Chemotherapy of an Experimental Glioma with Nitrosoureas

Charles H. Tator, Anna Day, Rodney Ng, and Leeba Liberman

Division of Neurosurgery, University of Toronto, Toronto, Ontario M5S 1A8 [C.H.T., A.D., R. N., L.L.J., and Division of Neurosurgery, Sunnybrook Medical Centre, Toronto, Ontario M4N 3M5, Canada [C.H.T.]

SUMMARY

Chemotherapy experiments were performed with 2-nitrosourea drugs in an experimental mouse brain tumor model. Cell suspensions of a transplantable mouse ependymoblastoma were injected i.c. by means of a stereotactic frame. The drugs used were 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea and 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea and were given by either i.p. or by direct intraneoplastic (i.n.) injection on the fifth day after tumor cell implantation. Injections i.n. of drugs were made with the stereotactic frame.

Both drugs were highly effective in increasing the median day of death and in yielding large numbers of long-term survivors. Effectiveness was evident after i.p. or i.n. injection. However, with certain dosage schedules such as every 2 hr for 5 injections daily on 2 consecutive days, i.n. injection was more effective and less toxic than i.p. injection. The reason why repeated i.n. injections produced less toxicity than repeated i.p. injections is not definitely known but may be due to local metabolism of the drugs in the tumors and surrounding brain to a less toxic form. This is the first laboratory report of direct i.n. injection of the nitrosoureas, and the authors consider these results encouraging.

INTRODUCTION

There have been numerous attempts to treat human brain tumors with chemotherapeutic agents, but the results in patients with malignant gliomas such as the glioblastoma multiforme have been poor. BCNU(3) has been the most frequently used nitrosourea in patients with malignant gliomas and has been shown to increase survival by only a few months in a large multicenter cooperative study (40). Recently, Calogero et al. (4) reported long-term survival of 4 to 5 years in 3 patients with brain tumors treated with BCNU. However, these patients did not have glioblastomas, but an ependymoma, an anaplastic astrocytoma, and an astrocytoma, and these favorable responses comprised less than 5% of patients treated with BCNU. Other nitrosoureas, including CCNU (10, 14, 24, 27) and methyl-CCNU (47), have also been used in patients with brain tumors, but the results in those with malignant gliomas have generally been poor. Thus, the numerous experimental evaluations of BCNU and other nitrosoureas which have shown excellent chemotherapeutic results in brain tumor-bearing animals (1, 2, 5, 20, 29, 31-35) have not led to improved results in most patients, and there is a need for further study of these agents. In our laboratory we have developed a brain tumor chemotherapy model in mice (42) with which it has been possible to evaluate chemotherapeutic agents given either i.p. or directly i.n. Direct i.n. injection of chemotherapeutic agents including methotrexate (12, 15, 23, 30, 44), cyclophosphamide (17, 25), triethylenetriaminepiperazine (22), and BCNU (13) has been performed in patients with brain tumors, but the results have not shown significant therapeutic benefit. However, to our knowledge, there have been no reports of laboratory chemotherapeutic studies of i.n. injection of nitrosoureas. Rosenblum et al. (26) have considered this approach and have prepared diffusion chambers containing methyl-CCNU for i.n. implantation in brain tumors. Injection i.n. of nitrosoureas may have advantages for brain tumor chemotherapy because there is evidence that even the lipid-soluble nitrosoureas may have impaired effectiveness against solid tumors due to their failure to reach all parts of the tumors after systemic administration (28). Many nitrosoureas including BCNU and CCNU have been shown to be less effective against i.c. than against i.p. implants of the same tumor (19). The present paper reports our experiments with CCNU and methyl-CCNU given i.p. or i.n. to mice bearing i.c. implants of a transplantable ependymoblastoma.

MATERIALS AND METHODS

Animals and Tumor. C57BI/6J female mice weighing 16 to 18 g were obtained from The Jackson Laboratory, Bar Harbor, Maine. A mouse ependymoblastoma obtained in 1963 and maintained in our laboratory since then by serial s.c. transplantation every 2 weeks was used. The tumor was originally induced by Zimmerman and Arnold (48) in mouse brain with i.c. implanted methylcholangthrene.

Tumor Cell Suspension and I.c. Implantation. A suspension of neoplastic cells was made from a sufficient number of mice bearing 2-week-old s.c. ependymoblastoma to yield 2 g of tumor tissue. The tumor was then trypsinized in calcium- and magnesium-free phosphate-buffered saline to
produce a suspension of tumor cells containing 100,000 cells/μl as previously described (42). The buffered saline contained 0.1% EDTA, 0.2% glucose, 0.002% KH₂PO₄, 0.0073% Na₂HPO₄, 0.03% KCl, and 0.8% NaCl. The final suspension contained penicillin and streptomycin in concentrations of 50 units and 50 μg/ml, respectively. Three μl of the tumor cell suspension were injected i.c. into the right frontal region of mice by means of a 30-gauge needle attached to a micrometer-syringe assembly in an especially designed stereotactic frame (42).

**Drugs.** CCNU and methyl-CCNU were provided in bulk form by the National Cancer Institute, Bethesda, Md. The drugs were injected as a suspension in 0.4% methylcellulose which was freshly prepared each day by mechanically grinding the mixture in a glass homogenizer of the Potter-Elvehjem type for 7 to 10 min. During preparation the drugs were chilled in an ice bath. The suspensions were ground fine enough to pass through a 30-gauge needle which was used for most of the i.n. injections. However, for concentrations of CCNU higher than 30 mg/ml or of methyl-CCNU higher than 40 mg/ml, a 26-gauge needle was required because of the thickness of the suspensions.

Several attempts were made to dissolve methyl-CCNU in various solutions of absolute alcohol, 0.9% NaCl solution, and Emulphor EL620 (GAF Corp., New York, N. Y.), a polyoxyethylated vegetable oil, as described by Davignon et al. (8). It was possible to dissolve small amounts of methyl-CCNU in 1:1:1 solutions of absolute alcohol, 0.9% NaCl solution, and Emulphor. For example, 1 ml of this mixture would dissolve 42.5 mg methyl-CCNU. However, in most experiments higher concentrations of methyl-CCNU were required for the i.n. injections where only 3 μl were injected. To dissolve larger amounts of the nitrosoureas it was necessary to use more alcohol and Emulphor and less 0.9% NaCl solution. These solutions usually produced marked toxicity after either i.n. or i.p. injection, and for this reason further attempts to inject methyl-CCNU or CCNU as solutions were abandoned. Thus, all the chemotherapeutic trials in the present report were performed with drug suspensions in 0.4% methylcellulose prepared as described above.

**Experimental Protocol.** A number of factors were considered in determining the total dose of drug to be administered and the frequency, volume, and route of injection. First, it was desired to use as large a total dose as possible for the i.n. and i.p. routes. With the i.n. route, previous studies (39) showed that volumes of 3 μl injected into the mouse brain as often as every 2 hr for 5 injections daily for 2 consecutive days were well tolerated by the mice. The limitations imposed by the use of drug suspensions and small-caliber needles often necessitated a multiple injection schedule in order to deliver the desired total dose of drug.

Tables 1 to 3 are representative of the experiments that were performed. With 1 exception all of the experiments were performed on 120 mice, all of which received the same tumor cell suspension. The exception was Experiment 2, methyl-CCNU (Table 2) which contained only 60 mice. Separate control groups were used for the i.p. and i.n. injection routes and for each variation in frequency of injection of drugs. The frequency of injection varied from a single injection on the 5th day after i.c. tumor implantation to repeated injections every 2 hr for 5 injections/day on the 5th and 6th days after implantation. Control animals received methylcellulose injections through the same gauge needle as received by treated animals. Penicillin and streptomycin in concentrations of 50 units and 50 μg/ml, respectively, were added to all treatment and control injections. All treatments were begun on the 5th day after i.c. tumor implantation. The volume of each control or treatment i.n. injection was 3 μl, and for each i.p. injection the volume was 100 μl.

**Statistical Analysis.** The median day of death was determined graphically by plotting the cumulative percentage of deaths against survival days after tumor implantation (Charts 1 and 2). Comparison was then made between the median day of death of treated and control groups, and the percentage increase or decrease in life-span was thus determined. LTS were defined as animals living more than 60 days from the time of tumor implantation. Significant prolongation (therapeutic) or shortening (toxic) of survival was assessed from the graphs by the Kolmogorov-Smirnov test as adapted by Tate and Clelland (38). To test the significance of differences between proportions as in the evaluation of the number of LTS, a table prepared by Csima and Reid (7) based on Fisher’s exact procedure was used.

**RESULTS**

**CCNU.** All the experiments performed with CCNU are shown in Table 1. Graphic analysis of Experiment 1 (CCNU) is shown in Charts 1 and 2. There was a marked therapeutic response from repeated i.n. injections of CCNU every 2 hr for 5 injections given on 2 consecutive days, for a total dosage of 60 mg/kg. In this group of 20 mice, there was a 128% prolongation of the median day of death, and there were 13 LTS. A single i.n. injection of the same strength
Two experiments were performed in which i.n. and i.p. routes of injection were used. All the animals in the same tumor cell suspension. Treatment began on the 5th day after tumor cell implantation. Each experiment received the increase or frequency of the total dose of injection of 60 mg/kg with repeated half-hourly i.p. injections with a total dose of 60 mg/kg both produced marked toxicity (\( p < 0.01 \)) (Table 1). The single i.p. injection also produced 7 LTS, which is significant (\( p < 0.01 \)). It was not possible to give a single i.n. injection of 60 mg/kg because the suspension was too thick to go through the 30-gauge needle which was used at the time.

**Methyl-CCNU.** Examples of the experiments performed with methyl-CCNU are shown in Tables 2 and 3. Table 2 shows that the i.n. schedule every 2 hr for 5 injections on 2 days was highly effective and produced a 112% prolongation of the median day of death and 13 LTS from the group of 20. In contrast, the comparable i.p. schedule was ineffective. However, a single i.p. injection at the same total dose of 40 mg/kg produced a 125% prolongation of the median day of death and 20 LTS from the group of 30 mice (Table 2, Experiment 2). In the experiments shown in Table 3, the value of the i.n. schedule every 2 hr for 5 injections on 2 days is again seen at a total dose of 40 mg/kg in contrast to the ineffectiveness of the same schedule given by the i.p. route. When the dose was halved to 20 mg/kg, effectiveness was present for both the i.n. and i.p. routes, indicating that at 40 mg/kg toxicity was present with the i.p. route. Other experiments with methyl-CCNU showed that 1- and 2-day schedules of injections every 2 hr for 5 injections were approximately equally effective when the drug was given i.n. and that the 1-day schedule of injections every 2 hr produced toxicity when the drug was administered i.p. to a total dosage of 30 mg/kg. There was less effectiveness when the drug was given once or twice daily i.n. for 4 days. The same was true when the drug was given once daily i.n. or i.p. every 4 days for 4 injections.

**DISCUSSION**

With respect to their effects on the nervous system, the nitrosoureas are an extremely interesting group of drugs.

![Chart 2. The survival of mice with i.c. tumors is plotted as the cumulative percentage of deaths against time after implantation. The 4 groups of 10 mice each received i.p. injections as outlined in Table 1 (Experiment 1, CCNU). There was no significant difference between treated and control groups, although the group receiving CCNU i.p. every 2 hr for 5 injections on 2 days (IP q2hx5x2) showed a slightly more rapid rate of death between 15 and 30 days after implantation.](chart2.png)

**Table 1**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Total dose (mg/kg)</th>
<th>Injection route</th>
<th>Frequency of injections</th>
<th>No. of mice</th>
<th>Median day of death</th>
<th>Treated control</th>
<th>Increase or decrease in life-span (%)</th>
<th>Significance</th>
<th>No. of survivors &gt;60 days</th>
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<tr>
<td>1 (CCNU)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
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<td>1</td>
<td>20</td>
<td>31</td>
<td>37/31</td>
<td>19</td>
<td>NS(^a)</td>
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<td>0</td>
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<tr>
<td>Control</td>
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<td>q2hx5x2</td>
<td>20</td>
<td>36</td>
<td>82/36</td>
<td>128</td>
<td>( p &lt; 0.01 ) Rx</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>i.n.</td>
<td>q2hx5x2</td>
<td>20</td>
<td>82</td>
<td>29/34</td>
<td>-15</td>
<td>NS(^a)</td>
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<td>0</td>
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<tr>
<td>Control</td>
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<td>10</td>
<td>29</td>
<td>29/34</td>
<td>-15</td>
<td>NS(^a)</td>
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<td>0</td>
</tr>
<tr>
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<td>i.p.</td>
<td>q2hx5x2</td>
<td>10</td>
<td>32</td>
<td>25/32</td>
<td>-22</td>
<td>NS(^a)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2 (CCNU)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>i.n.</td>
<td>ql/2hx5x1</td>
<td>20</td>
<td>29</td>
<td>34/29</td>
<td>17</td>
<td>NS(^a)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>60</td>
<td>i.n.</td>
<td>ql/2hx5x1</td>
<td>20</td>
<td>34</td>
<td>34/29</td>
<td>17</td>
<td>NS(^a)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
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<td>ql/2hx5x1</td>
<td>20</td>
<td>30</td>
<td>12/30</td>
<td>-60</td>
<td>( p &lt; 0.01 ) Tox</td>
<td>3</td>
<td>0</td>
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<tr>
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<tr>
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<td>20</td>
<td>10</td>
<td>10/30</td>
<td>-67</td>
<td>( p &lt; 0.01 ) Tox</td>
<td>7</td>
<td>0</td>
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\(^a\) Significance as compared with the appropriate control group in each series.

\(^a\) The abbreviations used are: NS, not significant; q2hx5x2, injections every 2 hr for 5 injections on each of 2 successive days after tumor implantation (5th and 6th days); Rx, therapeutic significance; q1/2hx5x1, injections every half hour for 5 injections on 1 day only, the 5th day after transplantation; Tox, toxic significance.
Two experiments were performed in which i.n. and i.p. routes of injection were used. All the animals in each experiment received the same tumor cell suspension. Treatment began on the 5th day after tumor cell implantation.

| Experiment | Total dose (mg/kg) | Injection route | Frequency of injections | No. of mice | Median day of death | Treated/control | Increase or decrease in life-span (%) | Significance* | No. of survivors >60 days |
|------------|-------------------|----------------|-------------------------|-------------|-------------------|----------------|---------------------------------|-------------|----------------|----------------|
| 1 (methyl-CCNU) |                   |                |                         |             |                   |                |                                 |             | 1              |
| 4          |                   | i.n.           | 1                       | 20          | 32                | 32/32          | 0                 | NS*          | 2              |
| Control    |                   | i.p.           | q2hx5x2                 | 20          | 70                | 70/33          | 112               | p < 0.01 Rx | 13             |
| Control    |                   | i.p.           | q2hx5x2                 | 10          | 32                | 23/32          | -28               | NS           | 0              |
| Control    |                   | i.p.           | q2hx5x2                 | 10          | 39                | 39/32          | 22                | NS           | 3              |
| 2 (methyl-CCNU) |                   |                |                         |             |                   |                |                                 |             | 0              |
| 40         |                   | i.p.           | 1                       | 30          | 32                | 72/32          | 125               | p < 0.01 Rx | 20             |

* Significance as compared with the appropriate control group in each series.

The abbreviations used are: NS, not significant; q2hx5x2, injections every 2 hr for 5 injections on each of 2 successive days after tumor implantation (5th and 6th days); Rx, therapeutic significance.

Table 3
Methyl-CCNU for chemotherapy of i.c. mouse ependymoblastoma after i.n. or i.p. injection

One experiment was performed in which the i.n. and i.p. routes of injection were used. All the animals received the same tumor cell suspension. Treatment began on the 5th day after tumor cell implantation.

| Experiment | Total dose (mg/kg) | Injection route | Frequency of injections | No. of mice | Median day of death | Treated/control | Increase or decrease in life-span (%) | Significance* | No. of survivors >60 days |
|------------|-------------------|----------------|-------------------------|-------------|-------------------|----------------|---------------------------------|-------------|----------------|----------------|
| 3 (methyl-CCNU) |                   |                |                         |             |                   |                |                                 |             | 4              |
| 40         |                   | i.n.           | q2hx5x2                 | 15          | 45                | >100/45        | >122              | p < 0.01 Rx | 12             |
| Control    |                   | i.p.           | q2hx5x2                 | 15          | 39                | >100/39        | >156              | p < 0.01 Rx | 9              |
| Control    |                   | i.p.           | q2hx5x1                 | 15          | 35                | 31/35          | -11               | NS           | 5              |
| Control    |                   | i.p.           | q2hx5x2                 | 15          | 37                | 66/37          | 78                | p < 0.01 Rx | 8              |

* Significance as compared with the appropriate control group in each series.

The abbreviations used are: q2hx5x2, injections every 2 hr for 5 injections on each of 2 successive days after tumor implantation (5th and 6th days); q2hx5x1, injections every 2 hr for 5 injections on 1 day only, the 5th day after tumor implantation; NS, not significant; Rx, therapeutic significance.

Some members of the group such as methylnitrosourea are selective neurotropic resorptive carcinogens capable of inducing a wide variety of tumors of the nervous system (9, 43), while others act as chemotherapeutic agents for brain tumors (1, 32, 35, 41, 46). As chemotherapeutic agents the nitrosoureas are cycle-phase nonspecific with selectivity for cell populations with a high growth fraction (3), and, although it was initially thought that they acted similarly to alkylating agents (16, 36, 45), recent evidence indicates that the tumor-inhibitory nitrosoureas show considerable differences in their mechanism of action as compared with alkylating agents (6). One of the main chemotherapeutic advantages of the nitrosoureas is that they are highly lipid-soluble compounds and therefore easily penetrate the blood-brain barrier (18, 21, 31). The original observation of Skipper et al. (37) that 1-methly-1-nitrosourea was almost equally effective against i.c. inoculated L1210 leukemia as against i.p. inoculated L1210 leukemia was the stimulus for the great interest in this group of compounds as chemotherapeutic agents for brain tumors.

Unfortunately, the clinical results achieved with the nitrosoureas in patients with brain tumors have generally been poor. Although temporary improvement has been observed in almost half the patients in 1 series (11), most studies have shown that the survival of patients with glioblastoma is only increased by a few months (24, 40, 47). A recent report of long-term survival in 3 patients with other types of gliomas who had received BCNU (4) is extremely difficult to evaluate because of the marked variability in the natural history of patients with these types of gliomas. Thus, there is a continuing need for further experimental study of this promising group of drugs. These poor clinical responses contrast markedly with the highly favorable chemotherapeutic responses seen in experimental brain tumor models after treatment with the nitrosoureas (1, 2, 5, 20, 29, 31-35), and thus an alternate route of injection was studied.

The mouse i.c. glioma model developed in our laboratory is a reliable, rapid, and inexpensive method for evaluating the chemotherapeutic effectiveness of these agents. It provides large numbers of animals with i.c. tumors with a median day of death of approximately 30 days and infrequent LTS (42). This model has previously been used to test
the therapeutic value and toxicity of radiotherapy and methotrexate (39). The model also provides the opportunity to evaluate the effectiveness of repeated i.n. injection of chemotherapeutic agents in brain tumor-bearing animals. In view of the limited value of systemically administered nitrosoureas in patients with brain tumors it was decided to study the local administration of these agents. Local injection of chemotherapeutic agents in patients with brain tumors has been shown to be a feasible route of administration (12, 13, 15, 17, 22, 23, 25, 30, 44), although major chemotherapeutic value has not been demonstrated. This route of injection has not been studied previously for the chemotherapy of experimental brain tumors with nitrosoureas.

In the present experiments, repeated i.n. injections of CCNU or methyl-CCNU were highly effective in prolonging the median day of death and in producing large numbers of LTS. Tables 1 and 2 show that both drugs were effective when given every 2 hr i.n. for 5 injections each day for 2 consecutive days. With methyl-CCNU effectiveness was also evident when the drug was given i.n. according to this schedule but for only 1 day (Table 3). CCNU was not effective, however, when given every half-hour for 5 injections on 1 day (Table 1).

Single i.n. injections were infrequently studied because high concentrations of drug were difficult to administer as a suspension through a 30-gauge needle with the injected amount limited to only 3 μl. Thus, with CCNU, the maximum dose given i.n. by a single injection was only 6 mg/kg (Table 1), and with methyl-CCNU only 4 mg/kg were given (Table 2). The better results with multiple i.n. injections as compared with single i.n. injection are probably due mainly to the higher total dose afforded by the former under the experimental conditions. Further work is in progress to determine whether there is any definite therapeutic advantage to multiple i.n. injection. This would require the use of either larger caliber needles or larger volumes of drug suspension. The possible increase in brain damage due to larger needles or volumes is being evaluated. If a nontoxic diluent were available, small-caliber needles and small injection volumes could continue to be used.

The i.n. route was frequently more effective than the i.p. route. This was usually due to the marked toxicity of repeated i.p. injections given according to the schedules used in these experiments (Table 1). However, when the total doses were reduced, repeated i.p. injections were better tolerated with little or no toxicity (Table 3). In fact, with methyl-CCNU (Table 3, Experiment 3), when the dose was reduced to 20 mg/kg given 5 times/day, a positive chemotherapeutic effect was noted. The reason for the reduced toxicity associated with the i.n. route is not apparent. It is possible that a significant fraction of the i.n. administered drug is metabolized in the tumor or surrounding brain to a less toxic or nontoxic form.

Thus, the present experiments show that CCNU and methyl-CCNU are highly effective against the mouse ependymoblastoma used in this glioma model. Marked increases in the median day of death and large numbers of LTS were found with both agents. These positive results contrast markedly with the chemotherapeutic ineffectiveness of methotrexate demonstrated previously in our laboratory with the same glioma model (39).

This is the 1st systematic study of the i.n. route of injection of CCNU and methyl-CCNU in experimental brain tumors, and the results appear highly encouraging. With both agents, some of the dosage schedules were more effective from the i.n. route than from the i.p. route. This was especially true for those schedules involving repeated i.n. injection every 2 hr. With this schedule i.p. injections were usually toxic.

These encouraging results have prompted us to continue our studies of the nitrosoureas for brain tumor chemotherapy. Other treatment schedules are being investigated, including ones comparable to those being used clinically where large doses are given with an interval of several weeks between doses (10, 24, 27). Before any consideration is given to direct i.n. injection of nitrosoureas in patients with brain tumors, the neurotoxicity of direct i.c. injections of these drugs would have to be studied in large animals.

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