Entry Routes of Malignant Lymphoma into the Brain and Eyes in a Mouse Model

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ABSTRACT

We have recently developed a novel mouse model for studying the infiltration of malignant lymphoma to the eye and brain. After i.p. inoculation of variant S49 mouse lymphoma cells into young mice (optimum: day 7 postnatal), specific homing of these cells (named Rev-2-T-6) to the brain and eyes took place. This model offers an opportunity to study the routes of infiltration to these sites and spread thereof, as well as the molecular mechanisms that govern this metastasis. By applying a time course histopathological analysis, we demonstrate that infiltration of the brain and eyes can be visualized as early as days 9 and 14 after inoculation, respectively. The lymphoma cells enter the brain preferentially through the choroid plexus and cranial nerves. Infiltration of the rostral part occurs before the caudal part of the brain. Once within the brain, the cells spread within it as well as migrate along the optic nerve sheath into the eyes, where they continue to migrate along the choroid, ciliary body, iris, and into the anterior chamber of the eye. The orbit is also infiltrated by the lymphoma cells. However, this occurs independent of the brain-optic nerve-intraocular route.

INTRODUCTION

A mouse model for infiltration of malignant lymphoma to the eye and brain has recently been developed in our laboratory (1). After i.p. inoculation of variant S49 mouse lymphoma cells into newborn mice (optimum: day 7 postnatal), specific homing of these cells (named Rev-2-T-6) to the brain and eye took place in 60% of inoculated mice (1). No such infiltration was observed in mice inoculated later than day 11 postnatal. Both postnatal and mature mice inoculated with Rev-2-T-6 cells developed systemic lymphoma (1). The infiltration of Rev-2-T-6 cells to the brain and eyes was first observed through clinical signs of eye and CNS involvement, including growth retardation (1). Subsequent histological analysis revealed tumorous infiltrates into a variety of structures in the orbit, intraocular tissues, along the optic nerve, and in the brain (1). Currently, to our knowledge, there is no other experimental model available whereby the lethal infiltration of malignant lymphoma cells to the brain and eye can be studied. Other experimental models of variable CNS metastasis, mostly in immune-deficient mice (2–5), as well as in normal rats (6), have been developed. However, none of these models demonstrated any ocular infiltration by the lymphoma cells.

Ocular lymphoma in humans is a lethal disease (7, 8) caused mainly by two clinically distinct forms of non-Hodgkin’s lymphoma: (a) non-Hodgkin’s lymphoma of the CNS or PCNSL and (b) systemic lymphoma metastatic to the eye. A great majority of the cases of ocular involvement in lymphoma occurs in conjunction with PCNSL. Furthermore, in ~25% of patients with PCNSL, the eyes are involved

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3The abbreviations used are: CNS, central nervous system; PCNSL, primary CNS lymphoma; SAS, subarachnoid space.

9. The PCNSL arises within the brain, spinal cord, leptomeninges, or the eye but then may spread throughout the CNS (10–12) with rare systemic spread outside the CNS (13, 14). Involvement of the eye often precedes the development of clinically evident disease of the brain parenchyma or SAS (15). The disease is aggressive, and most patients die within 1–5 years of diagnosis (16, 17). The incidence of PCNSL has increased significantly in immunocompetent as well as in immunocompromised patients over the last decades (18, 19).

The significance of our mouse model lies in its potential use for addressing a variety of issues including: (a) primary routes of lymphoma spread to the eye and brain; (b) molecular mechanisms (and genes) involved in metastasis to these organs; (c) providing a means to evaluate the impact of experimental treatment modalities such as pharmaceuticals, radiation, and chemotherapy, as well as immunotherapy on metastatic lymphoma behavior; (d) studying a variety of parameters (including, e.g., the kinetics of cytokine profiles) for early diagnosis of lymphoma spread in the eye and CNS; (e) investigating immune responsiveness of the eye and brain toward malignant lymphoma; and (f) delivering genes of interest to these sites.

The first issue above is addressed in the present work through a time course histopathological analysis of brain and eye infiltration after i.p. inoculation of Rev-2-T-6 cells into day 7 postnatal syngeneic BALB/c mice. It is demonstrated that infiltration of the brain and eye can be visualized as early as days 9 and 14 after inoculation, respectively. Furthermore, intraocular infiltration (as anterior to the iris and anterior chamber) is carried out by cells that migrate from the brain along the optic nerve sheath and uvea. Metastasis to the orbit, on the other hand, is independent of intraocular involvement and may take place through hematogeneous infiltration. Mice that develop clinical signs of brain and eye involvement demonstrate decreased survival when compared with mice that receive the same lymphoma cells but are devoid of any signs of ocular involvement.

MATERIALS AND METHODS

Cells. Rev-2-T-6 cells were derived from substrate-adherent, nontumorigenic (immunogenic) variants of the S49 mouse T-cell lymphoma, as described previously (1). These cells demonstrate cell-cell adhesion, growing in suspension culture as cell aggregates.

Cell Culture. Cells were maintained in DMEM (Biological Industries, Beit Haemek, Israel) supplemented with 10% horse serum, 50 units/ml penicillin, and 50 g/ml streptomycin.

Inoculation and Analysis of Mice. Rev-2-T-6 cells (3 106) were inoculated i.p. into syngeneic BALB/c mice on day 7 postnatal. Thereafter, mice were sacrificed (5 mice per time point) from days 7–31 after inoculation. Mice were checked daily for palpable abdominal tumors, as well as for signs of eye and CNS involvement as described previously (1). The principles of laboratory animal care were followed according to the NIH guidelines. Eyes and brains of sacrificed mice were fixed in buffered formalin, processed routinely, and embedded in paraffin. Sections (5 mm) were stained with H&E and subjected to histological analysis.

RESULTS

Onset of Clinical Signs and Survival of Mice. We have described previously a set of clinical signs which characterized the infiltration of
Rev-2-T-6 lymphoma cells to the eyes and brains of syngeneic mice (1). These signs included: unilateral or bilateral involvement of the orbit and eyelids; accumulation of lymphoma cells in the anterior chamber of the eye, thereby masking the posterior surface of the cornea; retardation of animal growth; ataxia; and spinning when held by the tail and arched backs. The last three neurological signs appeared in <10% of inoculated mice, whereas the former signs characterized >60% of inoculated mice. The above-mentioned findings were further substantiated by histopathological analysis of the eyes and brains of these mice (1).

A time course of the onset of the above-mentioned clinical signs, after inoculation of Rev-2-T-6 cells into day 7 postnatal mice (n = 38), is demonstrated in Fig. 1. Thus, involvement of the anterior chamber starts at day 19 after inoculation and reaches a maximum (18% of inoculated mice) at about day 30 after inoculation. Involvement of the orbit can be visualized as early as day 16 after inoculation, reaching a maximum (53% of inoculated mice) at about day 57 after inoculation. About 58% of inoculated mice demonstrated either one or both (intraocular or orbital) signs of eye involvement. Signs of growth retardation, defined previously (1), could be detected as early as day 17 after inoculation and reached a maximum (34% of inoculated mice) at day 30 after inoculation. None of the mice in this experiment demonstrated any of the additional signs of CNS involvement. The median survival of mice (Fig. 1) is about 53 days, with a range of 17–78 days after inoculation.

The survival curve in Fig. 1 includes mice that demonstrated clinical signs of eye involvement as well as mice that did not develop these signs. When the survival data are regrouped according to these two criteria (Fig. 2), it becomes evident that mice with clinical signs of eye involvement demonstrate a significantly shorter survival period than mice lacking these signs (median survival: 44 versus 68 days, respectively). Both groups developed systemic lymphoma.

Similar differences to those shown in Fig. 2 were observed when the survival of mice inoculated at day 9 postnatal with Rev-2-T-6 cells was studied: out of 35 mice, 18 developed clinical signs of eye involvement, and 17 were devoid of such signs. The median survival of the former group was 44 (range: 34–65) days, whereas that of the latter group was 63 (range: 34–90) days after inoculation.

**Infiltration Into and Within the Eye.** To study the preferred routes of lymphoma entry into the eye and brain, a histopathological time course study was applied. Rev-2-T-6 cells were inoculated i.p. into BALB/c mice at day 7 postnatal. At different days thereafter (starting as early as day 5 after inoculation, before the onset of any clinical signs), mice were sacrificed, and their eyes and brains were submitted to histological analysis. Table 1 demonstrates the results of such an analysis. The brain is the first site infiltrated by the lymphoma cells—as early as day 9 after inoculation. The earliest histological evidence for infiltration of the eyes is manifested at day 14 after inoculation, when the optic nerve, the uvea, and orbit are involved (see also Fig. 3). The first histological manifestation of anterior chamber involvement occurred at day 21 after inoculation (see also Fig. 3). The first clinical signs of eye (orbit and anterior chamber) involvement were evident on days 21 and 25 after inoculation, respectively. Thus, a gradient of lymphoma spread seems to be forming from the brain toward the anterior chamber of the eye.

These findings support our hypothesis (1) that intraocular infiltration starts in the brain followed by tumor cell migration along the optic nerve, through the uvea, and into the anterior chamber of the eye. Infiltration of the orbit seems to be independent of intraocular involvement, insofar as infiltration of the former is always higher than that of the latter. Furthermore, the instances whereby infiltration into the orbit take place with no evidence of intraocular involvement (Table 1) also support an independent route of orbit metastasis.

To further study patterns of infiltration into and within the eye, we investigated 40 mice (independent of their postinoculation age at the time of analysis) in which at least one site between the brain and the eye was involved histologically (Table 2). Different patterns of infiltration to the eye are apparent. In all patterns with intraocular (uvea + anterior chamber) involvement, there is also involvement of the ipsilateral branch of the optic nerve, as well as of the brain. We

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**Fig. 1.** Onset of clinical signs after inoculation of Rev-2-T-6 cells into day 7 postnatal BALB/c mice. 3 X 10⁶ cells were inoculated i.p. into 38 mice. ○, ○, survival; □, □, anterior chamber; Δ, Δ, growth retardation; ▲, ▲, orbit; O-O Eye (orbit + anterior chamber).

**Fig. 2.** Survival of mice with clinical signs of eye involvement, compared with mice devoid of such signs, after i.p. inoculation of Rev-2-T-6 cells into day 7 postnatal BALB/c mice. ●, ●, mice with eye involvement (nP = 22); ■, ■, mice without eye involvement (nP = 16).

**Table 1.** Histopathological involvement of eyes and brains during a time course study after inoculation of Rev-2-T-6 cells into day 7 postnatal BALB/c mice

<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>Braina</th>
<th>Optic nerve</th>
<th>Uvea</th>
<th>Anterior chamber</th>
<th>Orbit</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 (n = 5)4</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>7 (n = 5)4</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>9 (n = 4)</td>
<td>1/4 (25%)</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>11 (n = 5)</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>14 (n = 6)</td>
<td>4/6 (67%)</td>
<td>2/12 (17%)</td>
<td>2/12 (17%)</td>
<td>None</td>
<td>3/12 (25%)</td>
</tr>
<tr>
<td>17 (n = 5)</td>
<td>2/5 (40%)</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>1/10 (10%)</td>
</tr>
<tr>
<td>21 (n = 5)</td>
<td>5/5 (100%)</td>
<td>3/10 (30%)</td>
<td>2/10 (20%)</td>
<td>1/10 (10%)</td>
<td>3/10 (30%)</td>
</tr>
<tr>
<td>25 (n = 4)</td>
<td>2/4 (50%)</td>
<td>1/8 (13%)</td>
<td>1/8 (13%)</td>
<td>1/8 (13%)</td>
<td>2/8 (25%)</td>
</tr>
<tr>
<td>29 (n = 4)</td>
<td>2/4 (50%)</td>
<td>1/8 (13%)</td>
<td>None</td>
<td>None</td>
<td>2/8 (25%)</td>
</tr>
<tr>
<td>31 (n = 4)</td>
<td>2/4 (50%)</td>
<td>1/8 (13%)</td>
<td>1/8 (13%)</td>
<td>1/8 (13%)</td>
<td>4/8 (50%)</td>
</tr>
</tbody>
</table>

a For detailed analysis of brain tissues involved, see Table 3.  
b No. of mice.  
* First manifestation of clinical signs in this site.
have not encountered even a single pattern in which the uvea or anterior chamber was involved without involvement of the optic nerve and brain. Additionally, no pattern was observed in which the optic nerve was involved without involvement of the brain.

Metastasis to the orbit, however, seems to take a different route, because patterns can be seen where the orbit is involved with no involvement of the uvea and anterior chamber (e.g., numbers 8 and 9) and without intraocular and optic nerve involvement (e.g., numbers 4 and 5). There is even one pattern (number 17) where the orbit seems to be infiltrated without apparent involvement of the brain. Thus, the findings in Tables 1 and 2 support our previous preliminary findings (1) and demonstrate that the orbit is infiltrated independent of the intraocular tissues. Interestingly, some patterns demonstrate bilateral involvement of the eyes, whereas other patterns manifest only unilateral involvement (Table 2).

For statistical analysis of the proposed brain-to-anterior-chamber infiltration route, we analyzed 77 mice [including those analyzed in Tables 1 (18 mice) and 2 (39 mice, excluding the single mouse where only the orbit was involved), plus an additional 20 mice, independent of their age after inoculation at the time of analysis] where at least one component of the brain-to-anterior-chamber axis was involved histologically. These were assigned randomly by computer into 11 groups. Each group was tested for the frequency of brain, optic nerve, uvea, and anterior chamber involvement. The mean frequencies of involvement ± SD per each site (using the 11 groups) are shown in Fig. 4. Again, a gradient from the brain toward the anterior chamber is evident. All 77 mice demonstrated brain involvement. Of these, 19 mice showed both optic nerve and uvea involvement; 22 mice showed optic nerve but not uvea involvement; none showed uvea but not optic nerve involvement; and 36 mice showed neither optic nerve nor uvea involvement. We can test the null hypothesis of independent involvement of the optic nerve and the uvea in the mice against the one-
Infiltration Into and Within the Brain. During previous work (1), we have studied brain tissues infiltrated by Rev-2-T-6 cells. This was carried out on brains derived from mice about 7–10 days subsequent to the onset of clinical signs of eye involvement. Although the SAS demonstrated the highest frequency of involvement (±95%; see also Table 3), it was nevertheless hypothesized that the choroid plexus, cranial nerves, and cranial nerve ganglia were the primary sites for Rev-2-T-6 infiltration to the brain (1). To further probe the routes of brain infiltration, we carried out a detailed histological analysis of brain tissues from the time course study described in Table 1. Sections from both the anterior and posterior regions of the brain (derived from days 9–21 after inoculation) were studied with the following findings (Table 3): (a) The first histological evidence of brain involvement is manifested around day 9 after inoculation, when both cranial nerves and the SAS are infiltrated in the same mouse. Histological analysis of brains taken at days 5 and 7 after inoculation (n = 4 and 5, respectively) did not demonstrate infiltration of the brain. (b) Until day 21 after inoculation, the SAS is not the major site of infiltration. The choroid plexus, cranial nerves, cranial nerve ganglia, and the ventricular system take the lead in this respect. Thus, examples can be seen whereby these sites are infiltrated before the SAS. (c) The trend described above changes as of day 21 after inoculation, when the SAS becomes the predominant (≤100%) infiltrated site. (d) Furthermore, in all cases where lymphoma cells were detected in the SAS (before or after day 21 after inoculation), the choroid plexus and/or the cranial nerves and cranial nerve ganglia were also infiltrated. We have not encountered even a single case in which the SAS was infiltrated without involvement of at least one (or more) of the following sites: choroid plexus, cranial nerves, and cranial nerve ganglia. (e) More tissues (and to a larger extent) are infiltrated in the rostral part (i.e., lateral ventricle and basal ganglia) than in the caudal part of the brain (i.e., brain stem and skull base), at least until day 21 after inoculation. These findings are consistent with the former region being infiltrated before the latter region of the brain. Once the rostral part is infiltrated, the lymphoma cells can also migrate through the SAS to the caudal part of the brain without prior involvement of the choroid plexus or cranial nerves (see mouse number 5 from day 21 after inoculation).

In the same way, we can test the null hypothesis of independent involvements of the uvea and the anterior chamber in those mice against the one-tailed alternative. The alternative states that the conditional probability of uvea involvement given no optic nerve involvement is smaller than the conditional probability of uvea involvement given optic nerve involvement. Using Fisher’s exact test, the null hypothesis is strongly rejected (P = 4.8 × 10⁻⁴), indicating that the probability of uvea involvement in the absence of optic nerve involvement is smaller than the probability of uvea involvement in the presence of optic nerve involvement and in a very significant way. Indeed, no mouse showed uvea involvement without having optic nerve involvement.

Of these same 77 mice, 12 mice showed both uvea and anterior chamber involvement; 7 mice showed uvea but not anterior chamber involvement; none showed anterior chamber but no uvea involvement; and 58 mice showed neither uvea nor anterior chamber involvement.

In the same way, we can test the null hypothesis of independent involvements of the uvea and the anterior chamber in those mice against the one-tailed alternative. The alternative states that the conditional probability of anterior chamber involvement given no uvea involvement is smaller than the conditional probability of anterior chamber involvement given uvea involvement. Applying Fisher’s exact test, the null hypothesis is strongly rejected (P = 1.4 × 10⁻⁹), indicating that the probability of anterior chamber involvement in the absence of uvea involvement is smaller than the probability of anterior chamber involvement in the presence of uvea involvement, again in a very significant way. No mouse showed anterior chamber involvement without having uvea involvement.

Because no situation exists whereby the anterior chamber is infiltrated to a higher extent than the uvea, or the uvea involved more than the optic nerve, it is conceivable that no retrograde infiltration of Rev-2-T-6 cells from the eye to the brain takes place in the experimental model.

Taken together, the histopathological time course study and the statistical analysis demonstrate that the origin of Rev-2-T-6 lymphoma cells observed in the anterior chamber is the brain. These cells move from the brain along the optic nerve sheath toward the optic nerve head where they infiltrate the choroid (and vitreous). Once within the choroid, the lymphoma cells migrate through it to the ciliary body, iris, and finally, the anterior chamber (Fig. 3). Infiltration of the orbit is independent of this route.

**Table 2. Patterns of infiltration into the eye by Rev-2-T-6 cells**

<table>
<thead>
<tr>
<th>Pattern no.</th>
<th>No. of mice</th>
<th>Brain</th>
<th>Optic nerve</th>
<th>Intraocular</th>
<th>Orbit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4/40 (10%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>5/40 (12.5%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>3/40 (7.5%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>4/40 (10%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>3/40 (7.5%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>1/40 (2.5%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>1/40 (2.5%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>1/40 (2.5%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>5/40 (12.5%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>10</td>
<td>1/40 (2.5%)</td>
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<td>+</td>
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<tr>
<td>11</td>
<td>5/40 (12.5%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>2/40 (5%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>13</td>
<td>1/40 (2.5%)</td>
<td>+</td>
<td>+</td>
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<td>14</td>
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<tr>
<td>15</td>
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<tr>
<td>16</td>
<td>1/40 (2.5%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>1/40 (2.5%)</td>
<td>+</td>
<td>+</td>
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</tr>
</tbody>
</table>

* Each pattern demonstrates the findings from two eyes (orbit and intraocular), two branches of the optic nerve, and the brain, as observed at least in one mouse.

* Includes uvea and anterior chamber.
of clinical signs of eye (orbit and/or intraocular) infiltration. If cranial nerves and the SAS are already involved, it is conceivable that already of grade 2 (see Table 3, first column), and because both inoculation. Because the extent of infiltration found at this time is indeed lethal is demonstrated by the present findings that mice with infiltration to the eye and brain is demonstrated by the present findings that mice with clinical signs of eye involvement manifest increased mortality over mice that develop only signs of systemic lymphoma. The time intervals taken: (a) from the i.p. inoculation of Rev-2-T-6 cells until the onset of clinical signs (~20 days) and (b) from the onset of clinical signs until death (~35 additional days) suggest the mouse model as a convenient one for experimental intervention during the different phases of lymphoma metastasis to the brain and eye.

The histopathological time course study of brain infiltration, taken together with our previous data (Ref. 1 and Table 3, last column) demonstrate that the choroid plexus, the cranial nerves, and the cranial nerve ganglia are the primary sites of brain infiltration by Rev-2-T-6 lymphoma cells. In addition, the forebrain is infiltrated before the posterior fossa of the brain. The importance of the choroid plexus as a probable entry site for lymphoma in the brain was suggested by a previous study (20) where development of naturally occurring B cell lymphomas in mice with retroviral-induced immunodeficiency resulted (at a later stage of their growth) in brain infiltration. As the choroid plexus and the cranial nerve ganglia are anatomical sites that lack a functional blood brain barrier, our data indicate that an intact blood brain barrier may not be actively crossed by Rev-2-T-6 cells. The earliest manifestation of brain infiltration is evident on day 9 after inoculation. Because the extent of infiltration found at this time is already of grade 2 (see Table 3, first column), and because both cranial nerves and the SAS are already involved, it is conceivable that actual infiltration of the cranial nerves (and subsequently, the SAS) could have started 1–2 days earlier. Once within the brain, Rev-2-T-6 cells migrate into the eye along the optic nerve sheath. The histopathological time course study and the statistical analysis of ocular infiltration demonstrate that Rev-2-T-6 cells visualized within the anterior chamber arrive there from the brain after migration along the optic nerve, the choroid, ciliary body, and the iris. The cells can also spread from the optic nerve head into the vitreous (~15%; Ref. 1) and, to a much lesser extent, into the retina (~3%; Ref. 1). The orbit is infiltrated independently of the anterior chamber, probably through the hematogeneous route. Thus, in the present mouse model, visualization of lymphoma cells within the anterior chamber is a clear indication of prior metastasis to the brain, even with no clinical signs of CNS involvement. A point of interest related to our study is whether primary ocular tumors could spread to the brain. Although theoretically we cannot exclude this possibility, there are at present no indications (both clinical and from our experimental model) that such a reverse flow exists.

Once the preferred routes of infiltration and spread within the brain and into the eye have been established, it is of obvious interest to identify the in situ expression (on the metastasizing lymphoma cells, as well as on the host cells along the infiltration routes) of different candidate molecules that might regulate this metastasis. In vitro analysis of a variety of cell adhesion molecules on Rev-2-T-6 cells demonstrated enhanced expression of LFA-1 and ICAM-2 on the cell surface (1). Recently, we have also found4 that Rev-2-T-6 cells express in vitro the matrix metalloproteinase MMP-9, as well as the matrix proteinase inhibitor TIMP-2. Further in vivo experiments are presently under way to address these issues.

* Reference data taken from our previous work (1) and arranged according to decreasing frequency of tissues involved. Brains were derived from mice 7–10 days after the onset of clinical signs of eye (orbit and/or intracocular) infiltration.

* No. of mice per group.

* Numbers in parentheses identify specific mice in each group.

* Extent of lymphoma infiltration is graded on an increasing scale from 1–3 according to the histopathological analysis.

* N.A., not applicable.

* Tumor aggressiveness is defined as the sum of the individual extents of infiltration divided by the no. of mice per group.

**DISCUSSION**

Until recently, there has been no experimental model available whereby the lethal infiltration of malignant lymphoma to the eye and brain could have been studied. Our laboratory has now developed such a model in BALB/c mice, using Rev-2-T-6 cell variants derived from the S49 lymphoma (1). That infiltration to the eye and brain is indeed lethal is demonstrated by the present findings that mice with clinical signs of eye involvement manifest increased mortality over mice that develop only signs of systemic lymphoma. The time intervals taken: (a) from the i.p. inoculation of Rev-2-T-6 cells until the onset of clinical signs (~20 days) and (b) from the onset of clinical signs until death (~35 additional days) suggest the mouse model as a convenient one for experimental intervention during the different phases of lymphoma metastasis to the brain and eye.

The histopathological time course study of brain infiltration, taken together with our previous data (Ref. 1 and Table 3, last column) demonstrate that the choroid plexus, the cranial nerves, and the cranial nerve ganglia are the primary sites of brain infiltration by Rev-2-T-6 lymphoma cells. In addition, the forebrain is infiltrated before the posterior fossa of the brain. The importance of the choroid plexus as a probable entry site for lymphoma in the brain was suggested by a previous study (20) where development of naturally occurring B cell lymphomas in mice with retroviral-induced immunodeficiency resulted (at a later stage of their growth) in brain infiltration. As the choroid plexus and the cranial nerve ganglia are anatomical sites that lack a functional blood brain barrier, our data indicate that an intact blood brain barrier may not be actively crossed by Rev-2-T-6 cells. The earliest manifestation of brain infiltration is evident on day 9 after inoculation. Because the extent of infiltration found at this time is already of grade 2 (see Table 3, first column), and because both cranial nerves and the SAS are already involved, it is conceivable that actual infiltration of the cranial nerves (and subsequently, the SAS) could have started 1–2 days earlier. Once within the brain, Rev-2-T-6 cells migrate into the eye along the optic nerve sheath. The histopathological time course study and the statistical analysis of ocular infiltration demonstrate that Rev-2-T-6 cells visualized within the anterior chamber arrive there from the brain after migration along the optic nerve, the choroid, ciliary body, and the iris. The cells can also spread from the optic nerve head into the vitreous (~15%; Ref. 1) and, to a much lesser extent, into the retina (~3%; Ref. 1). The orbit is infiltrated independently of the anterior chamber, probably through the hematogeneous route. Thus, in the present mouse model, visualization of lymphoma cells within the anterior chamber is a clear indication of prior metastasis to the brain, even with no clinical signs of CNS involvement. A point of interest related to our study is whether primary ocular tumors could spread to the brain. Although theoretically we cannot exclude this possibility, there are at present no indications (both clinical and from our experimental model) that such a reverse flow exists.

Once the preferred routes of infiltration and spread within the brain and into the eye have been established, it is of obvious interest to identify the in situ expression (on the metastasizing lymphoma cells, as well as on the host cells along the infiltration routes) of different candidate molecules that might regulate this metastasis. In vitro analysis of a variety of cell adhesion molecules on Rev-2-T-6 cells demonstrated enhanced expression of LFA-1 and ICAM-2 on the cell surface (1). Recently, we have also found4 that Rev-2-T-6 cells express in vitro the matrix metalloproteinase MMP-9, as well as the matrix proteinase inhibitor TIMP-2. Further in vivo experiments are presently under way to address these issues.

* L. Wahl and J. Hochman, unpublished observations.
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Entry Routes of Malignant Lymphoma into the Brain and Eyes in a Mouse Model

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