Quantitative Determination of Disseminated Tumor Cells by \[^{3}H\]Thymidine Incorporation in Vitro and by Agar Colony Formation

Volker Schirrmacher and Catherine A. Waller

Institut für Immunologie und Genetik, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-6900 Heidelberg, Federal Republic of Germany

ABSTRACT

A new microradioassay for the detection and quantification of disseminated tumor cells in blood and organ samples of tumor-bearing animals has been worked out in a murine model system for tumor metastasis. In contrast to previous prelabeling or in situ labeling procedures, the basis of this assay is a postlabeling in vitro of tumor cell-containing material with \[^{3}H\]thymidine. Percoll gradient fractionation and autoradiography revealed that most of the isotope was incorporated into tumor cells. Titration curves with tumor cells from tissue culture were run in parallel and allowed to calculate from the radioactivity of a sample the actual number of proliferating tumor cells. The postlabeling assay correlated fairly well with an agar colony test which measures the clonogenic or stem cell potential of a tumor cell population. About 70% of syngeneic animals which had been inoculated s.c. with ESb tumor cells showed increased \[^{3}H\]thymidine uptake in their blood, particularly at certain time intervals (11 and 21 days). None of these animals lived for more than 2 days longer. The advantages of the new microradioassay and its possible prognostic significance will be discussed.

INTRODUCTION

The diagnosis of the degree of spread of a malignant disease presents a major problem to the clinician, and yet it is of crucial importance for therapy decisions. Many parameters of the multistep process of cancer dissemination and metastasis formation are still poorly understood; therefore, it is difficult to predict the outcome of a particular cancer therapy, especially with regard to its effect on the metastatic process. One reason for these difficulties is that there are hardly any reliable and sensitive methods available to identify and quantify disseminated tumor cells, especially those with metastatic potential, in the circulation or in internal organs. Such tests are much needed to detect disseminated tumor cells before they have established themselves as large secondary colonies.

In the present study, we introduce a simple postlabeling radioassay for the quantification of metastatic tumor cells in various tissues. Tumor cells are distinguished from normal cells on the basis of their higher proliferative activity and measured by their uptake of \[^{3}H\]thymidine.\(^{1}\) Our studies were performed in a murine tumor model system for metastasis (15–18, 20). The system consists of a methylcholanthrene-induced lymphoma (Eb) of the DBA/2 mouse and a spontaneous variant thereof (ESb) with increased metastatic potential. A third metastatic DBA/2 tumor, MDAY-D2, of independent origin (8) is included for comparison. At different times after s.c. tumor cell transplantation, blood samples were removed and labeled for 18 hr in vitro with \[^{3}H\]thymidine. Tumor cells in liver tissues were also tested, but these could only be measured after killing the animal and removing the organ. It was found that the assay can detect less than 0.1% of tumor cells within blood or liver cell suspensions. The postlabeling assay has allowed the study within individual mice of the kinetics of the spread of tumor cells from a local site to the blood and liver. A preliminary report on these results has been published (19).

In this report, qualitative and quantitative aspects of the postlabeling radioassay will be shown. The assay will also be compared with another quantitative test, the soft agar colony test which measures the clonogenic potential of the tumor cells.

MATERIALS AND METHODS

Tumor Cell Lines. The origin and characteristics of the chemically induced DBA/2 lymphoma Eb and its spontaneous metastatic variant ESb and of the MDAY-D2 tumor line have been described (6, 15–18, 20). Cloning was performed by limiting dilution in suspension culture in microtiter plates. The culture medium consisted of Roswell Park Memorial Institute Tissue Culture Medium 1640 plus 10% fetal calf serum, 7 mM glutamine, penicillin, and 5 x 10\(^{-5}\) M 2-mercaptoethanol.

Animals. DBA/2J mice were obtained from the Zentralinstitut für Versuchstierforschung, Hannover, West Germany. The animals were used at 2 to 6 months of age.

Preparation of Blood and Liver Samples. Tumor-bearing or control animals were bled from the eye (about 0.1 ml), and 10 units heparin per ml were added to prevent clotting. The blood samples were diluted 1:10 with culture medium (see above). Liver samples were prepared by removing the organ in a sterile fashion and mincing it finely with crossed scalpel blades. The minced tissue was then rinsed through a sterile 130-μm mesh using a syringe plunger and washed 3 times in Hank's balanced salt solution, and the number of live cells was determined by trypan blue exclusion.

\[^{3}H\]Thymidine Uptake in Vitro. Two hundred-μl test samples were incubated in round-bottom microtiter plates (Linbro), usually 6 replicates per group. They contained different numbers of tumor cells (see experiment in Chart 2). Blood diluted 1:10, or 10^5 viable cells isolated from liver tissue. After addition of 1 μCi [6-\[^{3}H\]thymidine (23 Ci/mmol; Amersham, England) (in 20 μl) per well, the plates were incubated for 18 hr at 37° in a 5% CO\(_2\) atmosphere. The samples were harvested with an automatic cell harvester (Skatron; Flow Laboratories, Inc.). The radioactivity content was determined by liquid scintillation counting using a Mark III beta counter (Searle, Heusenstadt, West Germany).

Soft Agar Colony Plating. Feeder layers were prepared by pipeting 1 ml of 0.4% Bacto-agar (Difco Laboratories, Detroit, Mich.) in culture medium and 20 μl of packed DBA/2 erythrocytes washed 2 times into 35-mm Falcon Petri dishes. These layers were allowed to set at room temperature, and then, a further 1 ml of 0.33% Difco agar in culture medium containing several dilutions of tumor cells (10^6 to 10^2) or several dilutions of organ cell suspensions prepared from tumor-bear-
ing animals was transferred by pipet on top. The Petri dishes were then cultured at 37° for 10 days in 10% CO₂ in air and stained according to the method of Salmon and Lin (13), and the number of tumor colonies was scored. The plating efficiency of the tumor cells was calculated for each experiment, and the number of tumor cells per organ was calculated from the formula:

\[
\text{No. of colonies} = \frac{\text{No. of cells plated} \times \text{plating efficiency}}{\text{total cell count for organ}}
\]

RESULTS

In Vivo Bioassay for the Detection of Disseminated Tumor Cells. An easy and reliable test for the presence of tumor cells in tissue samples is the in vivo bioassay, where the cellular material from the test tissue is transplanted into a normal syngeneic recipient. The results of such a test are illustrated in Chart 1. Samples from peripheral blood and from livers of DBA/2 mice which carried an s.c. transplant of the metastasizing tumors ESb or MDAY-D2 were removed, and cell suspensions were prepared. Viable WBC (10⁵) which were separated from RBC and tissue debris by the Percoll technique (see below) were inoculated s.c. into groups of normal DBA/2 mice.

Local tumor growth could be observed after 1 week (Chart 1, top), and the animals died after 2 to 3 weeks (Chart 1, bottom) with visible signs of liver and lung metastasis. These results demonstrate that disseminated tumor cells can be isolated from the blood of tumor-bearing animals. In this system, the tumor cells have retained their tumorigenic and metastatic potential.

Growth and [³H]dThd Uptake of Tumor Lines in Vitro. Since the in vivo bioassay has its limitations with regard to applicability for human tumor material and with regard to quantification, we concentrated on in vitro assays to determine the content of tumor cells, especially in tissues such as the blood and the liver. We first established a tissue culture medium (Roswell Park Memorial Institute Tissue Culture Medium 1640 plus 10% fetal calf serum plus 5 x 10⁻⁵ M 2-mercaptoethanol) sufficient to support the growth of the 3 tumor lines of our model system. The doubling times of the 3 cell lines in this standard medium during exponential growth were similar and varied between 12 and 15 hr.

Having established optimal culture conditions for tumor cell growth in vitro, we studied the uptake of [³H]dThd in relation to the tumor cell number seeded into wells of round-bottomed microtiter plates (Chart 2). In a dose range from 300 to about 30,000 cells per well, there was a linear increase of dThd uptake with the input cell number (see also Ref. 19). To see whether this assay could be used to quantify tumor cells from tissue samples, we investigated the [³H]dThd uptake of the tumor cells in the presence of normal blood (diluted 1:10) or of normal liver cells (10⁵/well). The titration curves were found to be shifted towards lower uptake, but areas of a linear relationship between tumor cell number and [³H]dThd uptake were still observed (see Chart 2).

Tumor Cells in Blood and Liver Detected by [³H]dThd Uptake in Vitro. The postlabeling [³H]dThd assay was therefore used to measure the content of tumor cells in tissues from animals bearing metastatic tumors. Table 1 shows the mean uptake of radioactive dThd in blood and liver samples from normal and tumor-bearing animals. The metastatic tissue samples were obtained from sick animals bearing a ESb tumor growing s.c. These animals usually died 1 to 2 days later.

The mean uptake from blood of normal animals was about 400 cpm (84 animals; 6 determinations per sample), while that of the tumor-bearing animals was about 10 times higher. The corresponding data for liver tissue were about 1,200 cpm for the normal animals and about 92,000 cpm for the tumor-bearing animals (increase by a factor of 80). Although these differences were highly significant, there was a great variation between...
individual tumor-bearing animals. We therefore graded the mice according to their uptake into 3 groups as shown in Table 1. About one-third of the animals had blood and liver values which were not significantly increased above normal. Two-thirds of the animals had Grade II blood values (10^3 to 10^4 cpm), and about 50% had highly increased liver values of Grade III (>10^5 cpm).

### Table 1

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>No. of animals</th>
<th>cpm</th>
<th>Frequency of distribution in grades</th>
<th>Factor of increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>Normal Metastatic</td>
<td>84</td>
<td>409 ± 336^b</td>
<td>Grade I. &lt;10^3 cpm; Grade II, 10^3 to 10^4 cpm; Grade III, &gt;10^5 cpm.</td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>36</td>
<td>4,017 ± 7,936</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Grade I</td>
<td>12</td>
<td>556 ± 239</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Grade II</td>
<td>21</td>
<td>2,919 ± 1,603</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>Grade III</td>
<td>3</td>
<td>26,943 ± 11,903</td>
<td>70</td>
</tr>
<tr>
<td>Liver</td>
<td>Normal Metastatic</td>
<td>6</td>
<td>1,158 ± 718</td>
<td>Grade I, 10^3 to 10^4 cpm; Grade II, 10^4 to 10^5 cpm; Grade III, &gt;10^5 cpm.</td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>19</td>
<td>92,832 ± 98,492</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Grade I</td>
<td>6</td>
<td>3,200 ± 2,293</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Grade II</td>
<td>4</td>
<td>29,625 ± 22,410</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Grade III</td>
<td>9</td>
<td>162,000 ± 69,882</td>
<td>47</td>
</tr>
</tbody>
</table>

^a Obtained from DBA/2 and DBA/2 x B10.D2 F, animals; the metastatic tissues were from animals which had been inoculated on Day 0 with 10^5 ES^b tumor cells s.c.; the samples were taken shortly before death of the animals (Days 10 to 20).

Density Distribution of [3H]dThd-labeled Cells on Percoll Gradients. The selectivity of the postlabeling assay for tumor cells was investigated by testing the tissue distribution of [3H]dThd uptake by 2 methods: (a) autoradiography and (b) radioactivity distribution profile on a Percoll density gradient. Autoradiography revealed that most of the grains were associated with tumor cells. These could be distinguished by their size from the much larger hepatocytes and from the smaller lymphocytes in the blood. Chart 3 illustrates the distribution of counts of [3H]dThd-labeled blood samples after separation on a 70 to 20% Percoll density gradient. In a previous study, we showed that ES^b tumor cells from tissue culture or from metastases had a buoyant density in Percoll of 1.060 ± 0.010 g/ml (1). The major radioactivity peak shown in Chart 3 coincided with this density and thus indicated the presence of the tumor cells. The second much smaller peak (density, 1.078 g/ml) showed the [3H]dThd uptake by normal lymphoid cells from tumor-bearing blood (1). Blood samples from normal control animals did not show any significant peak of radioactivity in the gradient. Similar results were obtained with cells isolated from the liver of tumor-bearing animals. The radioactivity peaks were usually broader than those from blood samples. This was possibly due to the higher number of tumor cells in the liver (see Chart 5).

### Comparison of the [3H]dThd Test with the Agar Colony

Chart 3. Blood samples from normal DBA/2 mice or from mice which had been inoculated 12 days previously with 10^5 ES^b or MDAY-D2 tumor cells were labeled with [3H]dThd overnight. Ten samples of 200 μl each were pooled, washed, and subjected to isopyknic gradient centrifugation in Percoll as described (1). The gradients were fractionated, and the radioactivity in each fraction was determined as in the [3H]dThd uptake assay. The buoyant density of representative fractions was determined by the inclusion of colored marker beads (Pharmacia, Uppsala, Sweden). NL, normal lymphoid; Ery, erythrocytes.
Test for Tumor Stem Cells. Soft agar cloning has proven a useful method in the study of the kinetics and biological properties of tumor stem cells (6, 7). We have tested the usefulness of this procedure for the quantification of disseminated tumor cells from tissue samples. We investigated first the plating efficiency of Eb or ESb tumor cells from tissue culture. Chart 4 Fig. 1 illustrate the results of titration experiments in soft agar. Both tumors gave a linear relationship between the numbers of colonies growing per plate and the number of cells seeded. The plating efficiency in the experiment of Chart 4 was about 0.30 for both tumor lines, while in the experiment of Fig. 1, it was as great as 1.0 (i.e., virtually 100%). The presence of cells from normal tissues such as liver or spleen in the agar did not significantly alter the plating efficiency of the tumor cells (Chart 4).

The agar colonies differed considerably in their morphology. Not only was there a heterogeneity in size but also in the intensity of spreading into the surrounding agar. The example of a titration experiment (Fig. 1) illustrates that colony size changed inversely with the number of cells plated. Both assays, the \([^{3}H]dTd\) postlabeling and the agar colony test, fulfilled the prerequisites of a quantitative test, namely the linear relationship of the test parameter with the tumor cell number. While one test measures short-term proliferation in suspension culture, the other measures the ability to form colonies in soft agar, a long-term test which is considered to measure the clonogenic portion of a tumor cell population (7).

Tissues from animals bearing the metastatic tumor ESb transplanted s.c. were removed at different times and investigated for their content of tumor cells by both of the above methods. The number of tumor cells determined by the postlabeling assay or the colony test was calculated by comparison with titration curves, such as those in Charts 2 and 4, which were run in parallel in each experiment. Chart 5 illustrates the correlation between the 2 assays when plotted on a double logarithmic scale. The results of both tests were not independent and correlated with a coefficient of \(t = 0.62\). The correlation between the 2 assays (Chart 5) and the high plating efficiency of the ESb tumor cells (Charts 2 and 4) suggest that virtually every cell of this highly malignant tumor which proliferates under the conditions of the postlabeling assay is also clonogenic in the agar colony test.

Analysis of the Prognostic Significance of the \([^{3}H]dTd\) Assay. Table 2 shows the results of an experiment in which we tried to follow the spread of EB tumor cells from an s.c. site to the blood in individual syngeneic animals. Twenty-five animals were inoculated on Day 0 with \(10^5\) ESb tumor cells. The mice were individually labeled and bled from the eye.
ml) on the days indicated in the table. The $[^3H]dThd$ uptake of the samples diluted 1:10 revealed the following: until Day 7, the values were quite similar and varied between 300 and 900 cpm. Between Days 9 and 11, there was an increased uptake in blood samples from many but not all of the animals. All of the animals with significantly increased blood values died shortly afterwards. On Day 21, 4 of 6 of the surviving animals showed increased blood values, followed shortly afterwards by death, while the remaining 2 survived the observation period. From the 25 animals studied, 17 (68%) showed increased blood values shortly before they died. From the titration curve in Chart 2, it can be calculated that the amount of tumor cells in these animals varied between $10^3$ and $10^5$/ml blood.

**DISCUSSION**

Separation of malignant cells from a primary cancer is an essential part of the metastatic process. Since metastases often arise in organs far distant from the primary lesion, the active and passive movements of cancer cells via lymphatics and blood vessels are of obvious importance. Both clinical and experimental experience indicates, however, that the presence of circulating tumor cells correlates poorly with blood-borne metastasis formation: more tumor cells enter the blood stream than are able to form metastases (4, 12). A decision about the biological significance and risk potential of tumor cells in the blood of cancer patients (14) seems to be hampered by the scarcity of reliable tests for the identification and quantification of the malignant cells in the circulation.

A variety of techniques has been developed to confirm the presence of tumor cells in the blood. The value of cytological methods in the detection of circulating tumor cells appears to be relatively limited, since many reports in the 1950’s and 1960’s on ‘malignant cells’ in the blood of tumor-bearing patients were erroneously describing abnormal but noncancerous cells, such as megakaryocytes and other immature bone marrow-derived cells (21). Electron microscopic studies can occasionally help to identify tumor cells if these have distinct morphological characteristics. Even then, however, biological assays would be needed to determine whether the tumor cells detected possessed the malignant potential necessary for the successful development into metastases.

Another serious problem in the ‘staging’ of a cancer is the determination of the extent of formation of micrometastases. In routine clinical practice where a pathologist examines selected areas of tissue first by eye and then selects small regions of these areas for histology, micrometastases consisting of a few cells may well be overlooked. Better methods are obviously needed to identify and quantify small numbers of disseminated tumor cells in the circulation and in internal organs. Such methods have to be developed first in animal model systems, even if such models, as is usually the case, do not exactly mimic the disease as it occurs and develops in a patient.

In experimental systems, metastases have been quantified by counting tumor nodules, for instance in the lung or liver (10, 22), or by the analysis of the organ distribution of $^{51}$Cr- or $^{125}$I-iododeoxyuridine-labeled tumor cells after i.v. inoculation (3, 5). Disseminated tumor cells in internal organs or body fluids have also been detected by a bioassay in vivo (2), by their outgrowth in tissue culture (9, 11), or by their ability to form colonies in soft agar (6, 7).
Quantitative Analysis of the Spread of Metastatic Tumor Cells

We describe here a new postlabeling radioassay for the detection of disseminated tumor cells in body fluids or internal organs. Tumor cells are distinguished from normal cells by the presence of disseminated tumor cells in body fluids or tissues. As an adjunct to histology, the assay may become useful to the clinician. The advantage of the postlabeling assay is perhaps its higher sensitivity and accuracy. Its disadvantage, however, is that it is time consuming and easily influenced by the quality of the reagents used (water, agar, etc.). The postlabeling assay seems less susceptible to changes in the reagents and has thus far been found to be highly reproducible.

A third demonstration of the potential of the postlabeling assay comes from a comparison with the agar colony test. The latter test has been considered to be particularly relevant because it measures the potential of a cell to give rise to a whole colony of progeny (i.e., the clonogenicity) possibly comparable to a metastatic focus. Both assays were used to determine the content of tumor cells in a number of samples from blood and liver tissue of ESb tumor-bearing animals. Titration curves with tumor cells from tissue culture were run in parallel, and the possible influence of the microenvironment of the blood or the liver on the in vitro tumor cell proliferation and growth was evaluated (see Charts 2 and 4). Both assays were found to correlate (Chart 5).
V. Schirrmacher and C. A. Waller

oncologist in providing him with information about the cell-proliferating potential within a given test specimen.

Note Added in Proof


REFERENCES


Quantitative Determination of Disseminated Tumor Cells by $[^3]H$Thymidine Incorporation *in Vitro* and by Agar Colony Formation

Volker Schirrmacher and Catherine A. Waller


**Updated version**

Access the most recent version of this article at: [http://cancerres.aacrjournals.org/content/42/2/660](http://cancerres.aacrjournals.org/content/42/2/660)

**E-mail alerts**

Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**

To request permission to re-use all or part of this article, use this link [http://cancerres.aacrjournals.org/content/42/2/660](http://cancerres.aacrjournals.org/content/42/2/660). Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.