Toxicity kinetics and clinical potential of subarachnoid lymphocyte infusions

EDWARD NEUWELT, M.D., AND DENNIS DOHERTY

Enzymology Section, Laboratory of Molecular Biology, National Cancer Institute, Baltimore Cancer Research Center, University of Maryland Hospital, Baltimore, Maryland

The feasibility of intrathecal lymphocyte infusions was examined since patients with gliomas are known to have circulating, tumor-specific, cytotoxic lymphocytes. Human (xenogenic) and syngenic lymphocytes were infused intrathecally into rabbits, and the toxicity and kinetics of the infused cells evaluated. Cerebrospinal fluid cell counts rose to as high as 70,000 lymphocytes/cu mm 12 hours after infusion and then dropped logarithmically over several days. No infiltration of host cells into the subarachnoid space in response to the lymphocyte infusions was detected. Evidence is presented that intrathecally infused lymphocytes may escape into the systemic circulation. Toxicity was minimal, especially following syngenic intrathecal lymphocyte infusions. A systemic allergic response, characterized by choroid plexitis and pulmonary edema was noted following a second xenogenic but not after a second or even a third syngenic lymphocyte infusion.

KEY WORDS • syngenic lymphocyte • xenogenic lymphocyte • toxicity • immune response • intrathecal infusion

MALIGNANT gliomas are universally fatal. Untreated patients with this disease survive about 6 weeks from onset of symptoms. Surgery, varying from needle biopsy to radical resection, is the most widely accepted therapeutic modality, but results in a median survival of only 6 months. Taveras, et al., have demonstrated that megavoltage whole head irradiation after surgical decompression can extend the median survival to 9 months. Wilson has shown that the nitrosoureas, BCNU (1, 3-bis(2-chloroethyl)-1-nitrosourea) and CCNU (1-(2-chloroethyl)-3-cyclohexl-1-nitrosourea), have some efficacy in malignant astrocytomas, but are not as effective as radiation. The combination of radiation and BCNU results in only an additional 6 weeks of median survival. Therefore, the combination of surgery, radiation, and chemotherapy is presently the best available therapy for malignant gliomas, but results in a median survival of only about 10 months.

Several factors are probably responsible for these dismal therapeutic results. First, the infiltrative nature of these tumors makes primary resection virtually impossible, and if resection is attempted, it can cause devastating deficit to the patient. Second, the brain is contained in a small, rigid, closed cavity, which means that even minor changes in tumor size may result in a marked elevation of intracranial pressure and herniation leading to death. Finally, the blood-brain barrier, which in large part results from the tight junctions present between endothelial cells in central nervous system (CNS) capillaries, is impermeable to most presently
available chemotherapeutic agents.

The blood-brain barrier is also important in regard to malignant gliomas in that it seems to partition the CNS from the body's immune surveillance system. Indeed, the blood-brain barrier and the absence of CNS lymphatics are the main reasons that the CNS is designated as an immunologically privileged site.\textsuperscript{11} Glioma patients have been shown to have circulating tumor-specific killer lymphocytes.\textsuperscript{3,4,7,10,20} Yet, as reported by Ridley and Cavanaugh,\textsuperscript{3} lymphocytes, although present in almost 50\% of malignant gliomas, are rarely able to penetrate the tumor parenchyma beyond the perivascular Virchow-Robin spaces. Takakura, et al.,\textsuperscript{16,17} demonstrated enhanced survival of glioma patients in a small series when autogenous white blood cells were placed in the tumor cavity. Thus, patients with malignant gliomas may derive significant benefit from direct exposure of the tumor bed to cytotoxic lymphocytes.

With this in mind we attempted to study the feasibility of circumventing the blood-brain barrier by injecting normal lymphocytes directly into the subarachnoid space. If this were successful, we felt that subsequent clinical trials might be indicated. A glioblastoma tumor bed might be surgically exposed to the subarachnoid cerebrospinal fluid (CSF), and, if feasible, to the ventricular CSF. Autologous lymphocytes could then be purified from whole blood obtained from the cell separator was then diluted 1:1 with normal saline and placed on Ficoll-Hypaque gradients.\textsuperscript{4} The gradients were then centrifuged at 500 times gravity in a Sorvall RC-5 centrifuge\textsuperscript{†} at room temperature for 40 minutes. The cloudy lymphocyte layer present at the plasma-Ficoll Hypaque interface was harvested and the highly purified lymphocytes were washed three times in normal saline, and then resuspended in 10 ml of synthetic CSF.\textsuperscript{‡} Cell counts were determined on a ZB-I Coulter Counter.\textsuperscript{§} The cellular composition of the purified lymphocyte suspension was evaluated by examining Wright's-stained smears. Lymphocytes were found to comprise 98\% or more of the leukocytes present. There was less than one erythrocyte per white blood cell (WBC), and 5 to 10 platelets/WBC cell. Viability was determined by dye exclusion, adding 0.05 ml of 0.4\% trypan blue to an aliquot of the purified lymphocyte suspension and examining the cells under a light microscope. All suspensions were cultured for bacterial and fungal contaminants but none were ever detected.

Preparation of Human Lymphocytes

Informed consent was obtained from normal healthy volunteers who were to be the source of xenogenic lymphocytes to be used in the experiments. Each donor had blood counts and white cell differential counts performed before each donation. Lymphocytes were obtained according to the method of Aisner, et al.,\textsuperscript{1} using the Haemonetics Model 30 cell separator.* The lymphocyte-rich

*Haemonetics Model 30 blood processor manufactured by Haemonetics Corp., Natick, Massachusetts.

Materials and Methods

Preparation of Human Lymphocytes

Preparation of Rabbit Lymphocytes

Syngenic lymphocytes were obtained from highly inbred, B strain female Dutch rabbits each weighing 2 kg.\textsuperscript{∥} For each experiment heparinized blood (35 cc) was obtained from each of nine rabbits by cardiac puncture, the blood pooled and the lymphocytes purified as described above. In contrast to purified human lymphocytes, the purified rabbit lymphocyte suspension contained up to 10 red blood cells (RBC)/WBC.

Intrathecal Administration of Lymphocytes

Female New Zealand white rabbits each weighing 4 kg were premedicated with 15 mg of Thorazine (chlorpromazine) and 0.15 mg of atropine. Fifteen to 20 minutes later, the rabbits were given approximately 50 mg of pentobarbital intravenously and a spinal nee-
Intrathecal lymphocyte infusions

dle was inserted into the cisterna magna per-

cutaneously. If the tap was atraumatic and

CSF could be easily aspirated with a syringe,

1 to 2 cc of CSF was obtained for baseline

studies, and 2 cc of the lymphocyte suspen-

sion infused. When B strain, inbred, female

Dutch rabbits were used, the dose of pento-

barbital was reduced to 30 mg.

Preparation of EL-4 Cells

EL-4 lymphoma cells were passaged

weekly in C-57 mice by injecting 10^6 cells in-

traperitoneally. Cells were harvested 7 to 10

days after passage by peritoneal lavage after

sacrificing the animal by cord transection.

The cells were purified as described above

using Ficoll-Hypaque gradients.

CSF Studies

Both a hemacytometer and the ZB-1

Coulter Counter were used to perform cell

counts on spinal fluid that contained less than

500 cells/cu mm. The Coulter Counter alone

was used on spinal fluid containing more than

500 cells/cu mm. Cellular composition was

determined after staining the cells with

methylene blue and examining them under

the light microscope. The dye exclusion

method was used for determining cell viability,

as described earlier. The CSF glucose was

determined by the glucose oxidase method and

the CSF protein by the Biuret method.

Autopsy

Autopsy specimens were taken at various

intervals following intrathecal infusion of

lymphocytes; the brain and cervical spinal

cord were removed intact. The specimens

were fixed in 10% formalin for 1 week before

being cut for gross and microscopic examina-

tion.

Analysis of Cells

Rabbit peripheral blood lymphocytes and

post-infusion CSF cells were analyzed on a

Model 6301 Cytograf.* Cell suspensions

were diluted to 10^6 cells/ml with isotonic

saline. Ammonium chloride (NH₄ Cl) (0.83%)

was diluted 9:1 vol/vol with 0.17 M

tris(hydroxymethyl)-aminomethane (Tris

HCl) pH 7.2. An aliquot (0.6 ml) of the cell

suspension was then added to 10 ml of the

NH₄ Cl in Tris HCl buffer to lyse any red

cells present. An aliquot of the RBC-free cell

suspension was then added to the instrument

and 10,000 cells counted. The instrument

assigned each cell to one of 100 different

channels, according to its absorbance of the

laser beam. After all 10,000 cells were

counted, a printout was obtained listing the

number of cells assigned to each channel.

Since cell size bears a direct relation to laser

beam absorbance, the instrument provided an

accurate profile of the size of various cells in a

suspension.

Results

Intrathecal Infusion of Xenogenic

Lymphocytes into Rabbit

Subarachnoid Space

The initial problem in these studies was ob-
taining sufficient quantities of normal, viable,
purified lymphocytes. Using a unit of whole

human blood was cumbersome in terms of the

number of donors needed and the time and

equipment required to purify the lympho-
cytes; this method yielded only 10^9 lympho-
cytes/unit of blood. If the blood was allowed
to sediment, yielding lymphocyte-rich

plasma, the volume to be processed was cut in

half, but the yield of lymphocytes was also

reduced by 20% to 40%. With the

Haemonetics Model 30 Cell Separator it was

possible to obtain 6 to 8 × 10^9 lymphocytes in

only 2 to 3 hours in a volume of 100 to 200 ml

and with an almost negligible red cell loss to

the donor.

Resuspending the purified and washed lym-

phocytes in synthetic CSF gave a concentra-
tion of 10^9 to 10^10 cells/ml. A cisternal tap

was performed under general anesthesia and 2 cc

of the lymphocyte suspension was injected

(Table 1). A larger inoculum was not prac-
tical since the entire volume of the rabbit sub-

arachnoid space is only 10 to 12 cc. A second

cisternal puncture was performed either 12 or

36 hours later and then again at 4 to 10 days,

and finally 4 weeks after infusion. In most

cases the second tap was performed at 36

hours, rather than 12 hours after infusion, to

avoid anesthetic complications. The results of

the diagnostic studies done on 0.5 ml of CSF

obtained by these cisternal taps are sum-

marized in Tables 2, 3, and Fig. 1.
### TABLE 1

**Intrathecal infusion of xenogenic and syngenic lymphocytes into New Zealand white rabbits**

<table>
<thead>
<tr>
<th></th>
<th>No. of Cells Infused (range)</th>
<th>No. of Rabbits</th>
<th>% Viability (range)</th>
<th>No. of Rabbits</th>
<th>% Lymphocytes (range)</th>
<th>No. of Rabbits</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Xenogenic lymphocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st infusion</td>
<td>$8.04 \times 10^8$</td>
<td>32</td>
<td>91.5 (80-100)</td>
<td>23</td>
<td>98.5 (95-100)</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>$(2.17 \times 10^9-2.1 \times 10^9)$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd infusion</td>
<td>$7.8 \times 10^9$</td>
<td>8</td>
<td>93.7 (80-100)</td>
<td>8</td>
<td>99</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>$(2.8 \times 10^9-1.25 \times 10^9)$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Syngenic lymphocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st infusion</td>
<td>$5.04 \times 10^7$</td>
<td>7</td>
<td>92.5 (85-95)</td>
<td>5</td>
<td>100</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>$(6.3 \times 10^7-6.8 \times 10^7)$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd &amp; 3rd infusions</td>
<td>$4.54 \times 10^7$</td>
<td>6</td>
<td>88 (85-90)</td>
<td>6</td>
<td>100</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>$(2.28 \times 10^7-6.8 \times 10^7)$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 2

**Intrathecal infusion of xenogenic and syngenic lymphocytes**

<table>
<thead>
<tr>
<th>Time of Measurement</th>
<th>Mean CSF Cell Counts* (cells/cu mm (range))</th>
<th>% Viability (range)</th>
<th>% Lymphocytes (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st administration of xenogenic lymphocytes:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre-infusion</td>
<td>n = 23</td>
<td>n = 17</td>
<td>n = 17</td>
</tr>
<tr>
<td>12 hrs post-infusion</td>
<td>n = 5</td>
<td>20,818</td>
<td>120 (0-1)</td>
</tr>
<tr>
<td></td>
<td>n = 2</td>
<td>20,818</td>
<td>120 (0-1)</td>
</tr>
<tr>
<td>36 hrs post-infusion</td>
<td>n = 23</td>
<td>4344</td>
<td>95 (40-100)</td>
</tr>
<tr>
<td></td>
<td>n = 23</td>
<td>4344</td>
<td>95 (40-100)</td>
</tr>
<tr>
<td>4 wks post-infusion</td>
<td>n = 13</td>
<td>7 (0-30)</td>
<td>7 (0-30)</td>
</tr>
<tr>
<td>2nd administration of xenogenic lymphocytes:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre-infusion</td>
<td>n = 4</td>
<td>12 (0-1)</td>
<td>12 (0-1)</td>
</tr>
<tr>
<td>36 hrs post-infusion</td>
<td>n = 7</td>
<td>348 (40-625)</td>
<td>348 (40-625)</td>
</tr>
<tr>
<td>4 wks post-infusion</td>
<td>n = 2</td>
<td>348 (40-625)</td>
<td>348 (40-625)</td>
</tr>
<tr>
<td>1st administration of syngenic lymphocytes:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre-infusion</td>
<td>n = 5</td>
<td>12 (0-1)</td>
<td>12 (0-1)</td>
</tr>
<tr>
<td>36 hrs post-infusion</td>
<td>n = 7</td>
<td>348 (40-625)</td>
<td>348 (40-625)</td>
</tr>
<tr>
<td>4 wks post-infusion</td>
<td>n = 2</td>
<td>348 (40-625)</td>
<td>348 (40-625)</td>
</tr>
<tr>
<td>2nd &amp; 3rd administration of syngenic lymphocytes:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre-infusion</td>
<td>n = 6</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>36 hrs post-infusion</td>
<td>n = 6</td>
<td>241 (81-500)</td>
<td>241 (81-500)</td>
</tr>
<tr>
<td>4 wks post-infusion</td>
<td>n = 2</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*Coulter counter used. Hemacytometer used for cell counts <500 cells/cu mm.
Intrathecal lymphocyte infusions

Pre-infusion rabbit CSF was found to be very similar to normal human CSF containing 0 to 3 lymphocytes/cu mm (Table 2). At 12 hours post-infusion the CSF cell counts ranged from 1150 to 70,000 cells/cu mm (Table 2). The variability of this figure was due to the different numbers of cells infused (Table 1), the quality of the cisternal tap, and possibly the amount of leakage of CSF and cells back through the needle tract. Differential cell counts and examination of viability by trypan blue dye exclusion method on the 12 hour post-infusion CSF revealed that the cells were all viable lymphocytes (Table 2). By 36 hours after infusion, the total cell count had dropped dramatically (Fig. 1), but the cells continued to be viable lymphocytes. No polymorphonuclear leukocytes were observed. At 10 days after infusion only a few hundred cells, again all viable lymphocytes, remained; by 4 weeks after infusion, the CSF cell count had returned to normal.

The high cell viabilities following intrathecal lymphocyte infusion suggest that cell destruction was not the cause of the rapid and marked drop in total cell count observed following intrathecal cell infusion. Furthermore, if a host response had occurred resulting in the destruction of the infused lymphocytes, systemic morbidity, hyperther-

<table>
<thead>
<tr>
<th>Time of Measurement</th>
<th>CSF Glucose (mg%)</th>
<th>CSF Protein (mg%)</th>
<th>Rectal Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st administration of xenogenic lymphocytes:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre-infusion</td>
<td>n = 23</td>
<td>n = 23</td>
<td>n = 22</td>
</tr>
<tr>
<td></td>
<td>75 (62-98)</td>
<td>70 (23-129)</td>
<td>38.4</td>
</tr>
<tr>
<td></td>
<td>(36.5-39.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 hrs post-infusion</td>
<td>n = 2</td>
<td>n = 2</td>
<td>n = 3</td>
</tr>
<tr>
<td></td>
<td>56.6 (53-64)</td>
<td>142 (52-233)</td>
<td>38.4</td>
</tr>
<tr>
<td>36 hrs post-infusion</td>
<td>n = 24</td>
<td>n = 24</td>
<td>n = 23</td>
</tr>
<tr>
<td></td>
<td>79.4</td>
<td>192.8</td>
<td>38.78</td>
</tr>
<tr>
<td></td>
<td>(60-112)</td>
<td>(40-618)</td>
<td></td>
</tr>
<tr>
<td>4 wks post-infusion</td>
<td>n = 14</td>
<td>n = 13</td>
<td>n = 20</td>
</tr>
<tr>
<td></td>
<td>69 (52-80)</td>
<td>64 (39-124)</td>
<td>38.5</td>
</tr>
<tr>
<td></td>
<td>(37.5-39.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd administration of xenogenic lymphocytes:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre-infusion</td>
<td>n = 4</td>
<td>n = 4</td>
<td>n = 6</td>
</tr>
<tr>
<td></td>
<td>64 (53-75)</td>
<td>11 (74-280)</td>
<td>38 (37-39.5)</td>
</tr>
<tr>
<td>36 hrs post-infusion</td>
<td>—</td>
<td>—</td>
<td>n = 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>38.5 (38-39.5)</td>
</tr>
<tr>
<td>4 wks post-infusion</td>
<td>n = 1</td>
<td>n = 1</td>
<td>n = 1</td>
</tr>
<tr>
<td></td>
<td>73 (73)</td>
<td>58 (58)</td>
<td>38 (38)</td>
</tr>
<tr>
<td>1st administration of syngenic lymphocytes:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre-infusion</td>
<td>n = 3</td>
<td>—</td>
<td>n = 4</td>
</tr>
<tr>
<td></td>
<td>83 (82-87)</td>
<td></td>
<td>38 (37.5-39)</td>
</tr>
<tr>
<td>36 hrs post-infusion</td>
<td>n = 2</td>
<td>n = 4</td>
<td>n = 4</td>
</tr>
<tr>
<td></td>
<td>75 (73-77)</td>
<td>68 (29-119)</td>
<td>38.1 (38-38.75)</td>
</tr>
<tr>
<td>4 wks post-infusion</td>
<td>n = 1</td>
<td>n = 1</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>95 (95)</td>
<td>58 (58)</td>
<td></td>
</tr>
<tr>
<td>2nd &amp; 3rd administration of syngenic lymphocytes:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre-infusion</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
</tr>
<tr>
<td></td>
<td>83 (91-86)</td>
<td>45 (7-76)</td>
<td>38 (37-38.5)</td>
</tr>
<tr>
<td>36 hrs post-infusion</td>
<td>n = 3</td>
<td>n = 14</td>
<td>n = 3</td>
</tr>
<tr>
<td></td>
<td>103 (82-142)</td>
<td>57 (44-82)</td>
<td>38 (37-39)</td>
</tr>
<tr>
<td>4 wks post-infusion</td>
<td>n = 2</td>
<td>n = 2</td>
<td>n = 2</td>
</tr>
<tr>
<td></td>
<td>38.3</td>
<td>38.5 (38.5)</td>
<td>38 (38)</td>
</tr>
</tbody>
</table>
submitted for publication.

E. Neuwelt and D. Doherty

Fig. 1. Graph showing changes in total CSF cell count, CSF protein, and CSF glucose following intrathecal infusion of human lymphocytes into New Zealand white rabbits. Brackets indicate the standard error of the mean; ●—● total CSF cell count; □—□ CSF glucose; ○—○ CSF protein.

mia, and a leukocytosis associated with hypoglycorrhachia and infiltration of the CSF with polymorphonuclear leukocytes might have resulted. None of these events was observed (Table 3). Indeed, the only parameter that seemed to change following lymphocyte infusion was a transient rise in the total CSF protein (Fig. 1). Therefore, it appeared that the rapid drop in CSF cell count following lymphocyte infusion was due to either sequestration in the CNS or escape from the CNS.

Only four of 32 rabbits showed any evidence of toxicity to a single infusion of xenogenic lymphocytes (Table 4). Two rabbits developed a transient monoparesis, one rabbit developed torticollis which resolved over 2 weeks, and one rabbit died after infusion. It is worth noting that the rabbits that developed monoparesis and torticollis could have developed these complications from the cisternal taps rather than as a direct result of the lymphocyte infusions. The single mortality was in a rabbit that died unnoticed 24 to 72 hours after infusion over a weekend; an autopsy was not possible.

**Toxicity of a Second Intrathecal Infusion of Xenogenic Lymphocytes**

Because of the possible need for multiple intrathecal infusions in clinical studies, the toxicity of a second infusion of xenogenic lymphocytes was evaluated. It was unfortunate that in the rabbit, second and third intrathecal infusions through cisternal punctures proved technically difficult. Although there was no histological evidence of inflammation in the cervical spinal cord and leptomeninges, it was often difficult to obtain a free flow of CSF after the initial cisternal punctures. It was possible in a few rabbits, nonetheless, to freely aspirate CSF and infuse cells a second time.

In one such study there were two rabbits that had previously received intrathecal xenogenic lymphocytes and two that had not; the latter two were given intrathecal xenogenic lymphocytes from a donor who had not been used previously. Over the next 24 to 48 hours, the two rabbits that had received xenogenic cells for a second time became noticeably tachypneic and appeared ill (Table 4). One of the ill and one of the asymptomatic rabbits were selected for autopsy 48 hours after infusion. The other ill rabbit recovered after 72 to 96 hours. The spinal fluid of all four rabbits 36 hours after infusion was of similar composition. No polymorphonuclear leukocytes were observed in any of the CSF samples, nor were there any changes in the CSF glucose. Similarly, gross and histological examinations of the CNS were unremarkable with one important ex-

<table>
<thead>
<tr>
<th>Toxicity</th>
<th>No. of Rabbits</th>
</tr>
</thead>
<tbody>
<tr>
<td>mortality</td>
<td>1</td>
</tr>
<tr>
<td>neurological status</td>
<td></td>
</tr>
<tr>
<td>transient monoparesis</td>
<td>2</td>
</tr>
<tr>
<td>transient torticollis</td>
<td>1</td>
</tr>
<tr>
<td>allergic reactions*</td>
<td>2</td>
</tr>
</tbody>
</table>

*Both allergic reactions occurred in rabbits receiving xenogenic lymphocytes for the second time. Two other rabbits received the same lymphocyte preparation without developing allergic symptoms, but had not previously been exposed to human lymphocytes.

TABLE 4
Toxicity of intrathecal infusion of lymphocytes in 43 rabbits followed for an average of 75 days (2 to 197 days)
Intrathecal lymphocyte infusions

In order to investigate the origin of the tachypnea in the symptomatic rabbits, a careful postmortem examination of the lungs of the two autopsied rabbits was performed. Grossly, the symptomatic rabbit's lung was congested in contrast to the asymptomatic rabbit's lung. Microscopic examination revealed pulmonary edema in the symptomatic rabbit in contrast to the asymptomatic rabbit (Fig. 3). Thus, the two rabbits that had received xenogenic cells a second time developed what appeared to be a prolonged systemic allergic reaction associated with marked pulmonary edema and choroid plexitis. The prolonged course of the reaction was consistent with continual escape of the xenogenic cells into the systemic circulation over 2 to 3 days. The rapid drop in CSF lymphocyte count over the first 2 to 3 days after infusion in the 32 rabbits studied supports such a pathogenesis (Fig. 1).

Fig. 2. Photomicrographs of the choroid plexus after intrathecal infusion of viable lymphocytes from normal humans into New Zealand white rabbits. H & E, X 75. Upper Left: Normal choroid plexus 48 hrs after infusion of $1 \times 10^9$ lymphocytes. Host rabbit had no prior exposure to human cells. The CSF cell count at time of autopsy was 2900 lymphocytes/cu mm. Upper Right: Marked lymphocytic infiltration of choroid plexus 48 hrs after infusion of $1 \times 10^9$ lymphocytes (same donor and cell preparation as in upper left). The host rabbit had a prior human lymphocyte infusion 6 weeks previously from another donor. The CSF cell count at the time of autopsy was 900 lymphocytes/cu mm. Lower Right: Mild lymphocytic infiltration of choroid plexus 9 days after intrathecal infusion of $1 \times 10^9$ lymphocytes. Host rabbit had no prior exposure to human cells. The CSF cell count at the time of autopsy was 200 lymphocytes/cu mm.

ception: there was a marked mononuclear infiltration of choroid plexus stroma in the rabbit that received cells for a second time (Fig. 2 upper right). This infiltration was not seen in the choroid plexus of the rabbit that received a single intrathecal lymphocyte infusion (Fig. 2 upper left). Indeed, a mononuclear infiltration of the choroid plexus was never seen 24 to 48 hours after infusion, but was seen to a mild degree in one rabbit 9 days after infusion when the total CSF cell count was returning to normal (Fig. 2 lower left). Therefore, infiltration of the choroid plexus by the rabbit's own lymphocytes (choroid plexitis) was a more likely explanation for the origin of these cells than infiltration of choroid plexus by infused lymphocytes from the ventricular space. Besides, few infused cells appeared to migrate into the ventricles on histological sections, making infiltration from the ventricle an even less tenable explanation.
E. Neuwelt and D. Doherty

Fig. 3. Photomicrographs of sections of the lung from two New Zealand white rabbits. H & E, X 100. Left: Absence of pulmonary edema 48 hrs after an initial intrathecal infusion of xenogenic lymphocytes. (Same rabbit whose normal choroid plexus is illustrated in Fig. 2 upper left.) Right: Pulmonary edema 48 hrs after a second intrathecal infusion of xenogenic lymphocytes. (Same rabbit whose choroid plexus is illustrated in Fig. 2 upper right.)

Intrathecal Infusion of Syngenic Lymphocytes

Since the objective of this study was to investigate the feasibility of intrathecal infusion of autologous lymphocytes into patients with malignant gliomas, early studies were performed to examine the toxicity of autologous intrathecal lymphocyte infusions. The use of autologous rabbit lymphocytes, however, permitted the infusion of only 10⁶ cells, since the lymphocytes had to be purified from whole blood. This problem was partially alleviated by infusing pooled syngenic lymphocytes obtained from several highly inbred B strain Dutch rabbits into a recipient rabbit. Even so, it was feasible to infuse only 6 × 10⁷ lymphocytes into the inbred rabbits (Table 1); this resulted in a maximum CSF cell count at 36 hours of 625 cells/cu mm (Table 2). As with the infusion of the xenogenic lymphocytes, the cell viability after infusion was always nearly 100% and there was a mild transient rise in total CSF protein (Table 3).

There was no change in CSF glucose (Table 3), mean systemic white cell count, or mean body temperature. No morbidity or complications were observed even after a second or a third syngenic lymphocyte infusion, and pathological examination 48 hours after the second and third infusions revealed neither choroid plexitis nor pulmonary edema. Thus, the allergic reaction seen following a second xenogenic lymphocyte infusion was not seen when syngenic cells were used.

Intrathecal Infusion of EL-4 Lymphoma Cells

Since it was impossible to differentiate donor and recipient lymphocytes morphologically, it was possible that there was a migration of host lymphocytes into the CSF in response to the intrathecal lymphocyte infusions. In an attempt to explore this possibility, it was first necessary to distinguish between infused and host cells. The EL-4 lymphoma cells which are larger than normal rabbit lymphocytes, were examined as described on the Cytograf, an instrument which can distinguish two cell populations of...
Intrathecal lymphocyte infusions

**FIG. 4.** Graph illustrating the use of the Cytograf to detect rabbit lymphocytes in a suspension of EL-4 lymphoma cells. The rabbit lymphocyte suspension (solid line) and the EL-4 lymphoma cell suspension (dotted line) were prepared as described, and the cell concentration of each adjusted to $1 \times 10^6$ cells/ml. An aliquot of each suspension and an aliquot of a mixture containing 95% EL-4 cells and 5% rabbit lymphocytes (dashed line) was added to the Cytograf as described. Each curve represents 10,000 cells and the Cytograf assigned each of these cells to one of 100 channels according to cell diameter. The channel numbers increase in proportion to cell diameter. The arrow indicates the peak due to the presence of 5% lymphocytes in the cell suspension containing 95% EL-4 lymphoma cells.

**FIG. 5.** Graph shows the absence of host lymphocytes in CSF and absence of EL-4 lymphoma cells in host blood 24 hours after intrathecal infusion of EL-4 lymphoma cells into a New Zealand white rabbit. This figure is analogous to Fig. 4 except the solid line represents normal, purified, New Zealand white rabbit lymphocytes; the dashed line represents host lymphocytes, purified from the blood of a New Zealand white rabbit 24 hrs after intrathecal infusion of EL-4 lymphoma cells; and the dotted line represents the CSF cells from the same host rabbit 24 hrs after intrathecal infusion of EL-4 lymphoma cells.

In *vitro* experiments (Fig. 4), the Cytograf could detect 5% rabbit lymphocytes in a cell suspension containing 95% EL-4 cells. The EL-4 cells were then infused intrathecally, and the Cytograf was used to detect any infiltration of host lymphocytes into the CSF. On this basis, $10^6$ EL-4 cells, purified from mouse ascites fluid on Ficoll-Hypaque gradients and resuspended in synthetic CSF, were infused into the rabbit subarachnoid space. Twenty-four hours after infusion, the CSF cell count was 30,000 cells/cu mm and over 80% of the cells were viable. At 72 hours, the CSF cell count was 500 and only 20% to 30% of the cells were viable, as determined by dye exclusion, and the CSF contained a large amount of cell debris. In order to examine viable EL-4 cells, CSF was obtained 24 hours after intrathecal EL-4 cell infusion and an aliquot examined on the Cytograf (Fig. 5). In addition, the host rabbit’s blood was obtained by cardiac puncture 24 hours after infusion, the lymphocytes purified, and an aliquot of this cell suspension examined on the Cytograf. These experiments revealed that at 24 hours after infusion no infiltration of the CSF by host lymphocytes was detectable and that no EL-4 cells were detectable in the rabbit’s systemic circulation (Fig. 5). Although
E. Neuwelt and D. Doherty

FIG. 6. Photomicrograph showing lymphocytic infiltration of the subarachnoid space of a New Zealand white rabbit 36 hrs after intrathecal infusion of $2 \times 10^9$ viable human lymphocytes. The CSF cell count just before autopsy was 3000 cells/cu mm; all of which were viable lymphocytes. H & E, × 350.

FIG. 7. Photomicrograph showing accumulation of lymphocytes around the superior sagittal sinus 48 hrs after intrathecal infusion of xenogenic lymphocytes into a New Zealand white rabbit. The mononuclear cells in the mass inferior to the sinus were all lymphocytes. H & E, × 100.

these experiments provide evidence that host cells do not appear to infiltrate the CSF in response to lymphoid cell infusion, the absence of detectable EL-4 cells in the systemic circulation could be explained in a variety of ways. Because of their large size or their rapidly decreasing viability, EL-4 cells might not have been able to escape from the CNS, or alternatively, they may have been escaping from the CNS and either the Cytograf was not able to detect them or they did not remain in the systemic circulation long enough to be detected. As a result, experiments are now underway to follow the migration of infused normal lymphocytes using radioisotopes.

Autopsy Studies

Animals were sacrificed at different times following intrathecal syngenic and xenogenic lymphocyte infusions in an attempt to find evidence of CNS parenchymal damage and to study the migration of the infused cells. On gross examination, after coronal slices were made of formalin-fixed brain, brain stem, and spinal cord, no abnormalities were seen in any of the specimens. There was no evidence of hydrocephalus or areas of pallor suggestive of demyelination. On microscopic examination, except for the findings in the choroid plexus discussed above, no abnormalities of the CNS parenchyma were noted. The subarachnoid space, but not the ventricles, was diffusely infiltrated at 12 to 36 hours after infusion with lymphocytes (Fig. 6), but the majority of cells had disappeared by 10 days. This is strong evidence that the rapid drop in total cell count following intrathecal lymphocyte infusion was not the result of sequestration of lymphocytes in the CNS. Although adequate sections of the superior sagittal sinus were difficult to obtain, cells did appear to accumulate around this structure transiently, suggesting a possible route of escape (Fig. 7).

Autopsy studies also revealed that the distribution of EL-4 lymphoma cells in the subarachnoid space and ventricles (Fig. 8)
Intrathecal lymphocyte infusions

Fig. 8. Photomicrograph showing infiltration of the subarachnoid space of a New Zealand white rabbit with EL-4 lymphoma cells 24 hrs after intrathecal infusion of $1 \times 10^9$ EL-4 lymphoma cells. Notice that the lymphoma cells in the subarachnoid space are larger than the lymphocytes illustrated in Fig. 6. The CSF cell count just before autopsy was 30,000 cells/cu mm, of which 85% were viable. H & E, X 350.

seemed to be identical to the pattern seen with normal xenogenic and syngenic lymphocytes. However, the EL-4 cell architecture was hard to discern and the cells fragmented, suggesting cell death at 72 hours after infusion. This finding is consistent with the dye exclusion studies of EL-4 cells at 72 hours noted earlier. Exactly why these cells did not remain viable in contrast to normal lymphocytes is not clear.

Microscopically, the only cells seen in the subarachnoid space following intrathecal EL-4 cell infusion were EL-4 lymphoma cells. No normal host lymphocytes were observed (Fig. 8). Thus, neither the Cytograf (Fig. 5) nor histological studies (Fig. 8) demonstrated any host cell infiltration of the CSF following lymphoma cell infusions. Up to 72 hours after intrathecal EL-4 lymphoma cell infusion no infiltrates of the choroid plexus were seen.

Discussion

The question asked in the present study was whether rabbits could tolerate intrathecal infusions of normal syngenic or xenogenic lymphocytes. Since preliminary studies using moderate numbers of autologous lymphocytes and later studies using larger numbers of syngenic lymphocytes revealed no toxicity, we wanted to determine if the animals would also tolerate the infusion of massive numbers of xenogenic lymphocytes. To investigate that question, we used the Haemonetics Model 30 Cell Separator which could furnish enormous numbers of normal lymphocytes from healthy human volunteers without a significant red cell loss to the donor.1 With this method, even though 1000 times the number of cells were used and species barriers crossed, toxicity after a single infusion was again minimal.

However, two rabbits did develop a systemic reaction to a second xenogenic lymphocyte infusion. No such reactions were seen with a second or even a third administration of syngenic lymphocytes. The systemic reaction might have been an allergic response to cells which escaped from the subarachnoid space over a 48 to 72-hour period into the systemic circulation of an already sensitized host. This could have resulted in the subacute pulmonary edema and the choroid plexitis we observed. The findings in the choroid plexus of several electively autopsied animals certainly support an immune mechanism, since after an initial xenogenic lymphocyte infusion, a mononuclear infiltrate in the choroid plexus was not seen until 9 days after infusion, which is consistent with the time sequence of primary immune sensitization. However, in the two rabbits with pulmonary edema who had a prior exposure to human lymphocytes, there was a marked mononuclear infiltration of the choroid plexus at 48 hours. Two other rabbits that received the same human lymphocyte preparation but did not have a prior exposure to human cells developed neither pulmonary edema nor choroid plexitis. Thus, xenogenic cells transplanted into the CNS were able to activate both limbs of the immune mechanism.

Scheinberg and Taylor16 reported that skin homografts transplanted into brain could accelerate rejection of subsequent systemic skin grafts from the same donor; this finding indicates stimulation of the afferent limb of the immune response. However, the actual mechanism of activation was not clear in their report. In the present study, escape of the infused lymphocytes from the subarachnoid space into the systemic circulation might ac-
count for sensitization of the immune system. The evidence that the infused lymphocytes were indeed escaping from the CNS is as follows. The infused lymphocytes disappeared very rapidly from the subarachnoid space; the total CSF cell count was as high as 70,000 cells/cu mm 12 hours after infusion and dropped to only 200 to 300 cells/cu mm 4 to 5 days later. It did not appear that the precipitous fall in total CSF cell count was due to cell death when normal lymphocytes were infused, since cell viability remained near 100%. In contrast, when EL-4 lymphoma cells were infused, viability dropped dramatically between 24 and 72 hours after infusion. If the infused normal lymphocytes were not dying, then they must have been either sequestered in or escaping from the CNS. Histological studies of autopsied animals essentially eliminated the former possibility. That is, although the subarachnoid space was filled with lymphocytes on histological sections 12 hours after infusion (Fig. 6), these cells had all but disappeared from histological sections obtained several days later. The only other remaining possibility, then, was that the lymphocytes were escaping from the subarachnoid space systemically. The accumulation of lymphocytes around the superior sagittal sinus (Fig. 7) suggested that this structure might be the avenue of escape. More detailed studies are presently underway to investigate further the migration of lymphocytes infused into the subarachnoid space and the possible role that the superior sagittal sinus may play in the escape of these cells into the systemic circulation. Regardless of the role of the superior sagittal sinus, however, the above evidence does strongly suggest that the infused lymphocytes were escaping from the CNS.

It should be noted that glioma cells shed from the main tumor mass in a glioma patient may escape to the systemic circulation by the same pathway as proposed here for lymphocytes. The presence of intravascular tumor cells in glioma patients has been reported previously. The escape of glioma cells into the systemic circulation may be a mechanism by which glioma patients develop immunologically specific, cytotoxic lymphocytes.

With respect to the therapeutic implications of this study, it appears that intrathecal or possibly intraventricular infusions of autologous lymphocytes from glioma patients may be a means by which specifically cytotoxic lymphocytes may gain direct access to a glioma tumor bed. Such an approach would eliminate the serum-blocking factors reported to be present in glioma patients which interfere with lymphocytotoxicity. Since the lymphocytes infused into the subarachnoid space of the rabbit in this study do not appear to penetrate the brain parenchyma, the therapeutic use of autologous lymphocytes probably would be most effective if a maximum surface area of a glioma patient’s tumor bed were first exposed to the subarachnoid, and, if possible, the ventricular CSF, before autologous lymphocytes were infused intrathecally or intraventricularly. On the basis of the present study, such a therapeutic regimen would be both feasible and probably nontoxic. If the intrathecal administration of lymphocytes into glioma patients does indeed prove to be nontoxic, patients with certain CNS viral or fungal infections might also benefit from this therapeutic regimen.

On the other hand, the apparent ability of intrathecally infused lymphocytes to escape from the CNS suggests that the repeated infusions of homologous as opposed to autologous lymphocytes in a glioma patient might evoke a systemic immunological reaction similar to that observed in the New Zealand white rabbits following repeated intrathecal xenogenic lymphocyte infusions. Therefore, even though lymphocytes from one individual can attack glioma tumor cells from a second individual with the same histological type of tumor in vitro, the use of such homologous lymphocytes in vivo should be approached with caution.

Acknowledgments

The authors would like to express their gratitude to Drs. C. Levy, K. Clark, R. Winchurch, and T. Ducker for their help and advice in the preparation of this manuscript; to Drs. C. Schiffer and J. Aisner, who were instrumental in obtaining lymphocytes from human donors using the Model 30 Cell Separator; and to Dr. M. Mardiney for the use of the Cytograf.

References

Intrathecal lymphocyte infusions


Address reprint requests to: Edward A. Neuwelt, M.D., Division of Neurosurgery, University of Texas Southwestern Medical School, 5323 Harry Hines Blvd., Dallas, Texas 75235.