Complete Regressions of an Established Murine Fibrosarcoma Induced by Systemic Application of Corynebacterium granulosum

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SUMMARY

C3Hf/Bu mice were given s.c. injections of cells of a syngeneic methylcholanthrene-induced fibrosarcoma, and 3, 7, or 13 days later were given i.v. injections of 0.25 mg Corynebacterium granulosum. The growth of tumors was followed for 100 days. C. granulosum, given at 3 and 7 days following transplantation of tumor cells, induced complete tumor regressions in 34 and 46% of the mice, respectively. Almost all regressions occurred during the second month from the tumor cell inoculation. The remaining tumors grew more slowly than those in untreated controls. Treatment of mice 13 days after tumor cell injection, when the tumors were between 4 and 9.5 mm in diameter, greatly slowed the growth of most tumors. Histology of regressing tumors revealed a heavy infiltration with lymphocytes, histiocytes, and macrophages.

INTRODUCTION

In a previous publication (15), we reported that mice treated with C. granulosum strongly resisted tumor growth induced by i.v., i.p., and s.c. injection of cells from a syngeneic FSA. A peculiar observation of that study was that s.c. injections of tumor cells generated tumors equally in normal and in C. granulosum-treated mice, but when the tumors in animals receiving this immunostimulant enlarged to a certain size (7 to 14 mm in diameter), more than 50% of the tumors regressed totally. The present investigation shows that complete regressions of s.c.-growing FSA can be achieved if C. granulosum is administered after tumor cells, even when the tumors have established their growth.

MATERIALS AND METHODS

Mice. The animals were 3-month-old male mice of inbred strain C3Hf/Bu from our own specific-pathogen-free mouse colony. (The mice carry only the following enteric bacteria: Clostridium sp., Peptostreptococcus sp., Bacillus sp., and Bacteroides sp.) During the experiments, 5 mice were kept to a cage.

Tumor. The FSA was induced in a young C3Hf/He female mouse by a single s.c. injection of 1 mg of methylcholanthrene suspended in peanut oil (19). The 1st- through 4th-generation isotransplants of this tumor had been kept in a liquid nitrogen refrigerator and the experiments were performed with tumors of the 5th generation. The tumor contains relatively strong tumor-specific antigen(s) as shown by different methods performed both in vivo (14, 18) and in vitro (11).

The suspension of single cells was prepared by digestion with trypsin of nonnecrotic tumor tissue; the method was described in detail previously (14, 15). Viability of the cells was more than 95% as assessed by trypan blue exclusion and by phase-contrast microscopy. To generate tumors, 4 x 10⁶ viable tumor cells were inoculated into the s.c. tissue of the right flanks of mice. The tumor cells were injected in a volume of 0.2 ml of Medium 199 (Difco Laboratories, Inc., Detroit, Mich.).

C. granulosum. Formol-killed C. granulosum bacteria were kindly supplied by Professor M. Raynaud, Pasteur Institute, Garches, France (15). The immunostimulant was diluted with Solution A (8.0 g NaCl, 0.4 g KCl, 1.0 g glucose, and 0.35 g NaHCO₃ in 1000 ml water) so that each mouse received i.v. 0.25 mg of bacteria in 0.4 ml.

Histology. A regressing tumor was removed and fixed in buffered formalin at pH 7.0. Sections of the tumor tissue (5 μm) were stained with hematoxylin-eosin.

Tumor Growth and Regression. To obtain tumor growth curves, 3 mutually orthogonal diameters of tumors were measured with vernier calipers and the mean values were calculated.

RESULTS

Regressions of FSA in C. granulosum-treated Mice. Fifty-three mice were each given injections of 4 x 10⁵ viable FSA cells. Groups of 14, 13, and 13 mice were given i.v. injections of 0.25 mg of C. granulosum 3, 7, and 14 days later, respectively. The remaining 13 mice were left as untreated controls. The size of tumors was checked twice weekly for 100 days. Chart 1 presents the percentage of mice with tumors in all 4 groups during the period of observation, and Charts 2, 3, and 4 trace the growth of individual tumors in treated mice. The appearance of tumors was similar in mice of all groups:
Chart 1. Percentage of FSA's in C3Hf/Bu mice following treatment with *C. granulosum*. Normal controls (—); *C. granulosum* given at Days 3 ( ), 7 ( — ), or 13 ( — ). Arrows, time of application of *C. granulosum*.

Chart 2. Growth of FSA in C3Hf/Bu mice, normal (—) or treated with *C. granulosum* 3 days after inoculation of tumor cells ( — ). Vertical bars on control curve, S.E. The curves for treated mice trace the growth pattern of individual tumors. Arrow, time of application of *C. granulosum*.

Palpable tumors had developed between 7 and 13 days following tumor cell inoculation. In control mice, the tumors grew progressively, killing 12 out of 13 recipients between 46 and 75 days (mean, 57 ± 3 days). The remaining mouse had, at 100 days, a tumor 42 mm in diameter. Tumors in mice given *C. granulosum* 3 days after tumor cells grew as those in untreated controls for about 15 days, but then most of the tumors began to regress. One tumor regressed completely by 23 days, and 6 more regressed by 61 days following transplanting of tumor cells. One tumor that had grown to 9.5 mm in diameter and then regressed to 5 mm was taken for histology and is excluded from data presented in Charts 1 and 2. No more regressions were noted after 2 months from tumor cell inoculation. At about this time, tumors that had partially regressed began to regrow. All mice, with the exception of 1 that died at 61 days, survived 100 days after tumor cell injection.

A similar pattern of growth of tumors was observed in mice treated with *C. granulosum* at 7 days. At this time, 9 out of 13 mice had developed tumors and the diameter of tumors ranged between 3 and 4.5 mm. Six of 13 tumors (46%) regressed...
completely within the 2 months of tumor cell inoculation. Three mice died before 100 days, at 71, 90, and 96 days, respectively.

All mice given *C. granulosum* 14 days after tumor cells had tumors measuring 4 to 9.5 mm in diameter (average, 7.4 mm). Treatment with *C. granulosum* slowed the growth of some tumors, induced temporary regressions of some others, but caused no complete regressions. Only 6 mice died before 100 days, and they died between 46 and 96 days (mean, 73 ± 7 days).

**Histology of the Regressing FSA.** Fig. 1 presents the histological features of FSA in normal, untreated mice. Many dividing cells are present reflecting progressive growth of the tumor.

Figs. 2 through 7 show the histology of FSA regressing in a mouse that received *C. granulosum* 3 days following tumor cell inoculation. Tumor tissue is almost totally replaced by lymphoid cells and fibrous tissue (Fig. 2). The cells are predominantly lymphocytes (Figs. 2 and 3), but numerous histiocytes (Fig. 3) and macrophages were found throughout the regressing tumor. Fig. 4 shows a concentration of macrophages; some of them contain phagocytosed material. Numerous polymorphonuclear cells (Fig. 5) and some plasma cells were also observed. Infiltration with lymphocytes and other cell types was particularly heavy at the margins of necrosis within the regressing tumor (Figs. 6 and 7). It can be seen that many macrophages entered necrotic regions where they engulfed cellular debris.

**DISCUSSION**

Non-specific stimulators of the reticuloendothelial system, in particular BCG and anaerobic corynebacteria, exert an antitumor adjuvant activity. Animals treated with the immunostimulants resist, in various degrees, subsequent inoculation of tumor cells, as evidenced by the reduced “tumor take,” slowed growth of the tumors, and prolonged survival of recipients (1, 7, 16, 17, 22, 23). Treatment initiated after injection of tumor cells usually resulted either in no effect or in a slight slowing of tumor growth. However, complete regressions of established tumors were described to occur after treatment with BCG (24) or *Corynebacterium parvum* (13), but only if the immunostimulants were injected into the tumor. This paper presented data showing that systemic application of *C. granulosum* can lead to complete regressions of s.c.-established tumors in mice, an observation not reported thus far for any other non-specific stimulator of the reticuloendothelial system. The effect of *C. granulosum* was greater if the interval between tumor cell inoculation and treatment with the immunostimulants was shortened; complete regressions were observed with treatment at 3 and at 7 days, but not at 13 days after tumor cell transplantation. Treatment at 13 days, however, caused partial, temporary regressions of some tumors and slowed the growth of most of the remaining tumors. Although slowing in the growth of tumors was already observable 11 days after treatment, almost all regressions occurred during the 2nd month following tumor cell inoculation.

Histology of the regressing tumor revealed heavy infiltration with lymphocytes, histiocytes, and macrophages and, to a lesser extent, with polymorphonuclears and plasma cells. The individual roles of these cells in causing the regression is not known, but most of them are presumably engaged at the effector level of tumor rejection. Thymus-derived lymphocytes are generally considered to play a predominant role in destruction of tumor cells (5, 6) but, recently, B-cells have also been found to be cytotoxic (12). Our recent report that spleen cells from mice immunized against FSA are more effective in transferring immunity against FSA pulmonary metastases suggests that B-cells might play a more crucial role in this tumor-animal system than do T-cells (14). That cells other than T-lymphocytes might be responsible for *C. granulosum*-induced antitumor activity is also suggested by the recent observations (to be published) that mice treated with this immunostimulant tolerate skin allografts longer than do untreated mice and that their lymphocytes exhibit a decreased response to stimulation by phytohemagglutinin.

Histiocytes are numerous in the regressing tumor. However, little is known about the role of these cells in immunological reactions. They are found to accumulate in tissues in which an immunological reaction of the delayed type occurs (21), even forming granulomas (4). Hanna *et al.* (8) have found that tumors and the draining lymph nodes are heavily infiltrated with histiocytes following injection of BCG into the tumor. The regression of tumors following this treatment was ascribed by the authors to the histiocyte response. Macrophages are also numerous in the regressing tumor and some of them were seen to contain cellular debris (Fig. 5). These cells are definitely able to destroy tumor cells, in either an immunologically specific (3) or a non-specific manner (10). We have observed recently that peritoneal macrophages from *C. granulosum*-treated mice are able to destroy Chinese hamster ovary cell cultures *in vitro* but are not able to destroy cultures of syngeneic fibroblasts (2).

Regression of the tumors was not observed after 2 months of tumor growth. Some tumors that had regressed from about 14 mm to as small as about 5 mm started to regrow at this time. There could be several reasons for this. First, the tumor bearer might have exhausted its lymphoid system during the 2 months of “struggle” with tumor cells. If this were so, it would be of great importance to determine whether multiple injections of the immunostimulant given to mice within the 1st 2 months would prevent the regrowth of regressing tumors. Another possibility would be that the tumors regrew from cells that are less antigenic and escaped immunological destruction. Immunological enhancement could be one further possibility, but if this were so then it is difficult to explain the regression that preceded the regrowth. Also, our previous observations (14) and those by Vaage (20) show that the humoral antibodies are unlikely to be responsible for the relentless growth of this tumor.

Some tumors that regressed were over 1 cm in diameter. According to Hewitt (9), a spherical tumor 1 cm in diameter that consists half of stroma and half of reproductively intact tumor cells having a mean cell diameter of 12 μm contains approximately 10^8-10^9 tumor cells. The elimination of such a large number of tumor cells could be anticipated only by an immunological reaction that is operative for a longer time. However, some non-specific nonimmunological events that accompany immunological attack might also be considered as
a cause for the observed regressions. During the immunological reaction, many nonspecifically toxic compounds are released and they can damage surrounding cells, both malignant and normal (1). Small blood vessels could also be damaged in this manner, causing small areas of necrosis.

These data, in addition to those previously reporting that C. granulosum induced complete regressions of pulmonary metastases of FSA (15), show that this immunostimulant exhibits very strong antitumor activity. The observations could be of great importance for immunotherapy of malignant tumors in man, i.e., tumors growing at remote sites that might be efficiently fought by systemic application of nonspecific immunostimulants.

REFERENCES


Fig. 1. Histology of FSA growing in a normal C3Hf/Bu mouse. No infiltration with lymphoid cell can be seen. X 115.

Fig. 2. Histology of FSA regressing in a C3Hf/Bu mouse treated with C. granulosum (3-day group). Heavy infiltration with mononuclear cells. X 115.

Fig. 3. Histology of FSA regressing in a C3Hf/Bu mouse treated with C. granulosum (3-day group). Infiltration with small lymphocytes and histiocytes. X 350.

Fig. 4. Histology of FSA regressing in a C3Hf/Bu mouse treated with C. granulosum (3-day group). Accumulation of macrophages, some of which contain phagocytosed cellular debris. X 350.

Fig. 5. Histology of FSA regressing in a C3Hf/Bu mouse treated with C. granulosum (3-day group). Accumulation of polymorphonuclears. X 350.

Fig. 6. Histology of FSA regressing in a C3Hf/Bu mouse treated with C. granulosum (3-day group). Heavy infiltration with mononuclear cells at margin with necrosis. X 115.

Fig. 7. Histology of FSA regressing in a C3Hf/Bu mouse treated with C. granulosum (3-day group); margin with necrosis. Macrophages are extending into necrotic area and contain cellular debris. X 350.

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