Chemotherapeutic implications of growth fraction and cell cycle time in glioblastomas

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Four patients received $^3$H-thymidine 4 to 7 days and vinblastine 4 to 6 hours prior to operation for recurrent malignant gliomas (three glioblastomas and one anaplastic astrocytoma). Tumor biopsies obtained at operation were fixed for routine histological studies and radioautography. The tumors' growth fractions averaged 0.28 with a range of 0.14 to 0.39. The tumor cell cycle time calculated in three patients had a mean duration of 57 hours with a standard deviation of 6 hours. The authors concluded that: 1) single short-term courses of cell-cycle specific chemotherapeutic agents alone will probably fail to achieve either significant reduction in tumor mass or dramatic clinical improvement; 2) cell-cycle phase-specific drugs should be administered to maintain effective blood levels over 2 to 3 days for maximal tumor cell kill. Tumor growth rate appears to correlate with the fraction of proliferating cells rather than the length of the tumor cell cycle. The scientific basis for combination drug and multimodality therapy is discussed.

Keywords: brain tumor · chemotherapy · growth fraction · cell kinetics · cell cycle time · glioblastoma · brain tumor growth

Major questions remain concerning the generation time of human malignant brain tumor cells and their proliferation kinetics in vivo despite the successful use of $^3$H-thymidine in defining cell proliferation kinetics of non-neural tumors. Although a correlation may exist between the generation times of glioma cells in vitro and in vivo, the proliferation kinetics of glioma cells multiplying in vitro cannot be strictly analogous to the growth of tumors in vivo because cell culture provides a different environment than that found in vivo. Several investigators have determined the labeling index of human gliomas, but an understanding of proliferation kinetics requires more information.

Information concerning the cell cycle time and the duration of each phase in the mitotic cycle is obtained by constructing a percent-labeled mitosis curve. This technique is suitable for neoplasia that can be sampled repeatedly, since under certain conditions time-lapsed sequential biopsies fall within the
limits of ethical practice. Clearly, a method requiring repeated sampling cannot be applied to brain tumors.

The present study is designed to determine the growth fraction (ratio of cycling to total cells) and median cell cycle time of malignant gliomas. Information on the cell cycle time and growth fraction is essential to understand glioma growth in vivo, select optimum chemotherapeutic regimens, and interpret results of investigations on brain tumor models.

Materials and Methods

Patient Selection and Experimental Procedure

Each year a small number of patients with recurrent glioblastomas fulfill the criteria for a second operation. This group has the following general characteristics: a previous histological diagnosis of glioblastoma; a significant interval between primary operation and recurrence; neurological symptoms and/or deficits assumed secondary to increased intracranial pressure; and a reasonable hope of worthwhile palliation following a second operation.

This study carried no increased risk or inconvenience for the patient apart from the indicated operative procedure. Permission to administer 3H-thymidine was obtained from the appropriate committees (Committees on Radioisotopes, Radiation Safety, and Human Experimentation), and each patient and/or responsible relative gave informed consent.

A dose of 5 mCi of 3H-thymidine (specific activity 0.36 c/mM)* was administered intraarterially to seven patients during cerebral angiography, 4 to 7 days prior to operation. Vinblastine sulfate (Velban, 0.1 mg/kg) was administered 4 to 6 hours before surgery to increase the number of mitotic cells and to calculate tumor cell cycle time. After the tumor had been exposed, representative samples were excised and fixed with 10% formalin, embedded in paraffin, and cut into sections 4 μ thick. Deparaffinized slides were stained by the Feulgen method, dipped in Kodak NTB-3 emulsion, and exposed at 4°C in light-tight boxes containing Drierite for 3 to 9 months.† The slides were developed in Kodak D 19 for 4 minutes at 18°C and fixed in Kodak acid fixer. The labeling index (percentage of cells labeled with 3H-thymidine), mitotic index (percentage of cells in mitosis), and ratio of labeled to total mitoses were determined. Only viable parts of the tumor with a fairly even distribution of labeled cells were analyzed. Five to 10 areas from each tumor were selected for determining the indices, and 1000 to 3000 neoplastic cells from each of these areas were examined. Cells containing more than four grains per nucleus were scored as labeled, since the average background was less than one grain per nucleus.

Calculations

Estimation of Growth Fraction. The growth fraction (GF) is an index of the relationship between the proliferating cell population and the total cell population. The possible existence of nonproliferating cells in a tumor cell population was first suggested by Baserga, et al., and the mathematical definition and the introduction of this factor into population kinetics was proposed by Mendelsohn. Growth fraction is expressed by the following formula:

\[
GF = \frac{\text{Proliferating pool cells}}{\text{Total cell population}} \quad (1)
\]

However, this equation has little practical value, since the number of cells in the proliferating pool cannot be measured. There is no way to separate the nonproliferating pool cells (G0 cells) from those in the G1 phase (pre-DNA-synthetic phase) or G0 phase (post-DNA-synthetic phase) in an asynchronous population. Since

\[
\frac{\text{cells in S phase}}{\text{cells in S phase}} = 1
\]

(where the cells in S phase are those undergoing active DNA synthesis), Equation 1 can be rewritten in the following manner:

*3H-thymidine obtained from Schwarz Bioresearch, Inc., Orangeburg, New York.

†Drierite made by W. A. Hammond Company, Xenia, Ohio. Kodak photographic supplies made by Eastman Kodak, Rochester, New York.
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\[ GF = \frac{\text{cells in proliferating pool}}{\text{total cell population}} \times \frac{\text{cells in S phase}}{\text{cells in S phase}}, \text{ or } \]

\[ GF = \frac{\text{cells in proliferating pool}}{\text{cells in S phase}} \times \frac{\text{cells in S phase}}{\text{total cell population}} \]

In this form, the right term represents the labeling index (LI) obtained by a pulse of \(^3\)H-thymidine, and the left term represents the reciprocal of the theoretical labeling index (TLI; the ratio of the cells in S phase to cells in the proliferating pool). Thus, the growth fraction may be expressed as the ratio of the observed labeling index to the theoretical labeling index:

\[ GF = \frac{\text{LI}}{\text{TLI}}. \]

Cleaver derived a formula for the calculation of the TLI, but its use requires knowledge of the cell cycle time and the duration of the G\(_1\) phase (two factors which have not been estimated for glioblastomas). However, Mendelsohn discovered that the ratio of labeled to total mitoses several generations after a single pulse of \(^3\)H-thymidine approaches the theoretical labeling index. With this information, the growth fraction may be estimated when the labeling index is known.

**Estimation of Cell Cycle Time.** A modification of the stathmokinetic analysis (a study of cellular proliferation kinetics using agents that block cycling cells in a certain phase) as reported by Puck and Steffen was used to estimate the cell cycle time. They reported that, for systems with a growth fraction approximating 1.0, the following two formulas estimated the number of cells in mitosis (Mt) at time (t) after administration of mitostatic agents (Equation 4) and the cell cycle time (Tc) (Equation 5).

\[ Mt = No \times GF \times 2^{\frac{t + tm}{Tc}} - No \times 2^{\frac{tm}{Tc}} \]

and

\[ Tc = \frac{t \log 2}{\log [1 + MI(t)]}, \]

where "No" is the total number of cells at the time of drug administration (t = 0), "tm" is the duration of the mitotic phase of the cell cycle, and "MI(t)" is the mitotic index (calculated as the number of cells in mitosis divided by the total number of cells) at time "t."

Solid tumors are composed of cells in proliferating (Np) and nonproliferating pools (Nnp), therefore

\[ No = Nnp + Np; \]

and growth fraction (GF) may be expressed as

\[ GF = Np/No, \]

or

\[ Np = No \times GF. \]

Since only proliferating cells will contribute to the number of mitoses at time "t," we may substitute "Np" for "No" in Equation 4, or

\[ Mt = Np \times GF \times 2^{\frac{t + tm}{Tc}} - Np \times 2^{\frac{tm}{Tc}} \]

The only cells that can divide after the administration of mitostatic agents are those in mitosis at the time of treatment. Therefore, when \( t > tm \), the total cell population [Nt] may be expressed as:

\[ Nt = Np \times 2^{\frac{tm}{Tc}} + Nnp, \]

or

\[ Nt = No \times GF \times 2^{\frac{tm}{Tc}} + (No - No \times GF). \]

The mitotic index may be defined as:

\[ MI(t) = \frac{Mt}{Nt} = \frac{GF (2^{\frac{t}{Tc}} - 1)}{GF + (1 - GF)/(2^{\frac{tm}{Tc}})} \]

Since the duration of mitosis is usually only a small fraction of the total cell cycle time (\( tm \ll Tc \), \( 2^{\frac{tm}{Tc}} \) will approximate 1. Thus, Equation 10 may be rewritten as:

\[ \text{J. Neurosurg. / Volume 43 / August, 1975} \]
MI(t) = GF \left( 2^{\frac{t}{T_c}} - 1 \right)

\text{and}

T_c = \frac{t \log 2}{\log \left( 1 + \frac{\text{MI}(t)}{\text{GF}} \right)}.

Therefore, if the GF, t, and MI(t) are known, the cell cycle time may be calculated.

Results

Although seven patients with recurrent malignant gliomas were investigated, the slides from three patients could not be analyzed. In two of these cases the specimens consisted almost entirely of necrotic tissue and those from the third showed very poor labeling for an unknown reason. A summary of the clinical data for the four patients evaluated is in Table 1. Biopsy specimens of Cases 1, 2, and 3 were collected at surgery, 5 to 11.5 hours after the administration of vinblastine sulfate. In Case 4, specimens were obtained at necropsy after administration of \(^{3}H\)-thymidine alone. Table 2 shows the number of cells scored, labeling index, mitotic index, and the ratio of labeled to total mitoses for all four cases. Using Equations 3 and 11, GF and Tc were calculated in Cases 1, 2, and 3; only GF was obtained in Case 4 since stathmokinetic analysis was not performed. The approximate GF's for the three glioblastomas were 0.29, 0.39, and 0.32, and the anaplastic astrocytoma GF was 0.14. The four tumors had a GF mean value of 0.28 with a standard deviation of 0.10. The cell cycle times calculated in three cases were 52.3, 64.1, and 55.4 hours, for a mean value of 57.2 with a standard deviation of 6.1 hours.

Discussion

Growth Fraction and Cell Cycle Time

There are several ways to determine growth fraction and cell cycle time. However, ethical considerations imposed by radiation hazards limit investigations performed on humans. The method used in this study appears the only way at present to determine these important cell kinetic parameters in patients harboring glioblastomas. Certain assumptions have been made in developing and evaluating this technique:

1. Over the 4- to 7-day period (from \(^{3}H\)-thymidine administration until sampling) the cells initially labeled in S phase evenly distribute throughout the cell cycle.
2. Cell loss during this period is negligible in viable tumor areas.
3. If transition of cells occurs between proliferating and nonproliferating pools, it is balanced.
4. The mitostatic drug (vinblastine sulfate) effectively penetrates into the tumor tissue and arrests cells in mitosis.

Most solid tumors are composed of a heterogeneous cell population possessing a range of cell cycle parameters. The dampening usually observed on labeled mitosis curves after one cell cycle time is attributable to this phenomenon. We have assumed that a similar dampening has occurred 4 to 7 days after a pulse of \(^{3}H\)-thymidine, and labeled cells, all initially in the S phase, have redistributed throughout the cell cycle. The finding of a 2- to 3-day cell cycle time supports this assumption.

Cell loss and transition of cells between proliferating and nonproliferating pools cannot be accurately measured. However, neither factor should greatly influence the distribution of labeled cells since we are analyzing only viable tumor areas in a non-perturbed tumor and the study is performed in a relatively short period of time.

Mitostatic agents such as colchicine, vinblastine sulfate, and vincristine have been used for stathmokinetic analysis in tissue culture. More recently, investigators have used mitostatic agents for cell cycle analysis of \textit{in vivo} systems. This study has applied similar methods for the study of human glioblastomas using vinblastine sulfate, a lipid-soluble agent that has been shown to cross the blood-brain barrier. However, our results must be interpreted with caution, since it is not known if the mitostatic agent itself will influence the tumor in an unexpected manner and affect the measured parameters.

Methodological factors that modify the results should be kept in mind when interpreting these data. A mean growth fraction of approximately 30%, though in reasonable agreement with growth fractions reported for other solid tumors, is probably an...
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TABLE 1
Clinical information on patients studied

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Sex</th>
<th>Age</th>
<th>Tumor Histology</th>
<th>Approximate Tumor Diameters (cms)</th>
<th>Time from First Operation (mos)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>65</td>
<td>glioblastoma</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>53</td>
<td>anaplastic</td>
<td>6</td>
<td>108</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>44</td>
<td>astrocytoma</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>39</td>
<td>glioblastoma</td>
<td>5</td>
<td>28</td>
</tr>
</tbody>
</table>

* Equivalent to secondary glioblastoma as defined by Scherer (26).

TABLE 2
Measured and calculated kinetic parameters of tumor cells studied *

<table>
<thead>
<tr>
<th>Case No.</th>
<th>No. of Cells Scored</th>
<th>Labeling Index (% ± SD)</th>
<th>Mitotic Index Fraction (% ± SD)</th>
<th>Labeled Mitoses (% ± SD)</th>
<th>Growth Fractions</th>
<th>Cell Cycle Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Labeling</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Scored (%) ± SD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(7o ± SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>16,697</td>
<td>9.8 (2.6)</td>
<td>0.043 (0.010)</td>
<td>32 (9)</td>
<td>0.32 (0.10)</td>
<td>55.4 (11.5)</td>
</tr>
<tr>
<td>2</td>
<td>5180</td>
<td>3.4 (1.0)</td>
<td>0.022 (0.010)</td>
<td>26 (9)</td>
<td>0.14 (0.06)</td>
<td>52.3 (26.5)</td>
</tr>
<tr>
<td>3</td>
<td>24,637</td>
<td>8.6 (3.1)</td>
<td>0.050 (0.009)</td>
<td>23 (7)</td>
<td>0.39 (0.10)</td>
<td>64.1 (12.9)</td>
</tr>
<tr>
<td>4</td>
<td>12,895</td>
<td>13.0 (3.2)</td>
<td>0.022 (0.006)</td>
<td>46 (7)</td>
<td>0.29 (0.07)</td>
<td>41.8–87.2</td>
</tr>
</tbody>
</table>

* Range of growth fraction and cell cycle times obtained from each group of 1000 cells from multiple areas within each tumor. SD = standard deviation.

overestimation since only microscopically “viable” areas were analyzed. Any evaluation of a growth fraction for the entire tumor should, by definition, also include necrotic areas. The inclusion of cells in these “nonviable” regions, occasionally containing 50% or more of the total number of tumor cells, would reduce the calculated growth fraction. Though exact determination of total tumor growth fraction is not methodologically possible, an estimate may be obtained by multiplying the calculated growth fraction by the ratio of viable to total tumor mass, assuming equal cell density in viable and nonviable regions. If cell density in these areas are different, the ratio can be estimated using the viable tumor volume (Vv), nonviable tumor volume (Vnv), and relative cell densities (DV and Dnv) according to the following formula:

\[
\text{viable tumor ratio} = \frac{(Vv \times DV)}{(Vv \times Dv)} + (Vnv \times Dnv)
\]  

For example, if 50% of all tumor cells were located in “nonviable” areas, the total tumor growth fraction would be 0.15 instead of the calculated 0.30.

Application of Model in Planning Treatment

The selection of anti-cancer drugs for use in patients with glioblastomas should utilize information on the agent’s mechanism of action in relation to tumor cell kinetics as well as its ability to cross the blood-brain barrier. Many chemotherapeutic agents predominantly affect actively proliferating cells and are classified as cell-cycle or cell-cycle phasespecific drugs. Since the measured growth fraction of glioblastomas is 0.3 and the total tumor growth fraction is often significantly lower, such agents can be expected to kill at the most 30% of tumor cells. If all these cells were immediately removed, the tumor diameter would only reduce by about 10%. However, intracerebral dead cell removal has been shown to be significantly retarded, and
tumor volume may increase due to the swelling of dead cells and proliferation of tumor cells transferred from the nonproliferating pool subsequent to treatment. These phenomena may obscure any decrease in tumor mass anticipated from short-term treatment with cell-cycle-specific drugs, and clinical improvement resulting from a decrease in tumor volume would not be expected.

Even though considerable variability exists in the cell cycle times calculated from different samples of each tumor, the average values for all three tumors were similar and when combined gave a mean of 57.3 hours with a standard deviation of 6.1 hours. This observation agrees with previously measured values for the duration of the S phase, found to be very similar (7 to 10 hours) in malignant as well as benign gliomas. In contrast to this agreement in cell cycle times, the growth fraction in Case 2 is markedly lower than for the other tumors. This same patient had a brain tumor diagnosed 9 years prior to this study. This pretreatment interval is much longer than in the other cases and demonstrates a good relationship between the lower growth fraction and the slower growing neoplasm. From these data it appears that the low labeling indices observed in slow-growing brain tumors (in comparison to rapidly growing glioblastomas) may occur as a result of a low growth fraction and not merely as a reflection of a prolonged cell cycle time.

The cell cycle time of 2 to 3 days, though considerably shorter than that reported by others, agrees with our previous observations. The tumor turnover time, defined as the time required for 100 tumor cells to produce 100 new cells, was calculated in the range of 4 to 7 days in a study of glioblastomas using double radioautography. In solid tumors with a growth fraction significantly less than 1.0, the cell cycle time should be less than the tumor turnover time. In the present study this was, in fact, observed.

Knowing the cell cycle time is essential in planning effective treatment with cell-cycle phase-specific chemotherapeutic agents. Drugs such as Vinca alkaloids, purine, and pyrimidine analogs will predominantly affect only those cells in a specific, sensitive phase of the cell cycle. Single doses of these agents may only kill cells in the sensitive cycle phase at the time of drug administration. However, if effective drug levels are maintained for a period (Tc) long enough for all cells in cycle to pass through the sensitive phase, the agent will affect all cycling cells and have its maximal effect. Furthermore, "continuous" drug administration (either by frequent injections or by continuous infusion) for periods significantly longer than the tumor cell cycle time may increase drug toxicity by affecting normal regenerating systems such as the bone marrow and intestinal epithelium, without increasing tumor cell kill.

General Considerations for Treatment of Glioblastomas

A symptomatic glioblastoma with a size of 50 to 100 gm contains approximately 0.5 to 1.0 × 10¹¹ cells. As already stated, a cell kill of 30% (the most that could be expected with a single course of a cell-cycle-specific agent in a tumor with a growth fraction of 0.3) would reduce a tumor diameter at the most by about 10%, with at least 1 × 10¹⁰ tumor cells remaining. It is postulated that cells in the nonproliferating pool move into the proliferating pool after selective reduction of the proliferating compartment. The amount of time required for this cell transition to repopulate the proliferating pool is unknown. Subsequent retreatment with cell-cycle-specific agents would result in a further tumor cell kill that, if equally as effective as the first course, would only reduce the number of tumor cells to 0.7 × 10¹⁰. However, because of the retarded dead cell removal, dead cell swelling, and proliferation of cells entering the cell cycle, much of the anticipated tumor mass reduction will not be observed. In addition, the information necessary for the exact timing of retreatment with cell-cycle-specific agents to effect a continual stepwise decrease in tumor cell population is not presently available.

Obviously, treatment should include some modality that can kill nonproliferating as well as proliferating cells. The percent cell kill would not then be limited by the growth fraction, since 100% of cells would be at risk, and a tumor cell kill of several logs may be achieved from a single course of therapy. (Tumor cell kill may be expressed in "log kill" where a log kill represents a tenfold reduction in the number of cells, that is, one log kill = 90% cell kill, two logs = 99%, and
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three logs = 99.9%). Chemotherapeutic agents, such as the nitrosoureas (BCNU, CCNU, and MeCCNU), cyclophosphamide, and the nitrogen mustards, as well as radiation therapy, fall into the category of cell-cycle-nonspecific (cytoidal) treatments. Nitrosoureas and radiation therapy have shown some effectiveness in the treatment of glioblastomas. Rarely has a cure been reported, and the median increase in lifespan has usually been less than 1 year. Either such trials have utilized suboptimal treatment doses and/or schedules, or it has not been possible to effect a cure for glioblastomas with a cell-cycle-nonspecific agent alone. Until further information is available on the post-treatment kinetics of tumors and normal tissues, the development of resistant tumor cell subpopulations, and cumulative drug toxicity, we favor the former explanation.

Another group of cell-cycle-nonspecific drugs, categorized as deoxyribonucleic acid (DNA)-binders by Schabel (adriamycin, daunorubicin, streptozotocin, bleomycin, mitomycin C, and so forth) may have potential for the treatment of glioblastomas. These agents may affect the DNA of non-proliferating cells in a manner that will not perturb ongoing metabolic processes, but will have cytoidal effects when the cell enters the proliferating pool and divides. The stimulus for cell transition between pools may be previous treatment with a different chemotherapeutic agent or surgery with subtotal tumor resection. Although the DNA-binder can affect non-neoplastic cells, these cells may be unharmed if division does not occur, as is the case with hepatic and peripheral blood cells.

Combination chemotherapy has greatly improved the life expectancy of patients with leukemia. Rational drug combinations, scheduled to take advantage of the information presently available, should be attempted for glioblastomas. Cell-cycle-nonspecific agents may be given first to decrease the total cell population. This could be followed with a cell-cycle-specific drug when the growth fraction of the remaining tumor increases secondarily to a decrease in tumor bulk. A DNA-binder may be given at the same time as the cell-cycle-specific agent to kill cells that would subsequently enter the proliferating from the nonproliferating pool. Tumor cell repopulation may then be retarded until normal tissues recover and subsequent retreatment with cell-cycle-nonspecific agents is possible. In addition, radiation therapy and surgery offer important selective methods of nonspecific tumor cell kill. These selective modes may be substituted for nonspecific chemotherapeutic agents, or used in conjunction with drug schedules limiting tumor cell repopulation or improving tumor cell kill while normal tissues recover from previous chemotherapeutic insults.

To obtain a sequential decrease in tumor size, repeated courses of therapy must be administered before the tumor cells repopulate. The time for tumor regrowth may be calculated from knowledge of the cell cycle time, growth fraction, cell loss, and tumor cell kill. (Cell loss represents the removal of cells from the tumor mass by various means during tumor growth, including dead cell removal from viable and necrotic areas and exfoliation of cells into the cerebrospinal fluid pathways.) An approximation of the minimal repopulation time for human glioblastomas may be computed if one uses a cell cycle time of 3 days, a growth fraction of 0.3, and no cell loss (since the actual rate of cell loss has not been determined). If a course of chemotherapy results in a 2 or 3 log cell kill, it will take a minimum of 53 or 78 days, respectively, for tumor cell repopulation. Therefore, to obtain a stepwise reduction in tumor mass a second course of therapy should be administered before that time. Furthermore, since the growth fraction increases after effective treatment (secondarily to a decrease in tumor size), the tumor repopulates more rapidly and retreatment should be given at shorter intervals. By applying the maximal growth fraction of 1.0, the minimum time for tumor regrowth is 20 or 30 days, respectively, and retreatment within this 3- or 4-week interval may eventually result in cure. If cell loss in the course of tumor regrowth is considered, the actual times might be significantly longer. In any case, the feasibility of repeated courses depends on the recovery of normal cell populations during the retreatment interval, a period during which selective treatment modalities may play an important role. Further advances in brain tumor chemotherapy depend on obtaining more information about the pretreatment and perturbed kinetics of tumors and normal tissues.
References

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