Facilitation of Metastasis by Antithymocyte Globulin

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SUMMARY

Antithymocyte globulin (ATG) increases the number, size, and rate of appearance of pulmonary metastases in mice from implants of the syngeneic Lewis lung carcinoma (3LL) without significantly increasing the weight of the primary tumor. An attempt was therefore made to determine whether this effect was due to an earlier release of malignant cells from the primary tumor or to facilitation of the subsequent implantation of the cells in the lungs. Counts of the numbers of malignant cells in the blood of mice with 3LL implants and experiments in which the primary tumor was excised at various times indicated that ATG caused an earlier release of malignant cells into the circulation. Examination of the blood of control tumor-bearing mice showed a maximum level of immunoblasts 4 days after implantation of 3LL tumor, while treatment with ATG abrogated this response. These findings suggest a possible correlation, in the ATG-treated mice, between the absence of the immunoblast response and the earlier release of malignant cells from the primary tumor.

INTRODUCTION

Clarification of the role of immunity in the development of primary as well as secondary tumors has been hindered by the fact that most potent immunosuppressives are also antitumor agents. In addition, there are few experimental systems in which metastases appear spontaneously, consistently, and predictably at the same time and in the same organ, so that attempts at evaluating the role of immunity in the development of metastases have not hitherto been practical. However, the 3LL in C57BL female mice has suitable characteristics for such a system (12, 15) and was therefore used by Hellmann et al. (13) to examine this problem. It has now been used to study the effects of heterologous ATG—a powerful immunosuppressive—on primary and secondary tumor development.

A study was also made of the effect of ATG on the release of 3LL tumor cells and immunoblasts into the circulation.

MATERIALS AND METHODS

Preparation of ATG. Rabbit ATS was made according to the method of Levey and Medawar (16). ATG was produced by Sephadex fractionation (DEAE-Sephadex A-50; Pharmacia Fine Chemicals AB, Uppsala, Sweden) of ATS. NRG was prepared in the same way from nonimmunized rabbits. ATG was active at dilutions of 1:256 against target thymocytes, as estimated by the trypan blue exclusion test (3), and increased the median survival time of histoincompatible skin allografts from 11 to 21 days.

Estimation of Primary and Secondary Growth. Specific pathogen-free animals were used in all experiments, including the preparation of sera. Twenty-g C57BL female mice were used s.c. implantations of 0.1 g of finely minced tumor in the flank according to the methods used routinely in the Department of Cancer Chemotherapy of the Imperial Cancer Research Fund (14). Mice were given s.c. injections of 0.25 ml ATG or NRG on Days −1, 1, 2, and 3, and thereafter on alternate days until termination of the experiment. Lungs were examined on Days 14, 17, and 21 after being filled with Indiana ink and fixed in Fekete's solution (19). Pulmonary metastases (measuring not less than 0.25 mm), which showed up as white nodules on the black lung surface, were counted.

Estimation of Circulating Abnormal Cells. Mice received implantations of 3LL tumor and injections of ATG, NRG, or 0.9% NaCl solution as described above. Daily, on Days 1 to 14, 6 mice per group were exsanguinated by cutting the subclavian vein; their blood was pooled, their tumors were excised and weighed, and their lungs were stained in the usual way. Pooled blood was collected in sodium edetate (Sequestrene: Stayne Laboratories, Bishop Auckland, Durham, England) and a nucleated cell concentrate was obtained by double centrifugation. Films were made of the whole nucleated cell layer, fixed in methyl alcohol, and stained with May-Grünwald-Giemsa. The films were coded and counted "blind" by means of light microscopy.

Excision of Primary Tumor. Mice received implantations of 3LL tumor on Day 0 and treated with ATG or NRG as described above. Each day from 1 to 11, primary tumors were removed under Avertin anesthesia (250 mg/kg body weight). A wide margin of skin overlying the tumor and the underlying part of the peritoneal wall were cut out with the tumor to minimize the possibility of regrowth. The peritoneal cavity and then the skin were sewn up with Ethicon mersutures (Ethicon Ltd., Edinburgh, Scotland), and the animals were allowed to recover under a heated lamp. Mortality was negligible in the NRG-treated animals, although the immunosuppressed animals showed a greater mortality (maximum, 2 of 7), probably due to a heightened susceptibility to infection. The mice were sacrificed at Day 23 and the 2% of mice with regrowths were discarded.
RESULTS

Estimation of Primary and Secondary Growth. Twenty-one days after 3LL implantation, there was a significant increase in the number of pulmonary metastases in the ATG-treated group (average number, 35; range, 24 to 52) compared to the NRG-treated group (average number, 14; range, 8 to 24) ($p < 0.01$ > 0.001 by Student’s t test). However there was no significant difference between the numbers of metastases in the ATG- and NRG-treated groups at Days 14 and 17 and no significant difference between the primary tumor weights of the treated and control animals at any time.

Estimation of Circulating Abnormal Cells. The time course of appearance and numbers of malignant cells and immunoblasts in the pooled blood from 6 mice implanted with 3LL tumor and treated with ATG, NRG, or 0.9% NaCl solution are shown in Chart I. The results are the mean of 3 separate experiments. In the 0.9% NaCl solution- and NRG-treated groups, malignant cells were never detected until 9 days after tumor implantation. In each of the 3 experiments, treatment with ATG caused an earlier and continuous release of malignant cells from the primary tumor which was effected from the 1st day after implantation.

Immunoblasts in the blood of tumor-bearing mice treated with NRG or 0.9% NaCl solution increased from Day 2 onward. Four days after implantation, the level reached a maximum, fell rapidly after Day 5, and then decreased slowly. In contrast, blood from tumor-bearing mice treated with ATG showed a complete abolition of the Day 4 and 5 immunoblast peak, and only a relatively small constant number of these cells was seen throughout the experiment.

**Table 1**

<table>
<thead>
<tr>
<th>Day after implantation when primary excised</th>
<th>No. of mice with metastases on Day 23/no. of mice treated with</th>
<th>Av. no. of metastases/lung on Day 23 in animals treated with</th>
<th>Mean tumor wt on day of excision (g) with treatment</th>
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<tbody>
<tr>
<td></td>
<td>ATG</td>
<td>NRG</td>
<td>ATG</td>
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<tr>
<td>1</td>
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<td>0/7</td>
<td>0</td>
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<tr>
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* Significance, $p < 0.01 > 0.001$ (by Student’s t test).
DISCUSSION

In a first attempt to determine the effect of immunosuppression on the spread of syngeneic tumors and the subsequent development of metastases, antilymphocyte serum raised against mouse spleen cells did not facilitate the spread of 3LL tumor (13). The present experiments indicate, however, that ATG facilitates the appearance of pulmonary metastases without significantly increasing the primary tumor growth. This phenomenon is not restricted to mice; such facilitation of tumor spread has been reported for a homotransplantable lymphoma in hamsters (11) and also has been described in other tumor systems (8, 9). Its mode of action has been attributed to many factors including its capacity to condition the tumor cell bed (18), to bring about a generalized "sterile activation" of lymphoid cells (16), to inhibit tumor-suppressive activity of peritoneal exudate cells (11), and to act directly on the tumor cells (10). What is not clear from these previous or our own studies is the underlying mechanism by which ATG or antilymphocyte serum accelerates the establishment of distant tumor deposits.

The present experiments have shown that treatment with ATG abrogates the appearance of immunoblasts which normally follows implantation of syngeneic 3LL tumor into SPF mice. In control tumor-bearing mice, large pyroninophilic blast cells or immunoblasts are released in large numbers into the efferent lymph from the stimulated local lymph node and reach a peak value 4 days after tumor implantation. This response was first described by Delorme et al. (6) when studying the thoracic duct lymph output in rats bearing irradiated syngeneic sarcoma grafts. Their presence in human blood after immunization of patients with various antigens has been described (5). However, this is the first time that immunoblasts have been demonstrated in mouse blood in response to a syngeneic tumor implant. Since they extravasate into the tissues very rapidly (2) and therefore will be present in the blood in only relatively small numbers at any one time, the results observed represent a small fraction of the total number of immunoblasts involved.

The period in which the immunoblasts are decreasing in the blood of control mice almost coincides with the time that tumor cells are first detected in the circulation (9 days after implantation of tumor). In the preceding week, however, the number of immunoblasts was rapidly increasing and during this time no tumor cells could be detected in the blood. When the development of this response was severely retarded by ATG treatment there was no delay before a small number of tumor cells escaped from the primary implant. The presence of malignant cells in the blood, however, is not necessarily indicative of metastasis formation (4, 17) and therefore the studies on primary removal on successive days with ATG or NRG treatment have some bearing on the problem. In NRG-treated tumor-bearing mice, pulmonary metastases were not evident at Day 23 if the primary was excised before Day 8. However, treatment with ATG caused the earlier release of malignant cells that ultimately became metastases, and this was demonstrated by primary excision at Days 6 and 7 when 1 of 5 and 3 of 5 mice, respectively, had secondaries in the lung.

It has been demonstrated, therefore, that ATG interferes with the immunoblast response that normally develops as a result of tumor implantation, and at the same time ATG facilitates the earlier escape of malignant cells from the primary tumor, so that some of these cells ultimately establish in the lung tissue and form secondaries.

At this time, it is difficult to decide whether the immunoblasts directly mediate immune destruction of the metastasizing tumor cells or whether their presence is simply an indication that an immunological response is under way. Studies on the cytotoxic action of these lymphoid cells against specific tumor cells both in vitro (7) and in vivo (1) in a rat tumor system suggest that a direct action is possible. Experiments to isolate these lymphoid cells and to test them against 3LL target cells both in vivo and in vitro are currently being performed. They should help to establish whether a direct correlation exists between the abrogation of the immunoblast response and the facilitation of the dissemination process in the 3LL system.

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REFERENCES

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