Interferon and Interferon Inducers in the Treatment of Malignancies

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ABSTRACT The mechanism of the antitumor action of polyinosinic-polycytidylic acid is probably multifaceted. The compound induces the synthesis of interferon, and interferon probably is active against some tumors. Poly I:poly C alters protein and RNA synthesis in tissue culture. It specifically inhibits such macromolecule synthesis in tumors in vivo, while having less inhibitory action on synthesis in normal organs, or it may actually enhance. Finally, poly I:poly C strongly enhances graft vs. host rejection mechanisms, which may play a role in the rejection of some tumors.

It was originally proposed by Isaacs that the interferon response is induced by the presence in a cell of a foreign nucleic acid. The net effect of this response is, in effect, to reduce the damage brought about by the presence of this foreign nucleic acid, which usually would be viral nucleic acid. Isaacs' hypothesis was supported by some data showing that foreign nucleic acids do indeed lead to the production of some slight amount of interferon. His data were not very convincing, and general acceptance of this concept was held in abeyance. The validity of this hypothesis has recently received support from the findings that certain double-stranded synthetic and natural ribonucleic acids, particularly polyinosinic-polycytidylic acid (poly I:poly C), can lead to the development by cells of a high level of resistance to virus replication and to the production of large amounts of interferon, both in vivo and tissue culture (7).

While interferon itself is in general not toxic to animals and does not inhibit cell growth, poly I:poly C has been found to inhibit the growth of a number of experimental tumors in animals (8, 9). The variety of tumors affected and the extent of inhibition are shown in Table I. In addition to those shown, carcinogenesis by dimethylbenzanthracene (DMBA) plus croton oil, or by DMBA alone, is strongly inhibited by suitable administration of poly I:poly C (11). This presentation will discuss this inhibition and the possible mechanism of its action.
TABLE I

EFFECT OF POLY I:C ON ANIMAL TUMORS

| Tumor                                           | Increase in median tumor survival over control |
|-------------------------------------------------|-----------------------------------------------|
| J96i32-Reticulum cell sarcoma (subcutaneous)    | 130*                                          |
| J96i32-Reticulum cell sarcoma (ascites)         | 96*                                           |
| Carcinosarcoma Walker 256                      | 100                                           |
| Reticulum cell sarcoma RCSL                     | 89                                            |
| Ehrlich ascites tumor                           | 70                                            |
| S91 Melanoma                                    | 55                                            |
| Fibrosarcoma                                    | 52                                            |
| B1237-Lymphoma (ascites)                        | 45                                            |
| L1210 leukemia                                   | 42                                            |
| Plasma cell YPC-1                               | 39                                            |
| B1237-Lymphoma (subcutaneous)                   | 28                                            |
| MT-1 tumor (subcutaneous)                       | 26                                            |
| Reticulum cell sarcoma ovarian                  | 20                                            |
| Leukemia F388                                   | 16                                            |
| Leukemia K1964                                  | 12                                            |

Treatment, in most cases, was 150-200 μg per mouse, three times weekly, by intraperitoneal route. With the exception of the J96i32 reticulum cell sarcoma, some Ehrlich ascites tumors, and a few Walker carcinosarcoma, all animals ultimately died.

* Mean day of death of the animals that died. About 30% of all the animals treated have survived, although treatment had been stopped at about day 30.

The mechanism of action of this compound appears to be complex. Viral interference (1), interferon (2), and interferon inducers (3) can inhibit the growth of oncogenic viruses in tissue culture (4) and can alter the course of murine leukemias in animals (3, 5, 6, 10, 13). It has been clearly shown by Gresser et al. that the murine leukemias, such as those induced by the Friend virus (5), are inhibited by exogenous interferon. Wheelock has shown that the interferon inducer, statolon, also inhibits the development of Friend virus leukemia (3). Cell transformation by oncogenic viruses can also be inhibited by interferon inducers (12). In addition Gresser has shown that the growth of a dimethylbenzanthracene-induced tumor is also inhibited by interferon (14), as shown in Fig. 1. In the animals bearing those tumors interferon treatment brought about an increased phagocytosis of tumor cells. The mechanism by which interferon may inhibit the tumors will be referred to later. Since poly I:poly C is a potent inducer of interferon in mice, it is likely that part of the antitumor action of the double-stranded RNA is through the interferon system.

A second aspect of the activity of poly I:poly C may be as a direct chemotherapeutic agent. Poly I:poly C is a double-stranded RNA, the homopolymers of which can code for the synthesis of polypeptides in a suitable cell-free test system. Poly C, for example, should code for the synthesis of
polyproline. If the poly C component of the poly I:poly C were to be responsible for the synthesis of polyproline in the cell one might imagine any number of cellular reactions to the presence of this "nonsense" protein. Poly I:poly C does stimulate the incorporation of proline into the proteins of L cells, but it also stimulated incorporation of a number of other amino acids for which it should not code. Further, polyadenylic-polyuridylic acid also stimulated proline incorporation for which it should not code. Neither nucleic acid has much effect on phenylalanine incorporation for which the poly U would code (Fig. 2). One can conclude that the double-stranded RNA's do stimulate amino acid incorporation into proteins but are not acting intracellularly as synthetic messenger RNA's. The nature of the proteins made is under study.

The increased amino acid incorporation is seen also in animals. Normal C57 black mice were injected intraperitoneally with 150 μg of poly I:poly C, the same dose as used in the treatment of the tumor-bearing mice. 16 hr later the animals received 10 μCi of proline-14C also intraperitoneally. 2 hr later the acid insoluble radioactivity in a number of organs was determined and compared with that found in animals that had received the radioactive proline but no poly I:poly C. The results are summarized in Fig. 3. One can see that many of the organs showed increased rates of protein synthesis.
Figure 2. Effect of poly I:poly C and poly A:poly U on amino acid incorporation into primary mouse embryo cells. Primary mouse embryo cells were exposed for 16 hr to 100 μg/ml of the RNA in the presence of BME and 5% fetal calf serum. The medium was changed to BME without serum for 1 hr, and then the cells were exposed to the labeled amino acid for 30 sec. Radioactivity was determined on the acid insoluble portion of the cells.

Figure 3. Effect of poly I:poly C on proline incorporation into various organs of a normal mouse. Three C57 black mice were treated with 200 μg per mouse of poly I:poly C, intraperitoneally. Three mice served as controls. After 16 hr, the mice were exposed to 10 μCi proline-14C for 40 sec, and the radioactivity incorporated into acid insoluble components of the indicated organs was measured. Ordinate is specific activity, expressed as dpm × 10^-3/mg protein.
Additional experiments were done with C57 black Kaplan strain of mice bearing the J96132 reticulum cell sarcoma, the tumor most sensitive to poly I:poly C. As before, the animals received poly I:poly C overnight and proline-\(^{14}\)C in the morning. As shown in Fig. 4 there was strong stimulation of proline incorporation into the various normal organs. However, poly I:poly C treatment caused marked inhibition of protein synthesis in the tumor. This inhibition ranged from 65 to 95% in different experiments. This inhibition was also seen with two other tumors. It appears reasonable to think that this inhibition of protein synthesis would be inhibitory to tumor growth. It is possible however, that the tumors had been damaged by some other mechanism and that the decreased protein synthesis is a reflection of this, rather than a cause of the tumor inhibition.

The effect of poly I:poly C on RNA metabolism in tissue culture is complex. Depending on dose and time, one can observe inhibition or stimulation. There are, however, profound effects on nuclear RNA metabolism, indicating that the effect of the compound on the host genome is much more than just stimulating the production of mRNA for interferon. The effect of the compound on RNA metabolism in organs of mice bearing the J96132 tumor is shown in Fig. 5. In general, RNA synthesis in mice is affected somewhat less by poly I:poly C than is protein synthesis. However, poly I:poly C treatment leads to a marked inhibition of RNA synthesis in the tumor.
The effect of poly I : poly C on thymidine incorporation into DNA can be either enhancement or inhibition depending on the tissue studied (Table II). It can be seen that there is inhibition in primary mouse embryo, rabbit kidney, and Vero cell monolayers. On the other hand, there appears to be stimulation in L cells, particularly in suspension culture.

A third facet of the antitumor action of poly I : poly C is its ability to enhance cell mediated defense mechanisms against foreign antigens. If spleen cells from adult mice are injected into newborn F1 hybrids, one of whose parents is the strain of the donor mice, the donor cells develop a graft vs. host reaction which affects the recipient animals in a number of ways (15). One of these ways is the establishment of a splenomegaly in the recipient (16, 17). The degree of spleen enlargement is a function of the activity and the logarithm of the number of donor cells. This test is useful in evaluating immune suppressive drugs and antilymphocytic serum. The depressed reactivity of the spleen cells from drug-treated donor animals results in a lesser splenomegaly in the recipient than that obtained from spleen cells from untreated donors. When donor mice are given poly I : poly C their subsequently removed spleen cells show enhanced immunological reactivity against the foreign antigens in the recipient and result in increased splen-
### TABLE II
EFFECT OF POLY I: POLY C ON THYMIDINE INCORPORATION INTO VARIOUS CELLS

| Cells                        | Incubation with PIC for: | Inhibition or enhancement | Dose |
|------------------------------|--------------------------|----------------------------|------|
|                              | hr | %      | µg |
| L 929 Y Spinner              | 3  | +24    | 100 |
|                              | 16 | +68    | 25  |
|                              | 16 | +42    | 100 |
| L 929 Y Monolayer            | 2  | No effect |     |
|                              | 48 | +37    | 30  |
|                              | 96 | +32    | 30  |
| Vero CCL 81 Monolayer        | 2  | -33    | 100 |
|                              | 16 | -34    | 50  |
| Rabbit kidney cells          | 3  | No effect |     |
|                              | 16 | -42    | 20  |
| P3 cells (Burkitt) Suspension | 2  | -32    | 25  |
|                              | 16 | No effect |     |
| Primary mouse embryo cells   | 2  | No effect | 50  |
|                              | 16 | -60    | 50  |

Cells were incubated with the indicated concentration of poly I:poly C. Cultures were exposed for 48 min to 2.5 µc of thymidine-14C per 100 ml of medium. The cells were washed twice with cold PBS, suspended in cold 10% TCA (trichloroacetic acid), washed with 0.1% cold TCA, and hydrolyzed for 15 min at 100°C in 5% TCA. Radioactivity and DNA concentrations were determined on aliquots of the hydrolyzate.

omegaly, as seen in Fig. 6 (18). Comparison of the position of the two parallel lines obtained from injecting different numbers of cells reveals that poly I:poly C at 1 mg per mouse causes greater than a tripling of the effectiveness of the cells in the graft vs. host reaction, while lesser amounts of poly I:poly C produced a definite but lesser stimulation. Enzymatic hydrolysis of the poly I:poly C destroys its enhancing ability. It is reasonable to think that enhancement by poly I:poly C of such cell-mediated rejection of foreign antigens could effectively act against the foreign antigens of the tumor, facilitating the rejection of the tumor. Indeed, the inhibition of macromolecule synthesis discussed earlier may be a manifestation of such cellular immunological activity. Such enhancement of immunological reactivity by polynucleotides has been observed repeatedly before (19).

It appears then that there are at least three different aspects of the action of poly I:poly C that bear on the antitumor activity of the compound: the activity of the interferon induced, possible direct chemotherapeutic action, and enhancement of immunological rejection mechanisms. The relative importance of each of these components and the interrelationships among

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1 Cantor, H., R. Asofsky, and H. B. Levy. Effect of polynosinic: polycytidylic acid on graft vs. host reaction. In preparation.
FIGURE 6. Effect of poly I:poly C on graft vs. host reaction. Balb-c mice were inoculated intraperitoneally with the indicated amount of poly I:poly C. 48 hr later they were sacrificed, and their spleens were removed and minced. The indicated number of spleen cells were inoculated into newborn F hybrids of Balb-c X C57 black mice. 9 days later these animals and their spleens were weighed, and the spleen weight was normalized to body weight. The spleen index is the ratio of this normalized spleen weight to the normalized spleen weight of animals not inoculated with spleen cells.

| Number of Grafted Cells x 10^6 | Spleen Index |
|-------------------------------|--------------|
| 1                             | 1.4          |
| 2                             | 1.8          |
| 3                             | 2.2          |
| 4                             | 2.6          |
| 5                             | 3.0          |
| 6                             | 3.2          |

Figure 6 shows the relationship between the number of grafted cells and the spleen index. The data indicates that as the number of grafted cells increases, the spleen index also increases.

them will be discussed. Since exogenous interferon in mice is effective against some tumors it is obvious that the interferon induced by poly I:poly C is important, but it is not understood how interferon inhibits tumors. It has been reported that interferon can inhibit cell growth in tissue culture (20), but the absence of such inhibition has also been reported (21, 22). Since all the testing has been done with interferons of varying degrees of impurity, it is hard to exclude the possibility that the observed inhibitions have been due to impurities. It may be that in an animal, interferon treatment increases the immune reactivity of the host. We are currently testing this possibility. There have been a number of reports suggesting a close association between the interferon system and the immunological systems. For example, lymphocytes from animals (including man) immunized with a specific antigen respond, in tissue culture, to exposure to that antigen by producing interferon (23, 24). Lymphocytes, on exposure to phytohemaglutinin, change to lymphoblasts, which then produce interferon. This is the same type of cellular transformation that occurs when these cells are stimulated to produce antibodies. It is possible therefore that there may exist a coordinated relationship between initiation of antibody response and initiation of interferon production.

2 Stinebring, W. Personal communication.
The ability of poly I:poly C to enhance cell mediated graft vs. host reaction could also account for part of its ability to inhibit the growth of tumors containing foreign antigens. It is worth noting that polyadenylic-polyuridylic acid has been reported to be as good an adjuvant for the production of circulating antibodies as is poly I:poly C (25). However, poly A:poly U is a much poorer inhibitor of tumor growth than poly I:poly C. It remains to be determined whether poly A:poly U enhances cell mediated graft vs. host reactions as well as does poly I:poly C. If it does, the implication would be that the immunological rejection mechanisms may not be very important in tumor inhibition by poly I:poly C.

Similarly the inhibition of macromolecule synthesis observed in tumors of animals treated with poly I:poly C may be attributable to a direct chemotherapeutic action, or it may reflect the beginning of the immune-rejection mechanisms. We have seen that by two days after injection of poly I:poly C there is maximum stimulation of the graft rejection capacity of the spleen cells of the treated animals. It might indeed be that the inhibition of macromolecule synthesis is just a reflection of the fact that these cells are dying from immune rejection mechanisms. In any case, 1–2 days after initiation of treatment of mice bearing the reticulum cell sarcoma there is marked alteration in the histological appearance of the tumor.

The effect of poly I:poly C on chemical carcinogenesis may throw some light on the mechanism of action (11). Dimethylbenzanthracene (DMBA) can be used to induce tumors in mice by two different procedures. One very small application followed by repeated treatments of the skin with coton oil will induce skin tumors, or a single application of a large amount of DMBA without croton oil will also do so. Poly I:poly C almost totally prevents the induction by the DMBA–croton oil system but only delays the production of tumors induced by DMBA alone. It has been shown that impairment of the host immunological processes will increase chemical carcinogenesis (26).

Two additional items are worth pointing out with particular reference to the possible use of poly I:poly C or interferon in the treatment of malignancies in man. One is the fact that certain sera hydrolyze poly I:poly C to split products that are altered in their biological properties (27). The split products no longer have the pyrogenic capability of the intact poly I:poly C, they do not induce interferon synthesis, and do not have any antitumor activity. Human serum is one such hydrolytic serum. This immediately poses the question as to whether in man poly I:poly C will be hydrolyzed before it can act. There are no data as yet on this point except that pre-

3 Baron, S., and H. B. Levy. Unpublished results.
4 Adamson, R., J. Nordlund, S. Wolff, and H. B. Levy. Unpublished results.
liminary evidence, with some other species of animals which have hydrolytic sera, indicates that poly I:poly C still can be strongly antiviral. The other point of importance deals with the toxicity of poly I:poly C. In monkeys and dogs, levels of 3 mg/kg are frequently fatal. 1 mg/kg is reasonably well tolerated and leads to the production of low levels of interferon. Toxicity is associated with intravascular coagulation in severe instances and with only transient elevation of serum transaminases and lactic dehydrogenase levels in mild instances. The levels that are lethal to mice approach 100 mg/kg, while levels that are effective in antitumor tests in mice are 5–10 mg/kg. Nothing is known as yet about the level that will either prove toxic to man or will induce interferon formation. The capacity of human serum to hydrolyze poly I:poly C may play a role here.

In summary, both interferon and synthetic inducers of interferon have been shown to increase host survival time in mice and rats bearing experimental tumors. Studies are currently underway in a number of laboratories to test the possibility that the inducer or exogenous interferon may be useful in the treatment of human viral disease and, at least for the inducer, in the treatment of malignancy. Interferon itself may prove to present less of a problem with regard to toxicity than any of the inducers, but its production in sufficient quantities, in a state sufficiently pure for use in man, may pose some real difficulties. The synthetic inducers of interferon, while expensive, are considerably cheaper than interferon itself, but they do present greater problems of toxicity. In addition to which, the synthetic inducers may (a) present hazards as yet undetermined, and (b) act by additional mechanisms to interferon induction. Further investigation will certainly be required before a compound like polyinosinic-polycytidylic acid should be used for minor illnesses. The treatment of a malignancy with nearly universally fatal outcome justifies a greater risk.

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