The Possible Role of the Properdin System in Transplantable Cancer

The Effect of Zymosan on Transplantable Human Carcinoma

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In a previous paper on heterologous transplantation of tumors, we (2) reported successful growth and serial propagation of a carcinoma of the human colon in irradiated and cortisone-treated weanling Wistar rats. The fact that 205 other malignant tumors failed to grow simply served to emphasize the well known presence of a highly effective protective mechanism normally possessed by animals against all such transplanted foreign tissues. In attempting to explain this mechanism, we suggested that the properdin system might play a leading role in this resistance phenomenon.

The theoretical implication of the properdin system in the protective mechanism against heterologously transplanted tumors was based on three important discoveries. The first discovery was the role of lymphocytes in maintaining the defenses of the host against foreign tissues. As early as 1914, Murphy (6) was able to break down the animal defenses by destroying lymphocytes with irradiation or benzol. In 1949, Toolan and Kidd (13) demonstrated invasion of transplanted C3H mammary carcinoma and lymphosarcoma 6C3HED grown in A strain mice by lymphocytes with subsequent necrobiosis and disappearance of neoplastic cells. In 1951, by treating host animals with cortisone and thus destroying lymphocytes, Howes (3) was able to grow adenocarcinoma E 0771 and 775 in resistant C57BL mice, and Foley and Silverstein (1) succeeded in growing lymphosarcoma 6C3HED in resistant CF1 mice. Using the irradiation technic of Murphy, Toolan (12) in 1951 succeeded, for the first time, in propagating malignant human tumors in the subcutaneous tissues of laboratory animals. Thus, while it was quite apparent that lymphocytes were concerned in maintaining resistance against heterologous tumors and that irradiation destroyed both the lymphocytes and the resistance, the precise mechanism of action still remained undetermined. The second pertinent discovery was that of Pillemer et al. (8). In 1954 they isolated a new euglobulin in serum which they called "properdin." In the presence of complement and Mg++ this protein (a) destroys bacteria, (b) neutralizes viruses, (c) lyzes erythrocytes, and (d) is readily destroyed in the animal by total-body irradiation. The third relevant discovery was that of Kidd (4, 5). In 1948 he demonstrated the presence of a "protein" in normal guinea pig serum that caused regression of subcutaneously implanted Gardner lymphosarcoma 6C3HED and lymphoma II in mice. Assembling these facts, we (2) theorized that because (a) total-body irradiation to animals causes a marked depletion of lymphocytes and properdin, (b) total-body irradiation to animals is necessary to destroy "something" in order that heterologous tumors may grow, and (c) a "protein" in normal guinea pig serum can destroy lymphosarcoma in mice, it might be possible (a) that lymphocytes may in some way be concerned with the formation of properdin, (c) that the "protein" in normal guinea pig serum could actually be properdin, and (c) that the properdin system may play a role in the natural resistance of animals to heterologously transplanted tumors.

The following experiment was designed to investigate the possible role of the properdin system in this protective mechanism. Because properdin is not available on the general market and because its manufacture comprises delicate chemical procedures and necessitates special equipment, zymosan was selected for use in this preliminary investigation. Zymosan is an insoluble carbohydrate complex derived from yeast cell walls that combines with properdin both in vivo and in vitro (11). In fact, so dependable is the union of zymosan with properdin that the use of zymosan actually led to the discovery and isolation of properdin in 1954 (8).
MATERIALS AND METHODS

The objectives of the experiment were to produce a low level of properdin in one group of animals, a high level of properdin in another group of animals, and then to compare the rate of growth of a transplantable tumor in each of these with its rate of growth in normal nontreated and in irradiated animals.

The animals used in the experiment were weanling female Wistar rats (purchased from Carworth Farms) weighing between 40 and 50 gm. The tumor was the transplantable human carcinoma of the colon (HR132) first implanted into our laboratory animals on December 29, 1954, and since propagated by subcutaneous transplantation every 10-14 days (2). Fleischmann's zymosan (batch 5B-171, purchased from Standard Brands, Incorporated) was prepared for administration by the method of Pillem et al. (9).

In the experiment, a new series of 64 rats was started each week. The animals were divided into four groups of sixteen. The rats in the first group received no treatment; those in the second group were given 500 r total-body irradiation (220 kvP, 36 r/min, 50 cm. TSD, 1.0 cu HVL)124 hours before the tumor was implanted; those in the third group received a single intravenous injection of zymosan 8 days before the tumor was implanted and subsequent intravenous injections on the 4th, 7th, and 11th days after the tumor was implanted. Following preliminary tolerance determinations, arbitrary dose levels of zymosan were selected at 10, 20, 40, 100, and 175 mg/kg of body weight. Each of these was administered to two successive groups of animals, with a total of 32 animals in each series (zymosan level). Tumor-bearing animals from the next to the last group of rats treated with 500 r served as donors. The donor tumor was thus 2 weeks old. Portions of the same tumor were implanted into an equal number of animals in each category, and implantation was made into the subcutaneous tissue of the right side of the animal by the usual trocar technic. Total leukocytic and lymphocytic counts on the peripheral blood were performed on control, irradiated, and zymosan-treated animals and also, for comparison, on animals treated with cortisone alone and with cortisone and 150 r combined. Animals were weighed at the beginning and termination of the experiment and, in addition, the zymosan-treated animals were weighed before each injection. All surviving animals were sacrificed on the 14th day after the tumor was implanted, and a complete autopsy was performed on each. Microscopic examination was carried out on all tumors, on all foci in the vicinity of the original implants, and on those organs that were grossly abnormal.

RESULTS

As indicated in Table 1 a total of 160 animals was used in each category. Out of this number, the implanted fragments of tumor grew in nineteen nontreated animals, in 136 given 300 r total-body irradiation, in 58 administered a single dose of zymosan, and in 90 treated with multiple injections of zymosan. Of the tumors that grew, the average diameters were as follows: 8 mm. in nontreated animals, 9 mm. in those given 300 r total-body irradiation, 7 mm. in those receiving a single dose of zymosan, and 8 mm. in those given multiple injections of zymosan. Thus, there is no doubt that the nontreated animals showed the poorest tumor "takes," that the irradiated animals revealed the best tumor "takes," and that the zymosan-treated animals disclosed tumor "takes" between these two extremes. It is also apparent that animals receiving multiple intravenous injections of zymosan showed, as a rule, a greater number of tumor "takes" than those receiving a single injection of the carbohydrate complex. As for dose levels, the best results were obtained in animals receiving 100 and 175 mg of zymosan/kg of body weight. This was especially true in animals treated with multiple injections of zymosan at 175 mg/kg, in which the number of tumor "takes" (25) almost approached that (29) in animals exposed to 300 r total-body irradiation.

Regarding toxicity, 175 mg of zymosan/kg was the maximum tolerated dose, because, in this category, five animals in the multiple zymosan group died within 24 hours after the second or subsequent injections. In comparison, there were no deaths in animals treated at other levels of zymosan.

Repeated peripheral leukocytic and lymphocytic counts were performed on a total of 24 nontreated animals, a total of 24 irradiated animals, and on sixteen zymosan-treated (by single and multiple injections) animals at each dose level. Since leukocytic counts were also performed on each irradiated and each zymosan-treated rat before treatment was instituted, the total number of counts on normal animals was over 300. In the group of animals examined, the average normal

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<td><strong>NUMBER AND SIZE (MM.) OF TUMOR “TAKES” OF HR132</strong></td>
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leukocytic count was 6,550, with extremes of 3,250 and 15,700, while the average lymphocytic count was 92 per cent, with extremes of 54 and 94 per cent. In animals given 800 r total-body irradiation, there was a consistent drop in total leukocytic count to as low as 500 and in lymphocytic count to as low as 5 per cent within the first 8 days following irradiation, after which the counts gradually rose toward normal within the next 7 days. Similar findings were noted in separate groups of sixteen animals treated with cortisone (6 mg/rat/2 days X 4) alone and cortisone (3 mg/rat/2 days X 4) and irradiation (150 r X 1) combined. Animals administered single or multiple injections of zymosan at each dose level revealed no significant alteration in either the total leukocytic or the lymphocytic counts.

The average weights of the animals at the start and termination, respectively, of the experiment were as follows: Nontreated, 47 and 100 gm. Irradiated, 47 and 72 gm. Single zymosan: 10 mg., 41 and 84 gm.; 20 mg., 50 and 86 gm.; 40 mg., 47 and 95 gm.; 100 mg., 47 and 95 gm.; and 175 mg., 46 and 95 gm. Multiple zymosan: 10 mg., 45 and 93 gm.; 20 mg., 50 and 93 gm.; 40 mg., 47 and 90 gm.; 100 mg., 47 and 88 gm.; and 175 mg., 47 and 76 gm. It is thus apparent that small amounts of zymosan do not appreciably retard gain in weight, while large amounts (175 mg/kg) impair weight gains to a degree comparable to that seen in 800-r total-body irradiation.

Pathologically the only noteworthy findings occurred in the tumor implants and tumors, spleens, lymph nodes, and livers. Tumor implants that failed to grow disclosed degeneration to complete disappearance of the neoplastic cells along with varying degrees of lymphocytic infiltration and fibroblastic proliferation. In some instances, the implants were ultimately absorbed completely, while in others they were represented by foci of necrosis and scar tissue. Tumors that grew were similar, regardless of treatment, to those previously reported (2). Histologically, they existed as solid sheets and cords of ill-defined polyhedral cells with moderate amounts of cytoplasm and round or oval, rather large hyperchromatic nuclei. The supporting connective tissue was usually scanty, well vascularized, and permeated with scattered leukocytes. Necrosis of neoplastic cells, even in tumors measuring as much as 20 mm. in greatest diameter, was inconspicuous. The spleens and lymph nodes were normal in nontreated and in zymosan-treated rats receiving the carbohydrate complex at levels up to and including 40 mg/kg of body weight. They were decreased to as little as one-quarter normal in rats exposed to irradiation and were increased to as much as 4 times normal in rats treated with single injections of zymosan at levels of 100 and 175 mg/kg and to as much as 10 times normal in rats given multiple injections of zymosan at the corresponding levels (Fig. 1). Histologically, the atrophic spleens and lymph nodes revealed lymphocytic depletion, erythrocytic extravasation, phagocytosis of blood pigments, and concomitant reticulum and reticulum-cell prominence, while the enlarged spleens and lymph nodes disclosed marked lymphoid hyperplasia with crowding of the underlying stromal structures (Figs. 2-7). The livers of twelve out of 160 animals treated with 800 r disclosed focal but diffuse acute nonspecific necrosis. Immediate toxic manifestations in some of the animals receiving 175 mg of zymosan/kg consisted of lethargy progressing to death. Pathologically, such animals showed nothing of note. Animals that died after subsequent injections disclosed loss of weight and, in two instances, a urinary tract filled with blood. Unfortunately, it so happened that they were not found early enough to be suitable for microscopic examination.

DISCUSSION

The transplantable carcinoma of the human colon (HR132) was selected for use in this experiment because (a) it is a human cancer, (b) the normal animal resistance to the tumor is exceptionally good, and (c) the neoplasm can be grown with any degree of regularity only by artificial prompting (2). Although the normal animal defense to HR132 can be broken down by treatment with cortisone alone (6 mg/rat/2 days X 4) or with combined cortisone (3 mg/rat/2 days X 4) and irradiation (150 r X 1), irradiation (300 r X 1) alone was selected as the destroyer of the animal resistance because (a) it is simple to administer, (b) growth of tumor following its use is most consistent, and (c) total-body irradiation has been shown by Fillmer et al. (9) to be followed by a severe depletion of the properdin level in the animal body. Wistar rats were used, because, from the onset, the tumor was adapted to this strain. Whether it will grow in other strains of rats has not yet been determined. The weanling stage of development was selected, because such animals are capable of surviving experimental procedures and, at the same time, support the growth of tumors more vigorously than do older animals. The female sex was used only for convenience. As a matter of fact, the HR132 grows as well in weanling male rats as it does in female rats of the same age.

From the data presented, there is no doubt that zymosan destroyed, or at least lowered, the resistance of the weanling Wistar rats to HR132.
and thereby permitted the tumors to grow. In fact, when administered in higher and multiple doses, the effect of zymosan was almost equal to the effect of 300 r total-body irradiation. Following the completion of our experiment and, actually, the writing of this paper, our attention has been drawn to an abstract published by Palm (7), wherein he also concluded that zymosan effectively conditioned rats for transplantation of human tumors. Because we were in no position to carry out properdin assays on the sera of the animals used in the experiment, we can only speak of properdin levels by inference. Using Fleischmann’s zymosan, Pillemer and Ross (10), however, did demonstrate that intravenous injections of small doses (5 and 25 mg/kg) of the carbohydrate complex caused a rapid fall (to 30 per cent) in properdin titer within 1–2 hours, followed after 2–14 days by a rise in titer to 300 per cent above normal level. They also demonstrated that intravenous injections of larger doses (125 mg/kg) produced a greater fall in properdin levels (to 20 per cent), with a return to only 75 per cent of the normal level after 6–10 days.

If a parallel can be drawn between this experimentally proved information and our own experiment, a single intravenous injection of zymosan at 10–20 mg/kg of body weight should have resulted in a high properdin level by the time the tumor was implanted (5 days later), and this high level should have been sustained for the duration of the experiment. If this were true, the resistance of the animals to the tumor implants should have been increased over that of normal, and the number of tumor “takes” should have approximated the number in the nontreated group. Actually, the number of tumors that grew in the 64 animals in these combined groups was eighteen, as compared with thirteen in the 64 animals in the corresponding controls. Similarly, a single intravenous injection of zymosan at 40, 100, and 175 mg/kg of body weight should have resulted in a properdin level of only 75 per cent of normal by the time the tumor was implanted (3 days later), and this lowered level should have been sustained for almost the duration of the experiment. Thus, the resistance of the animals to the implants should have been slightly reduced, and the number of tumor “takes” should have been increased over that obtained at normal properdin levels. Actually, this did occur. The number of tumors that grew in the 96 animals in this combined group was 40, as compared with six in the 96 animals in the corresponding controls. Thus, the theoretical considerations in relation to single injections of zymosan appear to be plausible.

Producing an initial low level of properdin is simple and, as already indicated, has been proved by actual chemical determinations (10). To maintain it at a low level, however, is not quite so certain. In personal communications with Doctor Ross, it was his opinion that this could best be accomplished by intravenous injections of zymosan at 3–5-day intervals. Accordingly, the first injection of zymosan was made 9 hours before the tumor was implanted, and subsequent injections were given on the 4th, 7th, and 11th days. That the resistance to HR132 was decreased in all animals receiving multiple injections of zymosan regardless of the dose is shown by the fact that in the entire series of 160 animals the tumor grew in 90 as compared with nineteen tumors that grew in the corresponding group of nontreated animals. Another significant finding, as would be expected, is that, with higher doses of zymosan (100 and 175 mg/kg), the resistance of the rats against HR132 was less than that with lower doses (10, 20, and 40 mg/kg), since tumors grew in 48 of 59 (surviving) animals in the former group and in 42 of 96 animals in the latter group. The theoretical basis for the better tumor “takes” at higher zymosan levels is lower properdin levels. One reason for lower properdin levels would be the increased amounts of zymosan available for direct union with circulating properdin. Another reason would be that, since larger doses of zymosan (125 mg/kg) produce “rebound” properdin levels of only 75 per cent of normal (10), there would be less properdin in the circulation to be neutralized. Thus, from the data and other points presented, the theoretical considerations in conjunction with multiple injections of zymosan also appear to be plausible.

If our theory regarding the possible role of the properdin system in the resistance to heterologously transplanted tumors is correct, the lymphocytes in the animal body appear to occupy a key position. If lymphocytes are in any way connected with the maintenance of a normal properdin level of the blood, the depletion of properdin in irradiated animals should be accompanied by destruction of lymphocytes in the circulating blood and in the regular lymphoid depots in the body. Such was the case, since the leukocytic counts dropped to as low as 500; the lymphocytic counts declined to as low as 5 per cent, and there was severe atrophy of the lymphoid tissue of the spleen and lymph nodes. Since, according to known facts, zymosan reacts directly with properdin after it has been manufactured, there should not only be no depletion of lymphocytes after its administration, but there should be, if anything, a hyperplasia of lymphoid tissue. Such was the case in our experiment. Both the leukocytic counts and the percentage of lymphocytes in the peripheral blood remained normal. As far as internal organs were
concerned, however, there were no appreciable changes in the spleens and lymph nodes of animals treated with 10, 20, and 40 mg of zymosan/kg. These organs, however, were increased in size as much as tenfold in animals receiving 100 and 175 mg of zymosan/kg. Histologically, they disclosed marked lymphoid hyperplasia.

It must, however, be stated again and emphasized that some of the foregoing discussion is based on facts and some is purely theoretical. The facts are (a) that intravenous administration of proper amounts of zymosan to rats results in an appreciable drop in blood properdin and that similar administration of zymosan to weanling Wistar rats decreases their resistance to the transplantable human carcinoma of the colon HR132 and allows the tumor to grow, (b) that total-body irradiation to rats similarly influences their blood properdin and their resistance to HR132, (c) that administration of zymosan is not attended by changes in the peripheral leukocytic or lymphocytic counts but that total-body irradiation is followed by severe leukopenia and lymphopenia, and (d) that administration of zymosan is followed by marked lymphoid hyperplasia of the spleen and lymph nodes, while total-body irradiation is followed by severe atrophy of these organs. The theoretical considerations are (a) that lymphocytes may in some way be connected with maintaining an adequate properdin level of the blood and (b) that the action of zymosan and irradiation, in decreasing the animal resistance to the heterologously transplanted tumor, may be mediated through the properdin system. Whether these theories are or are not correct can be determined only by further investigation.

SUMMARY

The possible role of the properdin system in the growth of heterologously transplanted tumors was investigated by observing the effects of zymosan on the transplantable human carcinoma of the colon HR132 in normally resistant weanling Wistar rats.

Out of a total of 160 animals in each category, the number of tumor “takes” was as follows: nineteen in nontreated animals, 196 in irradiated animals, 58 in animals given a single injection of zymosan, and 90 in animals given multiple injections of zymosan.

From the data presented, it seems reasonable to conclude that zymosan is effective in increasing the susceptibility of weanling Wistar rats to HR132. Whether this conditioning is mediated through the properdin system or through some other mechanism remains to be determined.

REFERENCES

Fig. 4.—Atrophic spleen from an irradiated rat. × 100.
Fig. 5.—Atrophic lymph node from an irradiated rat. × 100.
Fig. 6.—Hyperplastic spleen from a rat treated with a single injection of zymosan at 175 mg/kg of body weight. × 100.
Fig. 7.—Hyperplastic lymph node from a rat treated with a single injection of zymosan at 175 mg/kg of body weight. × 100.
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