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Review of Experience with Interferon and Drug Sensitivity Testing of Ovarian Carcinoma in Semisolid Agar Culture

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Summary. *Studies were performed with a semisolid agar culture system to determine the in vitro sensitivity of human ovarian carcinoma to human leukocyte interferon (IFN-α) and standard chemotherapeutic agents (adriamycin, cis-platinum, hexamethylmelamine, melphalan, and velban). Growth in culture occurred in 67% of samples derived from ascitic fluid and 71% of these exhibited reduction in tumor colony number by ≥ 25% in response to 300 units interferon/ml. The responsiveness of the ascitic fluid samples to interferon ran parallel to the overall responsiveness to the other agents tested. Sensitivity to interferon was not related to the histology or grade of the tumor or to the stage of the disease.*

As compared with cell suspensions prepared from ascitic fluid samples, solid tumor samples had markedly lower viability, 39% vs 89%, and had more tumor cells, 81% vs 28%. Also, colonies derived from solid tumor samples were less sensitive to interferon and more sensitive to cis-platinum and adriamycin than were ascitic fluid-derived colonies. Three of four ascitic fluid samples showed a reduction in tumor colony number of ≥ 25%, whereas none of the solid tumor samples obtained from the same donors were affected by interferon to that degree. Retrospective analysis of drug testing (exclusive of interferon) for in vitro: in vivo correlations revealed that in 58% of 12 evaluable situations, when a single drug (or at least one of a group of drugs) gave a positive response in vitro, stabilization or regression of the tumor occurred in vivo after treatment with the drug.

These studies prove the utility of the semisolid agar culture system for assessing the antiproliferative effects of interferon against ovarian carcinoma, and will be of uitility in the future for assessing whether various types of interferon have the same degree, range, and mechanism of action of antitumor effect.

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Introduction

Several years ago we were aware that clinical trials with human leukocyte interferon (IFN-α) were about to commence for the first time in our country, and that the supplies of this interferon, produced largely by Dr Kari Cantell in Finland, were limited. We were also aware of the work of Dr Sydney Salmon and his colleagues at the University of Arizona, who had developed a new technique for the short-term culture of human tumor cells from individual patients [5]. The ability of these investigators to test the sensitivity of the resulting tumor colonies to numerous conventional cancer chemotherapeutic agents was well documented [11], and their success in using their in vitro results to predict in vivo response to individual agents was eventually substantiated [12]. Recognizing that the same system could be used to evaluate the response of individual tumors to interferon as well as to more conventional agents, and perhaps to reflect in vivo response, we adapted the system to make this possible.

We chose ovarian carcinoma for study because it was known to grow well in the semisolid agar culture system [6], because it was a likely candidate for future clinical trials with interferon, and because there was absolutely no information concerning its sensitivity to interferon in vitro or in vivo.

Methods

Cultures were prepared as described previously [3, 4]. Agents to be evaluated for their antiproliferative capacity were either incorporated into the upper layer of the culture system for the duration of culture or preincubated with the cells in Hank's balanced salt solution for 1 h at 37° C.

Human leukocyte interferon (specific activity 2.3×10^5 units/mg protein) was obtained through the courtesy of the Viral Resources Branch, NIAID, NIH, Bethesda, Maryland. All other

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antitumor agents were obtained in pure form through the courtesy of the Drug Synthesis and Chemistry Branch and the Natural Products Branch of the Division of Cancer Treatment of NCI, NIH, Bethesda, Maryland. The final concentrations of each agent employed were: interferon, 30, 100, and 300 units/ml; adriamycin, 0.05, 0.1, and 1.0 μg/ml; melphalan 0.04, 0.1, 0.2, and 0.4 μg/ml; cis-platinum, 0.01, 0.1, and 1.0 μg/ml; hexamethylmelamine, 0.1, 0.5, 1.0, and 5.0 μg/ml; and Velban, 0.05, 0.01, and 0.2 μg/ml.

The cultures were maintained at 37° C in a humidified CO_2 incubator and the plates were examined with a Tiyoda inverted microscope at weekly intervals (usually 7, 14, and 21 days) after initiation of cultures. Tumor cell colonies were scored in three categories: (1) those which contained 10–30 cells; (2) those which contained > 30 cells; and (3) those which contained 10 or more cells, more than half of which showed evidence of degeneration by virtue of their darkened appearance.

Results

Studies on Ascitic Fluid Samples

In our first studies we examined the growth characteristics and response to interferon of 18 samples of ascitic fluid obtained from 15 patients with ovarian carcinoma [3]. The patients ranged in age from 29 to 76 years, and all presented initially in either stage III or stage IV of the disease. Two of the primary tumors were undifferentiated, three were classified as mucinous, and ten were classified as serous with good, moderate, or poor degree of differentiation. Viability of the ascitic fluid cells ranged from 86% to 97%, with the exception of one sample, which had a viability of 23%. The proportion of tumor cells as assessed by Pap smear ranged from 0 to 84%, with four of the fifteen patients having 1% or less tumor cells.

Growth was defined in these initial studies as an increase in total tumor colony number and/or by an increase in colonies with > 30 cells/colony with increasing duration of culture, and this was observed in 67% of the ascitic fluid samples with tumor cells demonstrated by Pap smear. No growth occurred in samples from patients treated with chemotherapeutic agents within 4 weeks of the paracentesis or in samples devoid of tumor cells as assessed by Pap smear.

We were able to follow with serial photomicrographs the actual growth of colonies of ovarian carcinoma cells with time. Increase in the size and volume of colonies was observed with time, and the formation of cystic-like structures within the colonies with degeneration of the central cells and overgrowth of the outermost layers was often observed. Scattered throughout the agar were small cells which under the inverted microscope appeared to be lymphocytes and macrophages. Collections of these same cells were also often seen at the outer margins of the tumor colonies.

Response to interferon was measured in cultures in which the interferon was directly incorporated into the agar. Response was defined as reduction in total tumor colony number by ≥ 50% and partial response by ≥ 25% reduction. The overall response rate for samples, the non-treated controls for which showed evidence of growth throughout the duration of the culture, was 71%. The overall response rate for samples which had distinct tumor colonies, but whose non-treated controls showed no increase in tumor colony number after the first week in culture, was 75%. Reduction was observed not only in total tumor colony number, but also in the size of colonies. Ascitic fluid samples which were sensitive to interferon were also sensitive to at least one other agent, and a unique profile of sensitivity or resistance to standard chemotherapeutic agents was observed for each sample [3].

Sensitivity to interferon was not related to the histology or grade of the tumor or to the stage of disease. In general, the responsiveness of the tumor cells to interferon ran parallel to their overall responsiveness to the other chemotherapeutic agents studied.

In other experiments in which we studied the comparative efficacy of interferon and other antitumor agents preincubated with the cells for only 1 h prior to their incorporation into agar, interferon and the other drugs, each at their highest concentration, appeared to have the same degree of effectiveness for a given donor. However, it should be noted that milligram for milligram the concentration of interferon employed is far lower than those of the other agents, which were employed at concentrations ranging from 0.005–5.0 μg/ml. Given the high specific activity of interferon, of $> 10^9$ units/mg, a concentration of 300 units/ml is equivalent to only 3×10^{-4} μg/ml.

Also, in no instance did interferon or the other antitumor agents completely destroy all the tumor colonies for any of the patients. In each case in which sensitivity to interferon or to the other drugs was demonstrated, residual tumor colonies were still present, presumably resistant to the antiproliferative effects of the interferon or the other agents.

Comparison of Solid Tumor and Ascitic Fluid Samples

In our next series of studies we examined the growth patterns and sensitivity to interferon of solid tumor samples from patients with ovarian carcinoma and compared them with data obtained from ascitic fluid samples [4]. The ten patients studied ranged in age

from 23 to 88 years and presented in stage I, III, or IV of the disease. With one exception, all samples represented either tumor taken from the primary site or from omental nodules at the time of initial laparotomy or at later exploratory operations. One sample was taken from a biopsy of lesions metastatic to the skin. One of the tumors was undifferentiated, one was classified as mucinous, two as endometrioid, and six as serous, with good, moderate, or poor degree of differentiation.

Tumor cell colonies were observed in eight of the ten samples, but sufficient numbers of tumor colonies to allow meaningful statistical evaluation of effects of interferon and other agents were obtained in only four samples.

Samples of both solid tumor and ascitic fluid were obtained from these four donors at the same time. There are important differences between these two forms of tumor samples. When the data from our total overall experience with ascitic fluid and solid tumor samples were considered, it was apparent that the mean value for the percentage of viable cells in the ascitic samples, 89%, was considerably higher than that observed in solid tumor samples, 39%. By contrast, the mean percentage of tumor cells in the ascitic fluid samples, 28%, was considerably lower than that observed for the solid tumor samples, 81%.

Thus, although an equal number (2×10^5) of viable cells from ascitic and solid tumor samples were used to seed the semisolid agar cultures, the cell composition of the inocula from the solid tumor samples differed considerably from those of the ascitic fluid. In the solid tumor samples in which viable cell number is low, large numbers of non-viable tumor cells are brought along in the inoculum; and in the ascitic samples in which the tumor cell number is low, many of the other cell types found in ascitic fluid, i.e. lymphocytes, macrophages, polymorphonuclear leukocytes, and mesothelial cells, are included in the inoculum. Thus one would not expect the colony numbers of solid and ascitic fluid samples from the same donors to be comparable, and in fact that is what we observed. Theoretically, the only definitive way to achieve equal number of colonies from ascitic and solid tumor samples would be to adjust the inoculum to contain comparable numbers of viable tumor cells and/or tumor stem cells.

When we examined the responses of tumor colonies derived both from solid and ascitic fluid samples to a final concentration of 300 units interferon/ml incorporated into the agar for the duration of culture, we found that in three of the four experiments, cultures derived from ascitic fluid were more sensitive to the antiproliferative effects of interferon than were the cultures derived from solid tumor samples. Similar experiments which were performed with final concentrations of interferon of 100 and 30 units/ml gave similar results.

To determine whether cultures derived from ascitic fluid samples were also more sensitive to other antitumor agents, similar experiments were performed with cis-platinum and adriamycin incorporated for the duration of culture. We found that the reverse situation obtained with both cis-platinum and adriamycin. The cultures derived from solid tumor samples were more sensitive to the antiproliferative effects of cis-platinum in three of three experiments and to adriamycin in two of three experiments than were the cultures derived from ascitic fluid samples [4].

Retrospective Studies

The chemotherapeutic agents that were employed in our studies were chosen either because the patients had previously been treated with them or because they were being considered for use in future therapy. Our studies were not designed specifically as prospective studies, nor was there any intention of predicting a given patient's response in vivo or of suggesting to the patient's oncologist the use in the future of drugs which gave a positive response in vitro. Nevertheless, we felt that it might now be possible, several years after the initiation of the original studies, to re-examine the records of the patients in an attempt to demonstrate any correlations between the results of the in vitro tests and the in vivo results. We could do these correlative analyses only for the standard chemotherapeutic agents, but not for interferon, because clinical trials of interferon for ovarian cancer had not yet commenced at the time of performance of our in vitro tests.

Ten of the patients were eligible for retrospective analysis because of demonstrated responses to chemotherapeutic agents in vitro, but the referring physicians and records of only eight of them were now available. The criteria for response was as follows: a positive reponse in the in vitro test was considered to be a $\geq 25\%$ reduction in total tumor colony number in response to a single agent. A response in vivo reflected subjective and objective improvement for at least 1 month, a plus–minus response reflected stabilization for at least 2 months, and a negative response indicated progression of the disease.

Twelve situations were evaluable. There were four instances in which a positive response to a single agent in vitro was followed by a positive response in

vivo and three in which a positive response in vitro was followed by stabilization. There were five instances in which a positive response was noted in vitro but progression of the disease in vivo occurred. Thus in a total of 7 out of 12 instances, or 58%, when a single drug (or at least one of a group of drugs) predicted a positive response, stabilization or regression of tumor occurred. In 42% progression occured.

With regard to negative in vitro tests, none of the patients received as therapy the agents which gave a negative response, or if they did, they received them along with other drugs which we did not test but from which they appeared to have a beneficial response. Thus in our studies we did not have data with which to evaluate the predictive value of negative in vitro tests.

Discussion

Our experience with retrospective analysis led us to conclude that the more stringent the in vitro test, i.e., the more profound the change in colony number to give a positive response, the higher will be the predictive value of a positive in vitro test. Also, it was difficult for us to draw conclusions in correlative follow-up studies, not only because of our small sample size, but also because often drugs were tested as single agents in vitro but were given to the patients as a part of a combination chemotherapy, which often included drugs not tested in vitro. Thus, the importance of studying combination chemotherapy as well as single agents in the in vitro system is obvious. Furthermore, when one is trying to do correlative studies based on the clinical impressions of many different oncologists in the community it is difficult to establish uniform criteria for evaluation of response to therapy to include common definitions of progression, stabilization, and improvement. Therefore correlative data can best be obtained from a population of patients seen within a given clinical group or center in which the clinical oncologists have uniform standards of evaluation. Alternatively, specific protocols could be distributed to physicians participating in any type of study of relationship of clinical outcome to the results of in vitro predictive tests.

With regard to testing with interferon we cannot yet say anything about the predictive value of the test without extensive correlative in vitro and in vivo studies. Hopefully, these studies will be performed in the near future when sufficient quantities of interferon become available to permit therapeutic trials to be done in sufficient numbers to make the information statistically significant.

Our studies [3, 4] and the subsequent studies of other investigators [1, 10] have proven the validity of using the semisolid agar culture technique for assessing the antiproliferative effects of interferon against various types of malignancy. Our studies have also indicated the potential for using this culture system to further understand the mechanism of the antiproliferative action of interferon for individual tumor types. For example, our observation that ovarian carcinoma colonies derived from ascitic fluid are more sensitive to the antiproliferative effects of interferon than are colonies derived from solid tumor cells might shed light on this mechanism. There are several possible explanations for our observation. The first is that lymphocytes and macrophages are far more abundant in the cultures derived from ascitic fluid than in those derived from solid tumors, and that the antitumor functions of these cells may be potentiated by interferon. It is well known that interferon can enhance natural killer (NK) cell activity in vitro [8, 9, 13] and in vivo [2, 7, 18] and also enhance the killing capacity of cells of the monocyte-macrophage series [15]. A second explanation might be that the presence of exogenous interferon may prime for the production of additional interferon by the lymphocytes, as a part of their reaction to the tumor cells. The priming by interferon for additional interferon production is a well-known phenomenon [14], and models for lymphocyte-tumor cell interactions which involve interferon production have also been reported [16, 17]. Finally, a third explanation might be that cells adapted for growth in the ascitic fluid of the host might in fact be more susceptible in their own right than tumor cells found in large solid masses to the action of interferon.

Confirmation by other laboratories of our observation of enhanced sensitivity of ascitic fluid-derived tumor colonies would be very important for physicians involved with clinical trials of interferon. Such an observation might provide a rationale for using interferon intraperitoneally for the treatment of patients with widespread intraperitoneal metastases, in addition to using it systemically. Furthermore, now that clinical trials of ovarian carcinoma with interferon have recently begun (J. Gutterman 1980; H. Strander 1980, personal communications), it would be of interest to determine whether a differential in vivo response to interferon occurs with solid tumor masses as compared with tumor cells in ascitic fluid, as we observed in our in vitro studies [4]. In addition, the challenging question of whether various forms of interferon, i.e., alpha, beta, gamma, and recombinant DNA-produced interferons have the same

degree, range, and mechanism of action of antitumor effects can now be answered with the use of this culture system.

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