

MECHANISMS OF ANTI-TUMOR ACTION OF *CORYNEBACTERIUM PARVUM*

I. Potentiated Tumor-specific Immunity and Its Therapeutic Limitations*

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Since the original description of the therapeutic action of *Corynebacterium parvum* in a syngeneic murine tumor system over a decade ago (1), many reports have appeared (2–4) describing the anti-tumor action of this agent against syngeneic and autochthonous tumors in animals, and against tumors in man. The efficacy of anti-tumor therapy with *C. parvum* varies greatly among different tumor-host systems and depends on such factors as tumor immunogenicity (3, 5), tumor size and location (2, 3, 6), the amount of *C. parvum* used (7, 8), and its route of administration (2). Depending upon the route of administration, *C. parvum* may elicit different anti-tumor mechanisms (2). Intravenous administration appears to favor the generation of nonspecific effector mechanisms, which are expressed by activated macrophages or natural killer cells (2, 3, 9–11). Intralesional therapy with *C. parvum*, on the other hand, is thought to favor the generation of T cell-mediated anti-tumor immunity, as evidenced by the finding that the anti-tumor action depends on the presence of a functional T cell system (8, 12, 13). Moreover, it has been shown that animals that have regressed their tumors after intralesional therapy with *C. parvum* display specific resistance to the growth of a tumor challenge implant (7, 14), and possess T cells that are capable of passively transferring anti-tumor resistance to normal recipients (12, 14). On the other hand, *C. parvum* has also been shown to enhance the growth of tumors, presumably because of its capacity to induce the generation of suppressor mechanisms (15, 16).

The main purpose of this paper is to describe a model currently being used to analyze the potential and limitations of tumor immunotherapy with *C. parvum*. It will show that when P815 tumor cells are admixed with *C. parvum* and implanted subcutaneously, the tumor that emerges grows for 9 d and then regresses. It will also show that the mechanism responsible for this regression is capable of causing the regression of an untreated test tumor growing at a distant site, and that this therapeutic action is based on a T cell-mediated immune response to tumor-specific transplantation antigens. It will show, finally, that the immunotherapeutic action of the *C. parvum*-tumor cell admixture against a distant test tumor is limited to a test tumor below a certain critical size. The companion paper (17) will deal with the reasons for this therapeutic limitation.

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Materials and Methods

Mice. B6D2 (C57BL/6 \times DBA/2) F_1 , CB6 (BALB/c \times C57BL/6) F_1 , DBA/2, and BALB/c mice of either sex were used when they were 8–12 wk of age. They were produced in the Animal Breeding Facility of the Trudeau Institute.

Tumors. The P815 mastocytoma syngeneic in DBA/2 mice was originally obtained from Dr. Virginia Evans of the National Cancer Institute, Tissue Culture Section, Bethesda, Md. A methylcholanthrene-induced tumor (Meth A fibrosarcoma)¹ syngeneic in BALB/c mice was obtained from Dr. Lloyd J. Old of the Memorial Sloan-Kettering Cancer Center, New York. The CaD2 mammary carcinoma syngeneic in DBA/2 mice was obtained from The Jackson Laboratory, Bar Harbor, Maine, and the BP3 fibrosarcoma was induced in C57BL/6 mice by subcutaneous injection of benzpyrene. The CaD2 and BP3 tumors were shown previously to be nonimmunogenic (18).

The P815 mastocytoma and the Meth A fibrosarcoma were subcultured for several weeks in Fisher's medium (Grand Island Biological Co., Grand Island, N. Y.) containing 15% heat-inactivated fetal calf serum (FCS) before being passaged several times as ascites in the peritoneal cavities of syngeneic mice. Ascites Meth A and P815 tumor cells, and BP3 and CaD2 cells grown in vitro were harvested and resuspended in Fisher's medium containing 20% FCS and 10% dimethyl sulfoxide, dispensed into a large number of small vials, and cryopreserved over liquid nitrogen. Before each experiment, a vial was thawed and the cells were washed in Dulbecco's phosphate-buffered saline (PBS). For experiments, the P815 mastocytoma and the Meth A fibrosarcoma were grown as ascites for 6 d in B6D2 mice, and harvested in PBS containing 10 U heparin/ml. After two washes in PBS, they were resuspended to the desired concentration in PBS for injection. The CaD2 and BP3 tumors were grown in vitro in RPMI 1640 containing 15% heat-inactivated horse serum. Confluent monolayers were harvested from disposable plastic tissue culture flasks (3024; Falcon Labware, Div. of Becton Dickinson & Co., Oxnard, Calif.) in trypsin-EDTA (Grand Island Biological Co.). The trypsinized cells were washed twice in PBS containing 1% FCS and resuspended to the desired concentration in PBS for implantation. Tumors were initiated in the hind footpads of mice by implanting the desired number of cells in a volume of 0.05 ml PBS with a 30.5-gauge needle. The growth of footpad tumors was monitored by measuring increases against time in the dorsoventral thickness of the foot with dial calipers.

Irradiation of Cells. P815 ascites tumor cells were harvested in PBS containing 10 U heparin/ml, and after several washes in PBS they were resuspended in RPMI 1640 containing 5% FCS at 10^7 cells/ml. The cells were then exposed to 4,500 rad of gamma rays from a cesium-137 irradiator at a midphantom dose rate of 29.5 rad/min. They were then washed twice in PBS and resuspended in PBS for implantation. Pilot experiments showed that 10^7 irradiated tumor cells implanted in a hind footpad failed to form a tumor during a 60-d period of observation.

T Cell-deficient Mice. 4-wk-old mice were rendered T cell deficient by thymectomy, followed 7 d later by lethal (900 rad) whole-body gamma irradiation. They were infused intravenously immediately after irradiation with 10^7 syngeneic bone marrow cells and used in experiments 6 wk later.

C. parvum. Formalin-killed *C. parvum* was supplied as a suspension (7 mg/ml) in physiologic saline by Dr. R. L. Tuttle of the Burroughs Wellcome Company, Research Triangle Park, N. C. Lot 771/A was used in all experiments. An admixture containing a known number of replicating, or gamma-irradiated, nonreplicating tumor cells with a known weight of *C. parvum* was injected subcutaneously into the hind footpads of mice.

Passive Transfer of Anti-Tumor Resistance. Cells were obtained from the spleens and draining lymph nodes of tumor-immune donors (see Results). To harvest the cells, spleens and lymph nodes were diced into small pieces and gently pushed through a 200-mesh stainless steel screen into cold PBS that contained 1% heat-inactivated FCS. The suspension was triturated with a Pasteur pipette to break up clumps, and passed through several layers of sterile surgical gauze. Cells were washed and resuspended to the desired concentration in PBS for intravenous infusion. Sera from the same tumor-immune donors were recovered from clotted blood and

¹ Abbreviations used in this paper: FCS, fetal calf serum; Meth A, methylcholanthrene A; PBS, phosphate-buffered saline.

stored at -70°C in 1.0-ml aliquots. The aliquots were thawed and injected intravenously as needed.

Recipient mice were infused intravenously with 1.5×10^8 spleen cells, or 5×10^7 lymph node cells from tumor-immune donors 1 h before receiving a challenge implant of 10^6 P815 tumor cells in a hind footpad. The protective ability of serum from tumor-immune donors was tested by giving recipients 0.5 ml intravenously 1 h before the challenge implant, and 1 and 2 d later. In some experiments, mice that received serum were pretreated 7 d earlier with an intradermal injection of $100 \mu\text{g}$ *C. parvum*. The growth of the challenge implant was monitored by measuring increases against time in the dorsoventral thickness of the foot with dial calipers.

Treatment with Anti-Thy-1.2 Antibody. Lyophilized anti-Thy-1.2 monoclonal IgM antibody (Sera Lab, Accurate Chemical and Scientific Corporation, Hicksville, N. Y.) was dissolved in a volume of distilled water equal to the original volume of ascites. The antibody solution was aliquoted and stored at -70°C until needed. When tested against mouse thymocytes, the antibody had a cytotoxic titer of $>1:50,000$. Lymph node cells were treated at $5 \times 10^7/\text{ml}$ with a 1:1,000 dilution of the antibody for 45 min at 10°C . The cells were then washed and incubated for 30 min at 37°C in the same volume of a 1:15 dilution of rabbit serum (Low-Tox-M rabbit complement; Accurate Chemical and Scientific Corporation) as a source of complement. After washing twice in PBS containing 5% FCS, the cells were resuspended to the desired concentration in PBS for intravenous infusion.

Results

Tumor Growth and Regression in the Presence of *C. parvum*. Earlier studies conducted in this laboratory (19) revealed that the implantation of 10^6 Meth A tumor cells admixed with $20 \mu\text{g}$ *C. parvum* resulted in 7–9 d of tumor growth, followed by tumor regression over the next 2–3 wk. Confirmation of this finding with the P815 is shown in Fig. 1, which compares the growth of 2×10^6 P815 tumor cells with 2×10^6 Meth A tumor cells when both types of tumor cells were implanted as admixtures with $100 \mu\text{g}$ *C. parvum*. It can be seen with both tumors that tumor growth in the presence of *C. parvum* was similar to the growth of control tumors over the first 9 d. After this time, however, the tumors containing *C. parvum* regressed, whereas control tumors grew progressively to kill their hosts. The percentage of regressed tumors that regrew after 30 d varied from 0 to 40% between experiments.

Therapeutic Effect of *C. parvum*-Tumor Cell Admixture on a Distant Tumor. As the first

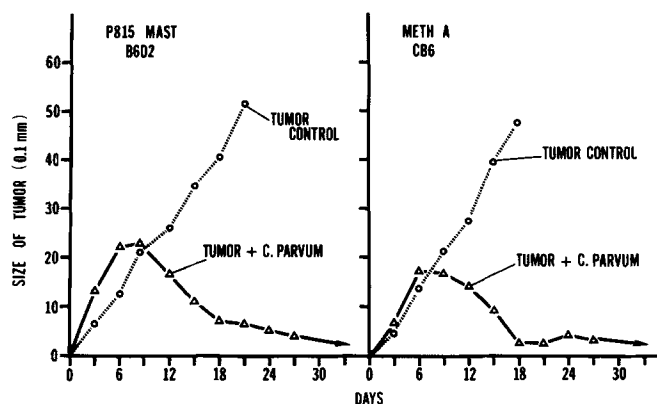


FIG. 1. Evidence that when 2×10^6 P815 mastocytoma cells are admixed with $100 \mu\text{g}$ *C. parvum* (left panel) or 2×10^6 Meth A fibrosarcoma cells are admixed with $100 \mu\text{g}$ *C. parvum* (right panel) and injected into a hind footpad, the tumor that emerges grows progressively for 9 d and then completely regresses. Means of five mice per time-point.

step in investigating the mechanisms of *C. parvum*-induced regression, experiments were designed to determine whether the regression of the tumor that contained *C. parvum* was a local phenomenon, or whether it was associated instead with the generation of an anti-tumor mechanism capable of causing the regression of an untreated test tumor growing simultaneously at a distant site. This involved injecting mice in the right hind footpad with an admixture of 2×10^6 tumor cells and 100 μg *C. parvum*, and in the left hind footpad with 2×10^6 tumor cells alone. It can be seen in Fig. 2 that the treated and untreated tumors behaved similarly. In both cases, a 9-d period of tumor growth was followed by progressive and complete tumor regression. Fig. 2 shows, in addition, that tumor cells needed to be admixed with *C. parvum* to cause the regression of a distant test tumor, because the injection of 100 μg *C. parvum* alone in the right hind footpad had no effect on the growth of a tumor in the contralateral side. As was the case with the tumors that arose from tumor-*C. parvum* admixtures, a variable proportion (0–30%) of the test tumors that regressed began to regrow after 28 d.

Requirement for Tumor Immunogenicity to Induce Systemic Anti-Tumor Resistance. The preceding results show that tumor cells and *C. parvum* must be injected as an admixture to cause a distant tumor to regress. The experiments in this section were designed to determine whether this therapeutic action of *C. parvum* depends on the tumor being immunogenic. To investigate this, the nonimmunogenic BP3 fibrosarcoma was used. Mice were injected in the right hind footpad with 100 μg of *C. parvum* admixed with 2×10^6 BP3 cells and in the left hind footpad with 2×10^6 BP3 cells alone. The results in Fig. 3 show that although the rate of growth of this tumor in the presence of *C. parvum* was slower after day 9, growth of the untreated tumor in the contralateral footpad was unaffected. Based on this result, and a similar result (data not shown) with the nonimmunogenic CaD2 mammary carcinoma, it is apparent that the therapeutic action of an admixture of tumor cells and *C. parvum* against a distant tumor is limited to immunogenic tumors. It is apparent that the reduced rate of growth of the tumor containing *C. parvum* was caused by nonspecific mechanisms (19) that developed locally in response to *C. parvum*.

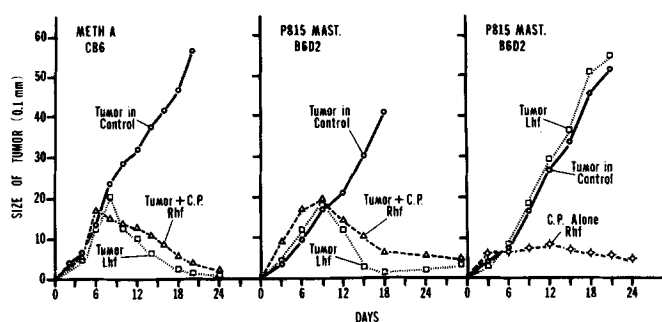


FIG. 2. Evidence that growth and regression of a *C. parvum*-treated tumor in the right hind footpad are concordant with growth and regression of an untreated tumor in the contralateral footpad. Mice were implanted with 2×10^6 tumor cells admixed with 100 μg *C. parvum* in the right hind footpad and with 2×10^6 tumor cells alone in the left hind footpad. Tumor growth and regression in these mice was compared with the growth of 2×10^6 tumor cells in control mice. The right panel shows that implanting 100 μg *C. parvum* alone in the right hind footpad had no effect on the growth of 2×10^6 P815 cells implanted at the same time in the left hind footpad. Means of five mice per time-point.

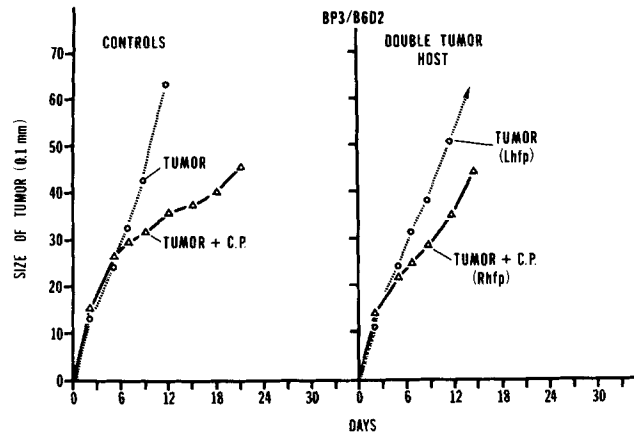


FIG. 3. Evidence that the therapeutic action of *C. parvum* depends on the tumor being immunogenic. The right panel shows that implanting 2×10^6 BP3 fibrosarcoma cells admixed with $100 \mu\text{g}$ *C. parvum* in the right hind footpad had no effect on growth of 2×10^6 BP3 cells implanted alone in the left hind footpad. The left panel shows the behavior of 2×10^6 BP3 cells implanted either alone or admixed with $100 \mu\text{g}$ *C. parvum*. Means of five mice per time-point.

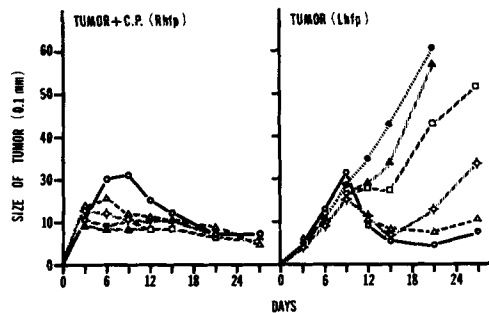


FIG. 4. Evidence that implanting gamma-irradiated, nonreplicating tumor cells admixed with *C. parvum* can induce regression of a distant test tumor. The effect of injecting decreasing numbers of irradiated tumor cells admixed with $100 \mu\text{g}$ *C. parvum* in the right hind footpad on the growth of a contralateral test tumor implanted at the same time in the left hind footpad is shown. Admixtures containing either 10^7 irradiated P815 cells or 2×10^6 replicating P815 cells caused regression of the test tumor. Progressively decreasing the number of nonreplicating tumor cells in the admixture resulted in progressive reductions in its therapeutic effect against the contralateral test tumor. Means of five mice per time-point. Irradiated P815 cells: Δ , 10×10^6 ; \diamond , 5×10^6 ; \square , 2.5×10^6 ; \blacktriangle , 1.2×10^6 ; \circ , 2×10^6 viable P815 cells.

Substitution of Gamma-irradiated Tumor Cells for Replicating Tumor Cells in the Therapeutic Admixture. Studies by others (8, 14) have shown that local implantation of either replicating tumor cells or nonreplicating tumor cells admixed with *C. parvum* can render mice immune to the growth of a tumor implant. The results shown in Fig. 4 are in keeping with these findings. They show that 10^7 irradiated, nonreplicating P815 tumor cells admixed with *C. parvum* were as effective as 2×10^6 replicating tumor cells in the admixture in causing the regression of a P815 test tumor growing in the contralateral footpad. Fig. 4 shows, in addition, that decreasing the number of irradiated tumor cells in the admixture resulted in a decreasing therapeutic effect against the contralateral test tumor. This result indicates that the strength of the

therapeutic response engendered by the admixture was determined by the quantity of tumor antigens.

Immunotherapeutic Action of C. parvum-Tumor Cell Admixture Is T Cell Dependent. The foregoing results indicate that the therapeutic action of an admixture of tumor cells and *C. parvum* against a distant test tumor is based on an immune response to tumor antigens. The experiments in this section were designed to determine whether this immune response is T cell dependent. The first experiment was designed to investigate whether the *C. parvum*-tumor cell admixture engendered a therapeutic response in mice made T cell deficient by thymectomy and irradiation, or in mice that were immunosuppressed by whole-body gamma irradiation. It can be seen in Fig. 5 that neither T cell-deficient mice, nor gamma-irradiated mice were capable of causing the regression of the tumor that emerged from the therapeutic admixture, or the test tumor growing in the contralateral side. These results clearly demonstrate the need for an intact T cell system for *C. parvum*-induced regression.

The second experiment was designed to determine whether the onset of *C. parvum*-induced tumor regression was concordant with the acquisition of T cells capable of adoptively immunizing normal recipients against the growth of a tumor cell implant. Mice were implanted in one hind footpad with an admixture containing 2×10^6 P815 cells and 100 μ g *C. parvum*, and in the contralateral footpad with 2×10^6 P815 cells alone. These mice served as donors of serum, spleen cells, and lymph node cells for passive transfer into recipients that received a tumor challenge implant in the left hind footpad. The results of an experiment that used cells harvested from donors at the onset of *C. parvum*-induced tumor regression on day 9, are presented in Fig. 6, where it can be seen that passive transfer of 1.5×10^8 spleen cells, or 5×10^7 lymph node cells adoptively immunized normal recipients against growth of the challenge implant. In contrast, recipients infused intravenously with 0.5 ml of "immune" serum at the time of giving the challenge implant, and 24 and 48 h later, failed to express anti-tumor immunity. Mice pretreated with 100 μ g *C. parvum* 7 d before treatment with immune serum also failed to suppress the growth of the challenge implant.

That the adoptive anti-tumor resistance was mediated by T cells is shown in Fig.

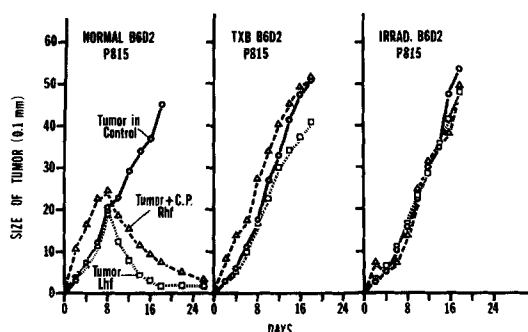


FIG. 5. Evidence that an intact T cell system is necessary for a *C. parvum*-tumor admixture to engender a therapeutic response. Mice made T cell deficient (TXB) by whole-body gamma irradiation and protected with bone marrow, or mice subjected to 900 rad irradiation 1 h before the experiment, were unable to regress the tumor that emerged from the admixture in the right hind footpad, or from P815 cells alone in the left hind footpad. Means of five mice per time-point.

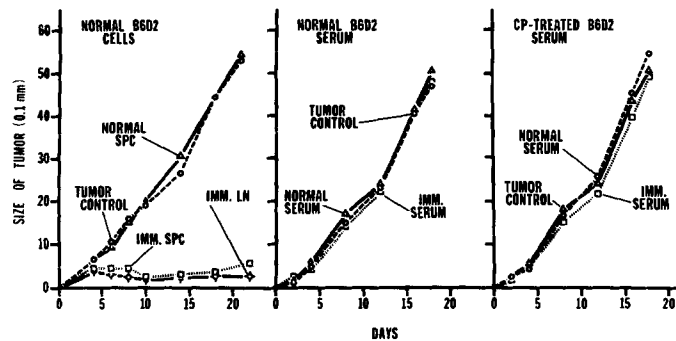


FIG. 6. Evidence that lymph node cells (LN) and spleen cells (SPC), but not serum, from donors that were in the process of simultaneously regressing a *C. parvum*-containing tumor and a contralateral test tumor were capable of adoptively immunizing normal recipients against the growth of a tumor implant. Recipients received either 1.5×10^8 spleen cells, or 5×10^7 lymph node cells intravenously 1 h before challenge with P815 cells in the left hind footpad. Recipients of serum received three 0.5-ml infusions of serum on d 0, 1, and 2 of tumor growth. Serum failed to transfer any anti-tumor immunity even when it was infused into mice pretreated 7 d earlier with a subcutaneous injection of 100 μ g *C. parvum* (right panel). Means of five mice per time-point.

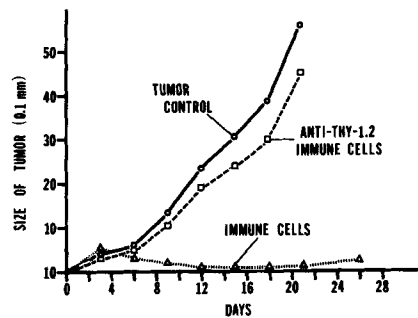


FIG. 7. Evidence that T cells are responsible for the therapeutic action of the *C. parvum*-tumor admixture against a distant test tumor. Lymph node cells (10^6) harvested from donors regressing their tumors failed to passively transfer immunity to growth of a tumor implant if the cells were treated with anti-Thy-1.2 and complement. Means of five mice per time-point.

7, where it can be seen that treatment of lymph node cells from 9-d donors with Thy-1.2 serum and complement completely abolished their protective capacity.

Specificity of *C. parvum*-potentiated Anti-Tumor Immunity. The foregoing results are consistent with the interpretation that *C. parvum*-induced anti-tumor resistance results in the generation of a population of T cells sensitized to tumor-specific transplantation antigens. The purpose of the experiments in this section was to determine whether these T cells are specific for the tumor that elicits their production. This was investigated by determining whether the response generated against the P815 mastocytoma admixed with *C. parvum* would cause the regression of an unrelated test tumor growing at a distant site on the same host. Mice injected intradermally in the belly region with 2×10^6 P815 cells admixed with 100 μ g *C. parvum* were injected with 10^6 P815 cells in the left hind footpad, and with 2.5×10^5 CaD2 tumor cells in the right hind footpad. Control tumor growth was measured in mice injected in a hind footpad with either test tumor alone. It can be seen in Fig. 8 that the therapeutic action of the *C. parvum*-augmented anti-tumor response was specific for the P815 test

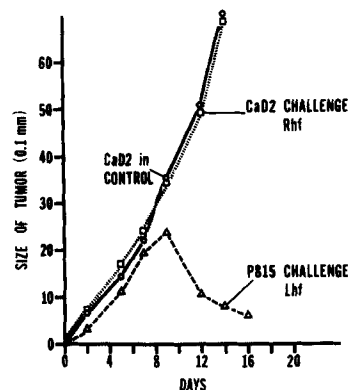


FIG. 8. Evidence showing that the anti-tumor response elicited by the admixture of *C. parvum* and P815 cells is specific for the P815 tumor. Mice injected intradermally with 2×10^6 P815 cells admixed with $100 \mu\text{g}$ *C. parvum* were implanted in the right hind footpad with 2.5×10^5 CaD2 cells, and in the left hind footpad with 10^6 P815 cells. The response to the admixture caused the P815 test tumor to regress after 9 d of growth, but had no effect on growth of the CaD2 test tumor. Means of five mice per time-point.

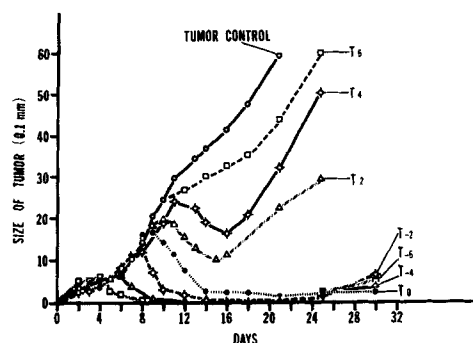


FIG. 9. Effect on growth of a test tumor in the left hind footpad when the therapeutic admixture containing 2×10^6 P815 cells plus $100 \mu\text{g}$ *C. parvum* was injected in the right hind footpad 6, 4, or 2 d before, or at the time of, or 2, 4, or 6 d after implanting the test tumor. The later the therapeutic admixture was given with respect to implanting the test tumor (10^6 P815 cells), the larger the test tumor grew before regression commenced and the less complete was regression. Except for the case of giving the admixture on day 6 of test tumor growth, the onset of regression always occurred between 8 and 10 d after injecting the admixture. Means of five mice per time-point.

tumor, in that the admixture of *C. parvum* and P815 cells had no therapeutic effect at all against the CaD2 tumor.

Limitation of Therapeutic Action of *C. parvum*-Tumor Cell Admixture. It has been reported on numerous occasions (2-4) that the intralesional injection of immunoadjuvants fails to cause the regression of tumors that have grown beyond a certain size. It was anticipated, therefore, that the immunotherapeutic effect of injecting an admixture of *C. parvum* and tumor cells would be similarly restricted to distant test tumors below a certain size. This was investigated by an experiment in which the therapeutic admixture was injected in the right hind footpad at different times before or after implanting the test tumor in the contralateral footpad. The results in Fig. 9 show that, whereas injecting the admixture before or at the time of implanting the test tumor resulted in complete regression of the test tumor, injecting the admixture after

implanting the test tumor resulted in partial or no regression. It can be seen that the onset of regression of the test tumor always occurred between 8 and 10 d after injecting the admixture. This meant that the earlier the admixture was given, the less time there was for the test tumor to grow, and the smaller it was at the time regression commenced. Practically no therapeutic effect was obtained when the admixture was given on the 6th d of growth of the test tumor. These results show, therefore, that immunotherapy with an admixture of *C. parvum* and tumor cells is limited to relatively small tumors.

Discussion

This paper shows that implanting P815 tumor cells, or Meth A fibrosarcoma cells admixed with *C. parvum* results in the emergence of a tumor that grows for a period of 9–10 d and then regresses. It shows, in addition, that regression of the *C. parvum*-treated tumor failed to occur in mice that were gamma-irradiated, or made T cell deficient by thymectomy and lethal irradiation, and restored with bone marrow cells. This evidence, together with the finding that the onset of *C. parvum*-induced tumor regression was associated with the generation in the spleen and draining node of T cells that were capable of adoptively immunizing normal mice against growth of a tumor implant, is consistent with the hypothesis that the therapeutic effect of *C. parvum* is based on its capacity to potentiate the generation of T cell-mediated anti-tumor immunity. The results are therefore in agreement with previous results from this (12, 14) and other laboratories (7, 8), showing that intratumor *C. parvum* favors the generation of T cell-mediated immunity. It will be shown in the following paper (17) that the onset of regression of a tumor containing *C. parvum* is preceded by an augmented production of T cells that are specifically cytolytic for P815 target cells in vitro.

This type of evidence does not prove, however, that the regression of the *C. parvum*-treated tumor was caused by T cell-mediated immunity. It is possible, instead, that the generation of T cell-mediated immunity was the result, rather than the cause, of tumor regression, in the same way that anti-tumor immunity develops after an immunogenic tumor is removed by ligation or excision (20, 21). It is possible, for example, that the tumor was caused to regress nonspecifically as the result of a mechanism directed against intratumor *C. parvum*. For this reason, it was significant to demonstrate that growth and regression of the *C. parvum*-treated tumor in one footpad was accompanied by concordant growth and regression of an untreated test tumor growing in the contralateral footpad. The close temporal relationship between the onset of regression of the treated tumor and distant test tumor favors the interpretation that the anti-tumor mechanism generated against the former was distributed systemically to cause regression of the latter. Because the passive transfer of large volumes of serum from mice whose *C. parvum*-treated tumors were regressing failed to confer any anti-tumor immunity on normal recipients, it is almost certain that the destruction of the distant test tumor was achieved by T cell-mediated immunity rather than humoral immunity. A similar therapeutic effect against a distant test tumor was demonstrated when gamma-irradiated, nonreplicating tumor cells were substituted for living tumor cells in the therapeutic admixture. The results indicate that this type of immunotherapy theoretically should be capable of specifically eliminating distant metastases.

Even so, the therapeutic effect of giving an admixture of replicating or nonreplicating tumor cells admixed with *C. parvum* is limited to test tumors of relatively small size. Indeed, in agreement with the findings of others (7, 8), the therapeutic effect of such an admixture was shown here to be greatest when the admixture was injected before the test tumor was implanted. The reason for this was shown by an experiment designed to determine the therapeutic effect of injecting the admixture at different times before and after implanting the test tumor in the contralateral foot. It was found that the onset of regression of the test tumor always occurred between 8 and 10 d after injecting the admixture. This meant that the earlier the admixture was given relative to implanting the test tumor, the less time there was for the test tumor to grow before the onset of regression. Consequently, injecting the admixture either before, or at the time of implanting the test tumor resulted in complete regression of relatively small tumors. In contrast, injecting the admixture after implanting the test tumor resulted in the partial regression of much larger tumors. It was not surprising to find, therefore, that practically no therapeutic effect was expressed at all against a 6-d test tumor. It is apparent, then, that immunotherapy of an established P815 tumor with an admixture of tumor cells and *C. parvum* is limited, at least in part, by the time it takes for T cell-mediated immunity to be generated in response to the admixture. The following paper (17) will show that it takes 9–10 d for the admixture to cause peak production of cytolytic T cells in the draining node and spleen. This means that injecting a therapeutic admixture of *C. parvum* and tumor cells with the hope of causing the regression of a 6-d test tumor represents an attempt to regress a tumor that will undergo an additional 9–10 d growth before enough cytolytic T cells are generated to destroy it. Considering the rapid rate at which most murine tumors grow, this would be an enormous tumor burden for immunotherapy to eliminate. Tumor burden, however, may not prove to be the major obstacle to immunotherapy. Recent work performed in this laboratory shows (22, 23), for example, that it is possible to cause the regression of very large tumors in recipient mice by the passive transfer of tumor-sensitized T cells from immune donors, provided the recipients have been made T cell deficient by thymectomy and irradiation. It was shown that passively transferred, sensitized T cells fail to cause regression of tumors in T cell-intact recipients because these recipients possess a tumor-induced state of T cell-mediated immunosuppression that inhibits the anti-tumor action of the infused effector cells. The possibility that this mechanism of immunosuppression is responsible for the failure of *C. parvum* to cause the regression of relatively large tumors must therefore be considered.

Summary

The anti-tumor mechanism in mice induced by a subcutaneous injection of syngeneic tumor cells admixed with *Corynebacterium parvum* was investigated. When mice were implanted in a hind footpad with 2×10^6 tumor cells admixed with 100 μ g *C. parvum*, the tumor that emerged grew progressively for about 9 d and then underwent progressive and complete regression. It was found that this *C. parvum*-induced regression was associated with the acquisition of a systemic, T cell-mediated mechanism of immunity to tumor-specific transplantation antigens, which enabled the host to cause the regression of an untreated test tumor growing simultaneously at a distant site. The generation of a *C. parvum*-potentiated anti-tumor response was

dependent on the presence of tumor cells in close association with *C. parvum*, tumor immunogenicity, and the quantity of tumor antigen in the admixture. The anti-tumor immunity was specific for the tumor in the therapeutic admixture and could be adoptively transferred to normal recipients with Thy-1.2-positive lymphocytes, but not with serum. Complete regression of a distant test tumor by the *C. parvum*-tumor admixture was limited to tumors below a certain critical size.

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References

1. Woodruff, M. F. A., and J. L. Boak. 1966. Inhibitory effect of injection of *Corynebacterium parvum* on the growth of tumor transplants in isogenic hosts. *Br. J. Cancer*. **20**:345.
2. Milas, L., and M. T. Scott. 1978. Antitumor activity of *Corynebacterium parvum*. *Adv. Cancer Res.* **26**:257.
3. Bast, R. C., B. S. Bast, and H. J. Rapp. 1976. Critical review of previously reported animal studies of tumor immunotherapy with nonspecific immunostimulants. *Ann. N. Y. Acad. Sci.* **277**:60.
4. Baldwin, R. W. 1978. Immunological adjuvants in cancer immunotherapy. *Dev. Biol. Stand.* **38**:3.
5. Parr, I. 1972. Response of syngeneic murine lymphomata to immunotherapy in relation to the antigenicity of the tumor. *Br. J. Cancer*. **26**:174.
6. Conley, F. K., and J. S. Remington. 1978. Effect of *Corynebacterium parvum* on tumor growth in the central nervous system of mice. *J. Natl. Cancer Inst.* **61**:827.
7. Scott, M. T. 1975. Potentiation of the tumor specific immune response by *Corynebacterium parvum*. *J. Natl. Cancer Inst.* **55**:65.
8. Bomford, R. 1975. Active specific immunotherapy of mouse methylcholanthrene induced tumors with *Corynebacterium parvum* and irradiated tumor cells. *Br. J. Cancer*. **32**:551.
9. Scott, M. T. 1975. *Corynebacterium parvum* as a therapeutic anti-tumor agent in mice. I. Systemic effects from intravenous injection. *J. Natl. Cancer Inst.* **53**:855.
10. Olivetto, M., and R. Bomford. 1974. *In vitro* inhibition of tumor cell growth and DNA synthesis by peritoneal and lung macrophages from mice injected with *Corynebacterium parvum*. *Int. J. Cancer*. **13**:478.
11. Ojo, E. 1979. Positive correlation between the levels of natural killer cells and the *in vivo* resistance to syngeneic tumor transplants as influenced by various routes of administration of *Corynebacterium parvum* bacteria. *Cell. Immunol.* **45**:182.
12. Tuttle, R. L., and R. J. North. 1976. Mechanisms of anti-tumor action of *Corynebacterium parvum*: replicating short-lived T cells as the mediators of potentiated tumor-specific immunity. *J. Reticuloendothel. Soc.* **20**:209.
13. Woodruff, M. F. A., and N. L. Warner. 1977. Effect of *C. parvum* on tumor growth in normal and athymic (nude) mice. *J. Natl. Cancer Inst.* **58**:111.
14. Tuttle, R. L., and R. J. North. 1976. Mechanisms of anti-tumor action of *Corynebacterium parvum*: the generation of cell-mediated tumor specific immunity. *J. Reticuloendothel. Soc.* **20**:197.
15. Bomford, R. 1977. An analysis of the factors allowing promotion rather than inhibition of tumor growth by *Corynebacterium parvum*. *Int. J. Cancer*. **19**:673.
16. Murahata, R. I., and J. Zigelboim. 1979. Inhibition of memory cell-mediated cytotoxic response by systemic administration of *Corynebacterium parvum*. *Cell. Immunol.* **42**:289.
17. Mills, C. D., R. J. North, and E. S. Dye. 1981. Mechanisms of anti-tumor action of *Corynebacterium parvum*. II. Potentiated cytolytic T cell response and its tumor-induced suppression. *J. Exp. Med.* **154**:621.

18. Berendt, M. J., R. J. North, and D. P. Kirstein. 1978. The immunological basis of endotoxin-induced tumor regression. Requirement for T cell-mediated immunity. *J. Exp. Med.* **148**:1550.
19. Tuttle, R. L., and R. J. North. 1975. Mechanisms of anti-tumor action of *Corynebacterium parvum*: nonspecific tumor cell destruction at the site of an immunologically mediated sensitivity reaction to *C. parvum*. *J. Natl. Cancer Inst.* **55**:1403.
20. Foley, E. J. 1953. Antigenic properties of methylcholanthrene-induced tumors in mice of the strain of origin. *Cancer Res.* **13**:835.
21. Prehn, R. T., and J. M. Main. 1957. Immunity to methylcholanthrene-induced sarcomas. *J. Natl. Cancer Inst.* **18**:769.
22. Berendt, M. J., and R. J. North. 1980. T cell-mediated suppression of anti-tumor immunity. *J. Exp. Med.* **151**:69.
23. Dye, E. S., and R. J. North. T cell-mediated immunosuppression as an obstacle to adoptive immunotherapy of the P815 mastocytoma. *J. Exp. Med.* In press.