Serial Passage of Tumors in Mice in the Study of Tumor Progression and Testing of Antineoplastic Drugs

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ABSTRACT

AKR lymphoma cells derived from spontaneous tumors were serially transplanted at identical inocula. The degree of malignancy and sensitivity to the polysaccharide levan and methotrexate were tested at each transfer by assessing the lag of development of primary and distant tumors and survival of mice.

A progressive increase in malignancy, accompanied by a loss of sensitivity to levan, were observed following serial passage of the lymphoma. Sensitivity to methotrexate was not affected. It is reasoned that, since serial passages permit a longer exposure of the tumor cells to selective forces of the internal milieu of the organism, better reflecting the situation in a long-life span animal, spontaneous and serial-passage tumors could serve as models for cancer therapy in humans.

INTRODUCTION

Tumors in humans and animals are known to change in morphology and biological behavior during their development, becoming more atypical and heterogeneous and gaining more and more aggressiveness. This process, studied and named progression by Foulds (8), probably involves a mutation-selection sequence which results in the dominance of more and more malignant clones (6). In experimental tumor systems, an increase in malignancy often occurs following serial transfers (5, 16). Although Foulds (8) considers this phenomenon as an artefact, it is suggested that it may have a biological sense. This procedure prolongs the time of exposure of the tumor cells to the selective forces of the organism such as the immune system, hormones, and growth factors. Tumors may differ following serial passage in sensitivity to drugs (1), antigenicity (2), and hormone dependency (16).

In a previous study, we found that, after successive transfers of AKR lymphoma during 2 years, it lost its sensitivity to the polysaccharide levan (13, 23). Levan exerts its antitumoral effect mainly by a host-dependent mechanism (12), although a direct effect on tumor cells is also present (14, 15). High-molecular-weight levan is an immunomodulator, affecting macrophages (19) as well as humoral (11) and cell-mediated (21) immunity. While losing its sensitivity to levan, the neoplasm also ceased to produce palpable tumors at the site of inoculation and caused a higher rate of mortality of the mice. Similar changes in growth characteristics were shown in the AKR system by Denton and Symes (5).

In the present study, changes in malignant behavior and sensitivity to levan were tested in serial passages of 2 spontaneous tumors. In one of the 2, sensitivity to MTX was also tested.

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3 The abbreviations used are: MTX, methotrexate; MST, mean survival time.

MATERIALS AND METHODS

Male 6- to 10-week-old AKR/Cu mice were obtained from the Weizmann Institute, Rehovoth, Israel. Tumor cell suspensions were prepared from mesenteric lymph nodes as described previously (13). The tumoral mesenteric lymph nodes were carefully separated, placed in Dulbecco-Vogt medium, minced, and filtered through several layers of gauze. Viable cells were counted in a hemacytometer with trypan blue exclusion as a criterion of vitality.

We considered that the majority of the cells of tumoral lymph nodes consisted of neoplastic cells. At the time of transfer, inguinal lymph nodes were markedly enlarged, and cachexia began to be apparent. At this stage, tumoral mesenteric nodes were very large compared to normal ones (a large mass or several smaller masses of about 3 x 15 mm in tumor-bearing mice, compared to a few nodes of less than 0.5 mm each in the normal animals), indicating that the large majority of cells were of neoplastic origin. The large size of the majority of the individual cells also indicated such an origin. Cells were inoculated s.c. in the back of mice.

Levan (M, about 2 x 10⁷), prepared according to the method of Hestrin et al. (9), was purchased in the Department of Biological Chemistry, Technical Unit, the Hebrew University, Jerusalem. A 5% solution was prepared according to the method of Shilo et al. (22). Levan was injected s.c. at the site of tumor inoculation, at a daily dose of 10 mg in 0.2 ml of 0.9% NaCl solution (saline), 6 days/week and continued during 2 months, e.g., about 50 doses.

MTX was purchased at Taro Pharmaceutical Industries, Haifa, Israel. Five doses of 50 µg/mouse were injected s.c. at the site of the tumor, every 2 days beginning on Day 0.

Control tumor-bearing mice were untreated in this series of experiments. Previous experiments showed that daily treatments with saline did not affect the tumor growth. Local and early application of levan were shown to be most effective in inhibiting the growth of AKR lymphoma, compared to late or systemic treatments (23). This treatment modality (intratumoral injection beginning on Day 0) was therefore chosen.

The experimental procedure consisted of several successive transfers of AKR lymphoma cells, the first transfer being from spontaneous tumors. Two spontaneous tumors, C and E, were separately transferred. The sequential transfers were designated C₁ to C₇ and E₁ to E₇. The inoculum was of 5 x 10⁴ viable cells in all transfers. Most experimental groups consisted of 10 mice. The occurrence and size of local tumors (s.c. tumors of site of inoculation), the lag in appearance of both local and distant growths (palpable enlarged inguinal lymph nodes), cachexia, survival of mice, and sensitivity to levan and MTX were tested.

Statistical evaluation for latency, size of tumors, and average survival time were done by the Student's t test.

RESULTS

Chart 1 shows the survival curves of tumor E at Transfers 1, 3, 5, 6, and 7 of nontreated and levan-treated mice, and Table 1 shows the growth characteristics and sensitivity to levan of this tumor at 7 successive transfers.

A gradual decrease in the size of local tumors was observed from the first to the fourth passages. Beginning with passage 5, no local tumors were formed (Table 1). The latency of metastatic growth was assessed by appear-
Table 1

Growth characteristics and sensitivity to levan of AKR tumor E at Transfers 1 to 7

| Transfer no. | Av. size of non-treated tumor at site of inoculation (mm) | Immediate size of tumor at inoculation | \( p \) (comparison to Transfer 1) | Time interval between first and last death of mice | No. of mice | Mean survival time (days) | Increased malignancy (MST, \( / \) MST) | Surviving mice on Day 100 | % of decrease in sensitivity
|-------------|-----------------------------------------------------------|--------------------------------------|-----------------------------------|---------------------------------------------------|-------------|-------------------------|-------------------------------|-------------------------|-------------------------|
| 1           | 20.0 ± 7.1 \( ^{a,b,c} \)                                | 45-53                                | 8                                 | 50                                                |             | 1.65                    | 5/10                          | 0                        | 0
| 2           | 8.4 ± 4.8 \( ^{a} \)                                     | <0.005                               | 25-51                             | 26                                                | 31          | 2.12                    | 7/10                          | 40                       | 20                      |
| 3           | 5.3 ± 2.4 \( ^{a} \)                                     | <0.005                               | 25-34                             | 11                                                | 24          | 2.04                    | 4/10                          | 20                       | 10                      |
| 4           | 3.0 ± 3.4 \( ^{a} \)                                     | <0.005                               | 15-54                             | 39                                                | 25          | 3.40                    | 3/10                          | 40                       | 20                      |
| 5           | Not present                                              |                                      | 15-35                             | 20                                                | 23          | 3.92                    | 0/10                          | 100                      | 10                       |
| 6           | Not present                                              |                                      | 16-19                             | 3                                                 | 15          |                         |                               |                          | 0                       |
| 7           | Not present                                              |                                      | 13-17                             | 4                                                 | 13          |                         |                               |                          | 0                       |

\( ^{a} \) The percentage of decrease in sensitivity in each transfer was calculated in comparison to the levan sensitivity observed in Transfer 1.

\( ^{b} \) Mean ± S.D.

\( ^{c} \) Size of tumor at maximum (Days 42, 31, 22, and 24 after tumor inoculation in Transfers 1, 2, 3, and 4, respectively). Statistical evaluation was done by Student's \( t \) test.

A progressive decrease of the MST is observed ranging from 50 days in the first to 13 in the seventh transfer (Table 1). The tumors in all transfers from 2 to 7 were significantly more malignant than the one in the first transfer, according to the average survival time (\( p < 0.005 \)). The increase in malignancy is gradual, as expressed by the relation between the MST in the first and each of the other passages. The increase in malignancy is 4-fold in the seventh compared to the first passage. It is also seen in Table 1 and Chart 1 that the kinetics of the life tables differ at the different transfers; kinetics are steep in Transfers 1, 3, 6, and 7 (resulting in short intervals between the first and last death) and slow in Transfers 2, 4, and 5 (resulting in long intervals). This could be due to differences in the degree of heterogeneity of tumor cell populations, slow curves reflecting high heterogeneity, and steep curves reflecting low heterogeneity.

Concomitant with the increased malignancy, a tendency to progressive loss of sensitivity to levan occurred during successive transfers as seen by the sharp decrease in MST of the levan-treated mice and by the decrease in the number of cured mice. The difference in latency of metastatic tumor appearance between levan-treated and untreated mice gradually decreased from 50 days in the first transfer to 13 in the third and 3 in the seventh.

Chart 2 shows the survival curves of Transfers 1, 2, and 4 of AKR tumor C-bearing animals in nontreated, levan-treated, and MTX-treated mice. As with tumor E, an increase in malignant behavior (transfer 2, \( p < 0.02 \); Transfer 3 and 4, \( p < 0.01 \)) is observed. Levan and MTX inhibit significantly the C tumor at the first transfer (\( p, \) very close to 0.05) but, in the following transfer, the inhibition is not statistically significant with either of the drugs. Although not significant, a progressive loss of sensitivity to levan is seen by a reduction in the number of long-term survivors (3, 2, 0, and 0 of 10 in the 4 consecutive transfers), while sensitivity to MTX, on the contrary, tends to be more stable (3, 3, 2, and 2 of 10 in the successive passages).

DISCUSSION

A progressive increase in malignancy was shown in AKR lymphoma following serial passages.
The increase in malignancy was expressed by: (a) a gradual loss in the ability of forming a local tumor at the site of inoculation; (b) a progressive decrease in the length of the period of appearance of both local and distant tumors; and (c) a progressive increase in mortality rate of the inoculated mice.

The increase in malignancy is accompanied by a loss of sensitivity to the polysaccharide levan.

Lentinan (a fungal polysaccharide) was found, by Bortin et al. (2), not to act on long passage AKR lymphoma but to exert some activity on spontaneous tumors. Loss of sensitivity to immunomodulators like levan and lentinan may indicate that the increased malignancy is due to the fact that the tumor succeeded to overcome some element of the immune reaction induced or enhanced by the polysaccharides.

Primary tumors were shown to be composed of heterogeneous populations of cells, and selection of more and more malignant clones are thought to be responsible for the increased malignancy observed with time in tumors of humans and animals (7, 18). Natural polyclonality was actually described in spontaneous AKR lymphoma (17). Tumor progression, like tumorigenesis, may be due to immunological escape. Loss of H-2 antigens was shown in AKR lymphoma cells and was believed to play a role in leukemogenesis (20).

Immunological escape is certainly not the only way for tumor progression. Progression may be due to a "biochemical escape," like loss of response to growth-regulatory factors. Such an alteration may endow tumor cells for example with resistance to cytotoxic agents.

It seems that, in our experiments, sensitivity to methotrexate has not been modified, while sensitivity to levan was altered. This may suggest a change in vulnerability of the tumor cells to some host defense mechanism. Preliminary results in our laboratory indicate a decreased vulnerability of AKR lymphoma cells of high malignancy, compared to cells of low malignancy, to cytotoxic macrophages.

Other authors proposed that increased malignancy following serial passages of AKR lymphoma could be due to a decrease in immunogenicity (3). Slater et al. (24) found, with another tumor, that serial passages caused development of resistance to cytotoxic agents, while sensitivity to interferon remained unaltered. Bortin et al. (3) found that spontaneous AKR lymphoma was sensitive to cyclophosphamide, while long-passage tumors were not sensitive. The opposite effect was observed with lentinan (2).

Burnet (4) suggested that rodents possess a less efficient system of DNA repair and that long-living organisms are more apt to resist neoplastic development. In one species, mice, Stutman (25) showed that there was not correlation between life span and incidence of tumors. Metastatic tumors are rare in mice, possibly because the life span of rodents is not long enough to allow tumors to pass all the stages of progression leading to metastasis. Metastatic tumors are possibly a disease more common in long-living organisms. It is suggested that there may be a difference, not in the incidence of tumors but, rather, in the type of tumors developed by animals with different life span, since those living longer have a higher probability to develop metastatic tumors.

It is proposed that serial passage of neoplasms in mice provides a model of tumor progression, by enabling tumor cells to live and divide in an artificially long-living animal, being thereby exposed longer to external and internal biological influence. The presence of the tumor in the host organism is artificially prolonged, creating a situation which resembles more to the tumor-host situation in humans.

Tumors that undergo progression following serial transfers in experimental systems are probably rare or, alternatively, their study might have been abandoned because of their frustrating changing properties. Nevertheless, a few examples are found in literature (5, 8, 16). In tumors transplanted during 20 to 30 years, such changes are more liable to appear (10), but such time intervals are hardly practical for research. Therefore, it is important to use those rare tumors which do change in malignancy during a "reasonable" number of transfers.

Tumor progression probably proceeds by a gradual enrichment in highly malignant tumor cells and probably does not involve a complete elimination of cells of low malignancy. Tumor progression is probably not a process analogous to cloning of malignant cells (namely, an event of selection of cells of high malignancy concomitant with disappearance of cells of low malignancy), but rather a process of gradual enrichment in highly malignant cells. Therefore, a natural model of tumor progression...
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as the one proposed here may be more biologically relevant than a cloning procedure.

Apart from the theoretical importance of such a model to the study of tumor progression, there is also a practical importance. It is known that tumors may be sensitive to chemotherapy for a period and may become resistant later. Some authors consider that only spontaneous tumors should be used for trial of new antitumoral drugs [e.g., Bortin et al. (3), Hewitt (10)], but if life span is a factor in tumor development, long-passage tumors in mice may reflect more faithfully the properties of highly malignant human tumors. Therefore, drugs should be tested on both spontaneous and long-passage tumors. Some drugs may be effective on spontaneous and others on long-passage tumors. Such results may be of importance in the treatment of neoplasia at its different stages of development.

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REFERENCES


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