly growing tumours in humans and animal models.

It is known that in a wide spectrum of solid tumours in mouse and in man, the number of immature myeloid cells (iMC) increases in peripheral blood and spleen (rewieved by Kusmartsev and Gabrilovich, 2005). These cells express CD11 and other myeloid markers (Gr-1), but do not compose a uniform population. More likely, it is a mixture of myeloid cell populations in different stages of maturation. The composition of this mixture differs in various tumours. It is clear now that these cells are able to kill activated T cells and promote tumour growth.

Compared with these facts, our results fit with the data about myeloid cells both in K2- and R5-28-bearing rats. The composition of leukocyte population in peripheral blood was monitored during the whole experiment. Starting from the first macroscopic manifestation of tumours, a significant increase of $CD11b^+$ myeloid cells was observed by flow cytometry. In blood smears, myeloid cells with high-segmented nuclei composed 50%–90% of all leukocytes in peripheral blood, dependent on tumour progression. The diameter of these cells was 10.5 μm, contrary to 9.3 μm in normal myeloid cells in control rats. That means a difference of about 10% of the cell size. However, this difference was not statistically significant, probably due to the low number of experimental and namely control animals and to the fact that myeloid cells compose only 20% of leukocytes in control rats. The bigger size of myeloid cells in tumour-bearing animals shows their activated status and may also represent lower differential stage of these cells.

A clearly visible vigorous increase of all CD11b-positive cells, either SSC^{hi} or SSC^{med}, was visible in flow cytometric analyses. At the same time the relative number of lymphocytes decreased dramatically. Detailed analysis shows that the population of cytotoxic $CD4^-$ $CD8^+$ selectively subsided, while the number of $CD4^+CD8^-$ helper cells remained constant. It is in agreement with the data of Kustmartsev et al. (2004), who brought evidence that iMC could directly kill T cells, especially activated cytotoxic T lymphocytes. As was described above, peripheral myeloid cells in tumour-bearing rats can be divided into two groups according to the SSC parameter. The SSC^{med} population in healthy rats is composed mainly of CD4-positive cells, which are generally known as monocytes. This monocyte population makes more than 2/3 of all $SSC^{med}CD11b^+$ cells; the remaining $SSC^{med}CD11b^+$ cells are $CD4^-$ in control rats. During the tumour progression the ratio between $CD11b^+CD4^+$ and $CD11b^+CD4^-$ changes and finally CD4-negative myeloid cells surpass $CD4^+$ monocytes in peripheral blood. All these data point to the fact that this myeloid population is very heterogeneous and this heterogeneity increases during tumour disease. The exact composition of this population as well as R5-28 tumour factors, which lead to such pathological events, would be subject to further research.

Acknowledgements

Authors thank Jaroslava Sestakova and Miluse Hokesova for technical assistance.

References

Alexander, K. R., Fishman, G. A., Swinfard, R. W., Misfeldt, M. L., Milam, A. H (1996) Sinclair swine with spontaneously regressing cutaneous melanomas do not have autoantibodies against retinal bipolar cells. *Exp. Eye Res.* **63,** 347–349.

Ayyoub, M., Taub, R. N., Keohan, M. L., Hesdoffer, M., Methez, G., Memeo, L., Mansukhani, M., Hibshoosh, H., Hesdorffer, C. S., Valmori, D. (2004) The frequent expression of cancer/testis antigen provides opportunities for immunotherapeutic targeting of sarcoma. *Cancer Immun.* **4,** 7.

Kusmartsev, S., Gabrilovich, D. I. (2005) Role of immature myeloid cells in echnisms of immune evasion in cancer. *Cancer Immunol. Immunother.*, Jul 27; 1-9 [Epub ahead of print]

Kusmartsev, S., Nefedova, Y., Yoder, D., Gabrilovich, D. I. (2004) Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. *J. Immunol.* **172,** 989-999.

Pokorna, E., Jordan, P. W., O'Neill, C. H., Zicha, D., Gilbert, C. S., Vesely, P. (1994) Actin cytoskeleton and motility in rat sarcoma cell populations with different metastatic potential. *Cell Motil. Cytoskeleton* **28,** 25-33.

Pokorná, E., Zicha, D., Chaloupková, A., Matoušková, E., Veselý, P. (2003) Two dynamic morphotypes of sarcoma cells, asymmetric stellate and triangle with leading lamella, are related to malignancy. *Folia Biol. (Praha)* **49,** 33-39.

Segal, N. H., Blachere, N. E., Guevara-Patino, J. A., Gallardo, H. F., Shiu, H. Y., Viale, A., Antonescu, C. R., Wolchok, J. D., Houghton, A. N. (2005) Identification of cancer-testis genes expressed by melanoma and soft sarcoma using bioinformatics. *Cancer Immun.* **5,** 2.

Veselý, P., Chaloupková, A., Urbanec, P., Urbancová, H., Boháč, L., Krchňáková, E., Franc, F., Sprincl, L., Vousden, K., Moss, R., Heaysmann, J., Dilly, P. N. (1987) Patterns of *in vitro* behaviour characterizing cells of spontaneously metastasizing K2M rat sarcoma. *Folia Biol. (Praha)* **33,** 307–324.