The Influence of Carbon Dioxide on the Cultivation of Human Neoplastic Explants in Vitro*

SHELDON ROVIN†

(Department of Oral Pathology, University of Michigan, School of Dentistry, Ann Arbor, Michigan)

SUMMARY

The watch glass technic of organ culture was successfully employed for the cultivation of human neoplastic tissues. A variety of neoplasms were grown under different gaseous atmospheres and with both biological (natural) and synthetic (chemically defined) media.

Some of the neoplasms survived for at least 19 days in vitro, and many exhibited cellular proliferation and mitosis. A 5 per cent level of CO₂ in the atmosphere was necessary for survival. Also, many of the neoplasms were well maintained in an oxygen level of approximately 1 per cent. The interpretations of these findings are discussed.

The purpose of this investigation was to employ organ culture methods for the investigation of human neoplastic tissue. These methods differ from other tissue culture procedures in that they concern themselves with the maintenance and development of the explant and neglect the cellular outgrowth. Actually, these technics are designed to inhibit cellular outgrowth (14). In using organ culture methods, one attempts to inhibit cellular outgrowth by shifting the explants every 2 or 3 days and by employing matrices and media which tend to promote maintenance rather than proliferation and migration. These methods have been used to a considerable extent by the embryologist and morphologist (4, 5, 11) and to a lesser extent by the oncologist.

During the course of the study it was found that the atmosphere in which the neoplasms were cultivated was of primary importance. Accordingly, the ensuing experiments were carried out in an attempt to delineate more clearly the nature of the gaseous requirement of tumors in organ culture.

MATERIALS AND METHODS

Human neoplastic tissues were obtained from surgical material, cut into fragments of no more than 1.5 mm. in the greatest dimension, and explanted by the watch-glass method (6) with biological, defined, and partially defined media. Six to ten explants were placed in each watch glass. The biological medium consisted of chicken plasma and chick embryo extract mixed to form a solid clot. The defined medium was CMRL-1066 (17), and the partially defined medium was Eagle’s medium (2, 3) and 20 per cent human serum. The glucose concentration of the Eagle’s medium was either 0.1 or 0.5 per cent. The choice of media was arbitrary and, hence, varied from tumor to tumor. Approximately 1 cc. of medium was used in each watch glass.

Matrices of either rayon acetate or stainless steel mesh were used to support the explants in the fluid media, and the clot itself served as a matrix or support for the explants in the biological medium.

Two dishes of explants, making a total of from twelve to twenty explants in each medium, were incubated at 35°–37° C. in one or more of three atmospheres which included:

1. An incubator atmosphere which presumably simulates that of room air and is composed of:
   a) approximately 20 per cent O₂
   b) approximately 0.3–0.4 per cent CO₂

2. An atmosphere provided by using a bell jar in which a candle was allowed to burn until the flame went out of its own accord and consisting of:
   a) approximately 1 per cent O₂
   b) approximately 5 per cent CO₂ (12)
The possibility of other cases, such as CO, being produced by the burning candle was not investigated at that time.

3. An atmosphere consisting of:
   a) 95 per cent O₂
   b) 5 per cent CO₂

Subculturing (transfer of explants to fresh nutrients) was generally carried out every 2–3 days. Samples (two to three explants) were withdrawn from culture for histological examination at the time of subculturing. The explants were fixed in either a formalin-alcohol-ascetic acid or formalin solution, sectioned at approximately 6–8 μ, and stained in hematoxylin and eosin.

The sections were studied microscopically, and the viability or growth of the tissue was based on the following histological and cytological criteria:
   (a) Mitosis;
   (b) cellular activity (neoplastic proliferation with the consequent formation of a type of capsule around the explant by the neoplastic cells);
   (c) an intact nucleus with a definite nucleolus or nucleoli and a definite chromatin dispersion;
   (d) intact cytoplasm and definite cell boundaries.

### RESULTS

Ten neoplasms were cultivated in one or more media, with rayon acetate and plasma clot surfaces used as matrices and with an incubator atmosphere. The results are summarized in Table 1.

Except for a melanoma, the results were uniformly poor. Nine of the ten cultivated neoplasms survived only 2–3 days, regardless of the media employed. The melanoma remained viable for 7 days before any significant degeneration appeared. The medium in which the melanoma was maintained consisted of a chicken plasma-chick embryo extract clot.

#### TABLE 1

**ORGAN CULTURE OF NEOPLASMS IN INCUBATOR ATMOSPHERE**

The matrices were plasma clot and rayon acetate. Incubator atmosphere was approx. 20% O₂ and approx. 0.4% CO₂. The figures indicate the number of days in culture before progressive degeneration began.

<table>
<thead>
<tr>
<th>Tumor*</th>
<th>Eagle + 40% human serum (0.1% glucose)</th>
<th>1066</th>
<th>Chicken plasma + chick embryo extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ameloblastoma</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Melanoma</td>
<td>5</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Meningoma</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Adenocarcinoma of large intestine</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Scirrhous carcinoma of breast</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Poorly diff. neoplasm of large bowel</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Odontogenic myxoma</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Poorly diff. neoplasm of parotid gland</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

* Two watch glasses per tumor containing from six to ten explants apiece were cultivated in each medium.
* Approximately 1 cc. per watch glass.

Because of the uniformly poor results obtained with the first group of neoplasms, it was decided to utilize a different gaseous environment, one which had been successfully used for normal tissues in organ culture (12). Thus, seven neoplasms were cultivated in one or more of the same media utilized with the first group of neoplasms and in a bell jar atmosphere. The results may be found in Table 2.

When cultivated in Eagle’s medium (0.1 per cent glucose) plus 20 per cent human serum, six of the neoplasms survived a minimum of 7 days. Both thyroid adenomas were viable when withdrawn from culture after 19 and 17 days, respectively. Each exhibited some central degeneration within the first few days but then stabilized so that no further degeneration was apparent upon termination of the culture. Moreover, instead of the irregular border noted initially, the thyroid
expiants demonstrated a smooth, cellular border or capsule which appeared to be composed of the tumor epithelial cells (Figs. 1, 2).

In addition, the carcinoma (Figs. 3, 4) and adenofibroma (Figs. 5, 6) of the breast were viable when taken out of culture after 7 and 12 days, respectively. Also, the adenofibroma demonstrated proliferation of neoplastic epithelial cells which formed a capsule around the expiants (Fig. 7).

None of the tumors cultivated in CMRL-1066 medium survived more than 3 days.

It was necessary to ascertain whether it was the elevated CO₂ or the decreased O₂ that was required for the maintenance of the tumors. Therefore, ten neoplasms were cultivated in three different gaseous environments (bell jar, incubator, and 95 per cent O₂–5 per cent CO₂) with the same media used as before except for one addition: Eagle’s medium and human serum with a higher (0.5 per cent) glucose concentration. Table 3 summarizes the results.

Four of the neoplasms exhibited little or no survival in any of the media or gaseous atmospheres. Five of the remaining six tumors survived at least twice as long in both the bell jar and 95 per cent O₂–5 per cent CO₂ atmospheres than in the incubator environment. Only one neoplasm survived as long in the incubator atmosphere as in the other two atmospheres. This was a hypernephroma (clear-cell carcinoma of kidney), and the medium used was Eagle’s with increased glucose. However, the same neoplasm, in the same medium, maintained viability 2 days longer in a higher CO₂ environment.

The metastatic squamous-cell carcinoma expiants demonstrated two different types of activity. Some of the expiants exhibited proliferation of cells in nests similar to that of the control (Figs. 8, 9). In others the neoplastic cells formed a capsule around the explant (Fig. 10) similar to that described for the thyroid adenoma and breast adenofibroma.

All the successful cultures demonstrated maintenance of cell detail and morphology. Also, many of the various expiants exhibited mitoses (Figs. 11, 12).

In addition, a detailed record was kept of the pH changes which occurred when synthetic or partially synthetic media were employed. There was no correlation between pH changes and cellular activity or growth. In no instance was a pH change to an alkaline range observed. Moreover, a

### Table 3

**Organ Cultures of Neoplasms in Three Different Gaseous Atmospheres**

<table>
<thead>
<tr>
<th>Tumor*</th>
<th><strong>Incubator (approx. 80% O₂ and approx. 0.4% CO₂)</strong></th>
<th><strong>Bell Jar (approx. 5% CO₂ and approx. 1% O₂)</strong></th>
<th><strong>Special Equip. (95% O₂ and 5% CO₂)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Media†</td>
<td>Media†</td>
<td>Media†</td>
</tr>
<tr>
<td></td>
<td>Eagle+ 20% human serum 0.1% glucose</td>
<td>Eagle+ 20% human serum 0.1% glucose</td>
<td>Eagle+ 40% human serum 0.5% glucose</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>1066</td>
<td>1066</td>
<td>1066</td>
</tr>
<tr>
<td>Oncocytoma</td>
<td>5</td>
<td>0</td>
<td>18‡</td>
</tr>
<tr>
<td>Liposarcoma</td>
<td>7</td>
<td></td>
<td>13†</td>
</tr>
<tr>
<td>Sq.-cell ca. metastatic to vagina</td>
<td>2</td>
<td></td>
<td>13†</td>
</tr>
<tr>
<td>Poorly diff. ca. of parotid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastatic ca. to liver</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypernephroma</td>
<td>2</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Adenocarcinoma of large bowel</td>
<td>2</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Adenocarcinoma mucosum breast</td>
<td>2</td>
<td>2</td>
<td>2‡</td>
</tr>
<tr>
<td>Chondrosarcoma</td>
<td>2</td>
<td>2</td>
<td>2‡</td>
</tr>
</tbody>
</table>

* Two watch glasses per tumor containing from six to ten explants apiece were cultivated in each medium.

† Approximately 1 cc. per watch glass.

‡ Culture was ended at this time.
FIG. 1.—Thyroid adenoma. Control. H. & E., X120.

FIG. 2.—Thyroid adenoma. Seventeen days in bell-jar culture. Eagle + human serum. Note epithelial capsule composed of cells similar to those of the neoplastic thyroid follicles. H. & E., X192.

FIG. 3.—Breast carcinoma. Control. H. & E., X480.

FIG. 4.—Breast carcinoma. Seven days in bell-jar culture. Eagle + human serum. H. & E., X480.

FIG. 5.—Breast adenofibroma. Control. H. & E., X480.


FIG. 7.—Breast adenofibroma. Twelve-day bell-jar culture. Eagle + human serum. Note peripheral proliferation and the formation of an outer membrane or capsule by the neoplastic epithelial cells. H. & E., X480.

FIG. 8.—Squamous-cell carcinoma metastatic to vagina. Control. H. & E., X192.

FIG. 9.—Squamous-cell carcinoma metastatic to vagina. Thirteen days in bell-jar culture on plasma clot and in bell-jar atmosphere. H. & E., X192.

FIG. 10.—Squamous-cell carcinoma metastatic to vagina. Thirteen days in bell-jar atmosphere on plasma clot. Note encapsulation of the explant by the neoplastic epithelial cells. H. & E., X48.

FIG. 11.—Fibrosarcoma. Control. H. & E., X480.

FIG. 12.—Fibrosarcoma 7 days in bell-jar culture. Eagle + human serum. Note mitoses. H. & E., X480.
pH change to an acid range did not necessarily indicate that cell growth had occurred. A change to an acid pH range was noted in instances when the explants were viable and also when no viability was present. Conversely, some explants which demonstrated a high degree of activity effected no observable pH changes. This was especially true of those explants which survived for longer periods of time.

**DISCUSSION**

The results of the present study suggest that, in some manner, the level of CO₂ is important for the survival of some neoplasms in vitro. Moreover, the evidence presented herein shows that high O₂ levels are not essential for neoplastic survival in vitro and that neoplastic tissues are capable of surviving and reproducing in an atmosphere of approximately 1 per cent O₂. It has been suggested elsewhere that neoplastic cells in vitro do not require a high atmospheric content of O₂ (7, 18-20).

The primary manner in which CO₂ affected the neoplasms may not have been through pH control. Some workers believe that increased CO₂ tension is necessary to prevent media from becoming alkaline (9, 16). In the present study, none of the media used became alkaline, as shown by the phenol red indicator, regardless of the partial pressure of CO₂. However, this may have been the result of the amount of tissue used in each dish. Moreover, in a synthetic medium, which is more sensitive to pH change because it lacks the buffering capacity inherent in serum or other proteins, no differences in pH were noticed in cultures grown in high and low partial pressures of CO₂.

Recent work indicates that CO₂ may be implicated in differentiation (13, 15). Hoff and Breckenridge (10) suggested that CO₂ participates in cellular metabolism on a molecular basis and not simply as a regulator of pH. They reported that CO₂ can be metabolized by cells and may increase the rate of O₂ consumption in certain tissues and increase glycolysis in other tissues. Other work has shown that CO₂ is an essential for the synthesis of metabolites in human cell culture (1, 8).

In the current work, the apparent requirement for an exogenous source of 5 per cent CO₂ might suggest that these tumors were not producing sufficient quantities of this metabolite. It might follow, as suggested by Warburg (18, 19), that there is a decrease in the respiratory capabilities of tumors, with a consequent decrease in CO₂—an end-product of respiration. It is postulated that tumors in vivo may depend upon other somatic cells for this metabolite.

**ACKNOWLEDGMENTS**

The author wishes to acknowledge Drs. Donald A. Kerr and Raymond H. Kahn for their advice and encouragement during the writing of the thesis.

**REFERENCES**

The Influence of Carbon Dioxide on the Cultivation of Human Neoplastic Explants *in Vitro*

Sheldon Rovin


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/22/3/384

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, contact the AACR Publications Department at permissions@aacr.org.