IN 1964 Breyere and Williams (7) observed that syngeneic skin transplants taken from animals infected by an oncogenic virus are rejected. Many experiments designed to explore the mechanisms of this rejection have been carried out in our laboratory (2–7). One of these experiments, in which the variable manipulated was time, revealed that rejection occurred when the skin transplanted was taken from a donor which had been inoculated with Gross passage A (GPA) ascites tumor only 12 hours before (6). In another experiment, a decreased rejection frequency and a delayed development of tumor at the graft site were observed when the tumor cells in the donor or skin transplant itself were destroyed by irradiation.

The most recent work deals directly with the tumor cell. Tumor cells were introduced directly into newly placed skin grafts exchanged between syngeneic animals of the same sex. The results of these experiments indicate that viable tumor cells must be present for graft rejection to take place.
Moreover, it appears that a critical number must be present for the rejection phenomenon to occur. These data add support to our postulate that tumor cells are a major factor in the mechanism of syngeneic skin-graft rejection in the GPA virus system.

MATERIALS AND METHODS

The inbred C3H/Bi, DBA/2, and F1 hybrids of the mice were originally obtained from the colonies of the late Dr. John Bittner and of Dr. Carlos Martinez. Rigorous inbreeding procedures have been carried out. To eliminate the mammary carcinoma milk-borne agent, both strains were originally foster-nursed on C57BL mice.

The Gross lymphoma in the ascites form was induced in both male and female (C3H/Bi X DBA/2)F1 mice with techniques previously reported (3). The ascitic fluid (39th passage) was aspirated under aseptic conditions on the 11th day after transplantation of the ascites lymphoma. Cell count indicated >89% cell viability. The cells counted were those morphologically intact and capable of excluding trypan blue (0.5%). Phase-contrast microscopy was used on diluted samples to determine the count. The pooled fluid was then diluted with Hanks' balanced medium supplemented with 5% fetal calf serum to the requisite cell number.

Skin was always transplanted male-to-male and female-to-female by techniques described in (3). The criteria for rejection were little or no hair growth or complete sloughing of the grafted skin. The criterion for acceptance was luxuriant hair growth.

Normal (C3H/Bi X DBA/2)F1 mice received skin grafts from normal syngeneic hosts. Twenty-four hours after transplantation, the skin grafts on some of these animals were inoculated subdermally in the center of the grafted site with the following numbers of tumor cells: $1 \times 10^6$, $1 \times 10^5$, $1 \times 10^4$, $1 \times 10^3$, $1 \times 10^2$, $1 \times 10^1$, and 4 tumor cells in a 0.1 ml suspension. Another group of grafted animals was inoculated with 0.1 ml of 20% suspension of cell-free filtrates from leukemic tissues. Two control syngeneic skin grafts were inoculated with Hanks' solution (0.1 ml).

RESULTS

This study, designed to determine the number of tumor cells required for syngeneic skin graft rejection in the GPA virus system, involved more than 50 transplants (table 1). Tumor suspensions in quantities of $1 \times 10^6$, $1 \times 10^5$, $1 \times 10^4$, $1 \times 10^3$, $1 \times 10^2$, $1 \times 10^1$, and 4 cells were injected into the skin grafts 24 hours after transplantation. The frequency of skin graft rejection by recipients receiving tumor cells of $1 \times 10^6$, $1 \times 10^5$, $1 \times 10^4$, and $1 \times 10^3$ quantities (Groups I, II, III, IV) was 60-100%, and the rejection times were means of 14-16 days. The animals given injections of $1 \times 10^2$ tumor cells (Group V) showed a lowered rejection rate of 40% and a mean rejection time of 18 days. When large quantities of tumor cells were used, the rejection rate was 60-100%. This high incidence agrees with our previous findings in which syngeneic grafts obtained from donors bearing tumors for 4-14 days were regularly rejected and contained tumor cell clusters in the skin (3, 6). Among the animals given injections of $1 \times 10^1$ and 4 tumor cells (Groups VI, VII), none rejected the graft. Apparently, more than 10 cells of GPA lymphoma must be present in the graft to obtain syngeneic skin rejection at all. The critical number of tumor cells separating acceptance from rejection appears to be in the range of 10-100. The critical cell number range where rejection regularly takes place is $1 \times 10^2$-$1 \times 10^3$ tumor cells.

On the other hand, tumors at the graft site developed in all animals receiving more than 10 tumor cells (Groups I, II, III, IV, V, VI). Tumors were produced at the graft site in 100% of the animals inoculated with $1 \times 10^6$, $1 \times 10^5$, $1 \times 10^3$ cells (Group I, II, IV); in 80% of those with $1 \times 10^4$ cells (Group III); in 90% of those with $1 \times 10^2$ cells (Group V); and in 80% of those with $1 \times 10^1$ cells (Group VI). In contrast, only 10% of those animals inoculated with 4 tumor cells (Group VII) had tumor at the grafted site. All animals in the control group accepted the skin grafts and tumors did not grow (Group IX).

Latent period for tumor development at the graft site (table 1) was progressively delayed as tumor cell dosages were decreased—the means increased from 13-19 days. This delay was particu-
Table 1.—Number of viable tumor cells, syngeneic skin graft rejection, tumor development and host survival time in (C3H/Bi X DBA/2)F1 recipients

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of tumor cells*</th>
<th>Skin graft</th>
<th>Tumor</th>
<th>Survival time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number rejected</td>
<td>Days</td>
<td>Number developed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total No. (%)</td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td>I</td>
<td>$1 \times 10^6$</td>
<td>$3/5$ (60)</td>
<td>13-15</td>
<td>14</td>
</tr>
<tr>
<td>II</td>
<td>$1 \times 10^5$</td>
<td>$3/5$ (60)</td>
<td>13-18</td>
<td>15</td>
</tr>
<tr>
<td>III</td>
<td>$1 \times 10^4$</td>
<td>$5/5$ (100)</td>
<td>14-20</td>
<td>17</td>
</tr>
<tr>
<td>IV</td>
<td>$1 \times 10^3$</td>
<td>$5/5$ (100)</td>
<td>15-17</td>
<td>16</td>
</tr>
<tr>
<td>V</td>
<td>$1 \times 10^2$</td>
<td>$4/10$ (40)</td>
<td>16-19</td>
<td>18</td>
</tr>
<tr>
<td>VI</td>
<td>$1 \times 10^1$</td>
<td>$0/10$ (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>4</td>
<td>$0/10$ (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIII</td>
<td>Control†</td>
<td>$0/15$ (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IX</td>
<td>Control‡</td>
<td>$0/2$ (0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Inoculated 24 hours after skin transplantation; 0.1 ml suspension in Hanks' balanced medium supplemented with 5% fetal calf serum.
†Injected with 0.1 ml of 20% suspension of cell-free filtrates from leukemic tissues.
‡Injected with Hanks' solution (0.1 ml).

larly significant for the 18 animals (Group V, VI, VII) in which small numbers of tumor cells ($1 \times 10^6$, $1 \times 10^4$, 4 cells) were injected into the skin graft. Similar findings were obtained for the survival times of the animals. The survival time increased as tumor onset was delayed—the means increased from 26-42 days.

All animals that developed tumors at the graft site ultimately died of leukemia as determined by autopsy. The pathologic findings showed a disseminated lymphomatous process similar to that previously described (3).

All animals given injections of the cell-free filtrate from leukemic tissues accepted the skin graft and produced no tumors at the graft site (Group VIII).

DISCUSSION

This study, oriented specifically toward the tumor cell and its function in the syngeneic graft rejection phenomenon, revealed that approximately 10-100 tumor cells are necessary to produce any syngeneic skin graft rejection in the GPA virus system. The frequency of graft rejection decreased significantly when the number of tumor cells injected was reduced from $1 \times 10^6$ to $1 \times 10^2$ cells. Similar findings were obtained when the dosage level was still fur-
ther reduced from $1 \times 10^2$ to $1 \times 10^1$ tumor cells. A high incidence of tumor occurred at the graft site at all the dosages used except at the lowest dilution—the 4 cell dose. In addition, the onset of tumor was significantly delayed as the quantity of tumor cells was decreased. Because of this delay in tumor growth, host survival time was greatly prolonged. These features of lymphomatous tumor cell behavior are well known and have been accurately documented (8). Clearly they are fully applicable to the system being tested in this study.

The role of virus in the rejections obtained in these experiments must also be considered. Virus was present in the ascitic fluid used for the cell suspensions. It seems justifiable to assume that the virus would be a constant factor in all the cell suspensions and hence would have the same effect on all the animals. But different results were obtained in the different groups. It appears then that the tumor cell, the factor being manipulated in these experiments, was of greater importance in the rejection phenomenon and tumor development at the graft site than the virus. The tumor cell and the time of growth into a visible tumor mass determined the fate of the skin graft. When the cell number was large and tumor outgrowth early, the graft was sloughed or rejected. Conversely, when the cell number was small and the tumor outgrowth delayed, the graft was accepted, though tumor ultimately appeared at the skin-grafting site.

This importance of the tumor cell agrees with the results in our earlier work in which suspensions irradiated at levels that would eliminate the tumor cell but not affect the virus failed to produce either rejection or graft-site tumors (6). Apparently, viable and probably replicating tumor cells are necessary to induce skin graft rejection and tumor growth at the graft site.

Many mechanisms may be factors in syngeneic skin graft rejection. Some kind of virus activity may be one of them (3, 6). But neither the postulated virus-induced antigenic alteration of normal tissue nor any form of virus-tumor cell interaction is indicated by our data. Rather, our data implicate the tumor cell as prime agent in the rejection phenomenon. But the tumor-cell-related mechanism needs to be clarified. Potentially, this mechanism is a response by the immune system to the tumor cell. Analyzing the inter-involvement of immune responses and graft rejection may uncover the explanation for this phenomenon.

REFERENCES