MECHANISMS OF ANTI-TUMOR ACTION OF *CORYNEBACTERIUM PARVUM*

II. Potentiated Cytolytic T Cell Response and Its Tumor-induced Suppression*

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It was shown in the companion paper (1) that a correctly timed subcutaneous injection of *Corynebacterium parvum* admixed with P815 tumor cells on one side of a mouse can cause the complete regression of a P815 tumor growing subcutaneously on the contralateral side. In addition, it was shown that the expression of this therapeutic effect was associated with the generation by the host of a population of tumor-sensitized T cells that were capable of passively transferring a state of anti-tumor immunity to normal recipients. This evidence, together with the finding that the *C. parvum*-tumor admixture had no such therapeutic effect against a tumor growing in T cell-deficient mice, allowed the conclusion that the therapeutic action of C. parvum is based on its capacity to augment the generation of T cell-mediated immunity. It was also shown, however, that the therapeutic action of the *C. parvum*-tumor admixture was limited to tumors below a critical size, in that the admixture was without effect against a contralateral test tumor that had been growing for 5–6 d. There are two possible reasons for the loss of the test tumor's susceptibility to *C. parvum* immunotherapy. Either the tumor becomes physically too large for a *C. parvum*-potentiated immune response to cause its regression, or the tumor evokes the generation of a mechanism that actively inhibits the *C. parvum*-potentiated response.

The purpose of this paper is to show that the capacity of an admixture of *C. parvum* and tumor cells to cause the rejection of a small test tumor growing at a distant site is associated with the augmented production of cytolytic T cells in the lymph node draining the site of injection of the therapeutic admixture. It will also show that a potentiated cytolytic response fails to develop when the admixture is injected into mice bearing tumors that have been growing for 6 d. The evidence is consistent with the hypothesis that tumor immunotherapy with an immunopotentiator is limited to an early period of tumor growth before the tumor causes the host to generate a mechanism that inhibits a potentiated immune response to tumor-associated antigens.

Materials and Methods

Most of the procedures used were the same as those described in the preceding paper (1), except for those used to measure cellular cytotoxicity in vitro, and to deplete lymph node or spleen cell suspensions of B cells.

* Supported by grant CA-16642 from the National Cancer Institute, grant IM-266 from The American Cancer Society, and grant RR-05705 from the Division of Research Resources, National Institutes of Health.

‡ Supported by a fellowship from the J. M. Foundation.

^{51}Cr Release Assay. Cellular cytotoxicity was measured using a modification of the ^{51}Cr release assay described by Brunner et al. (2). Target tumor cells were grown in RPMI 1640 containing 15% heat inactivated horse serum (HS),[1] 100 U/ml penicillin, and 100 μg/ml streptomycin. These and other tissue culture reagents were purchased from Grand Island Biological Co., Grand Island, N. Y. Tumor cells to be used as targets were harvested during log-phase growth, and 10^6 cells in 0.4 ml tumor growth medium were labeled with 100 μCi sodium chromate (CJS. 11; Amersham Corp., Arlington Heights, Ill.) at 37°C for 1 h. The tumors used included the P815 mastocytoma (H-2^d) and the EL-4 thymoma (H-2^b), originally obtained from Dr. Virginia Evans, National Cancer Institute, Tissue Culture Section, Bethesda, Md. The YAC-1 lymphoma (H-2^a) was obtained from Dr. G. Cudkowicz, State University of New York, Buffalo, N. Y., and the L5178Y (H-2^d), L1210 (H-2^d), and P815Y (H-2^d) were obtained from Dr. E. F. Wheelock, Thomas Jefferson University, Philadelphia, Pa.

Lymph node or spleen cells were obtained by pressing finely diced pieces of spleens and lymph nodes through a stainless steel screen using cold Hanks' balanced salt solution containing 10 mM morpholinopropane sulfonic acid buffer (MOPS; Sigma Chemical Co., St. Louis, Mo.) and antibiotics. Cells were then centrifuged and resuspended in an assay medium that consisted of RPMI 1640 containing 10% HS, MOPS, and antibiotics. The assay was performed in triplicate with plates containing 96 round-bottomed wells (Flow Laboratories, Inc., Rockville, Md.). Each well contained 10^4 ^{51}Cr-labeled targets and various numbers of effector cells in a total volume of 200 μl of assay medium. Unless indicated, the effector to target cell ratio used was 50:1. After 6 h incubation at 37°C in an atmosphere of 7% CO_2, 50 μl of the supernate was removed from each well and counted in a Rack Gamma II gamma counter (LKB Instruments, Inc., Rockville, Md.). Controls for the assay included labeled target cells alone (spontaneous release), and labeled target cells lysed with 0.5% Triton X-100 (total release). Triton X-100 caused the release of >97% of the total incorporated ^{51}Cr, whereas spontaneous release ranged from 8 to 12% of the total release for all the targets employed. The percent specific ^{51}Cr release was calculated as follows:

$$\frac{\text{experimental cpm - spontaneous cpm}}{\text{total cpm - spontaneous cpm}} \times 100.$$

T Cell Enrichment. Lymph node or spleen cell suspensions were enriched for T cells using the procedure of Mage et al. (3), which depletes B cells by adherence to plastic dishes coated with antisera to mouse immunoglobulin (Ig). Plastic dishes (3025; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) were coated with goat anti-mouse Ig (N. L. Cappel, Cochranville, Pa.) by adding 20 ml of 0.05 M Tris-HCl buffer, pH 9.5, containing 20 μg antibody/ml, and incubating the dishes at 25°C for 30 min. Unbound antibody was removed by washing three times with cold Dulbecco's phosphate-buffered saline (PBS) containing 5% HS. Spleen cells were added in a volume of 20 ml PBS-HS at a concentration of 1.5×10^6 cells/ml and incubated at 4°C for 45 min. Unattached cells were recovered, washed in PBS-HS, and resuspended in the appropriate medium for assay.

Results

C. parvum-induced Tumor Regression Is Associated with an Augmented Production of Cytolytic Cells. It was shown in the preceding paper (1) that implanting B6D2 F_1 mice with P815 tumor cells admixed with *C. parvum* results in a 9–10-d period of tumor growth, followed by complete tumor regression. The experiments described in this section were designed to determine whether this *C. parvum*-induced regression was associated with a *C. parvum*-augmented production of cells capable of lysing P815 tumor cells in vitro. The experiments involved injecting the individuals of one group of mice in a hind footpad with 2×10^6 P815 cells admixed with 100 μg of *C. parvum*, and those of another group with 2×10^6 P815 cells alone. The injections of admixture and tumor

[1] *Abbreviations used in this paper:* HS, horse serum; MOPS, morpholinopropane sulfonic acid buffer; PBS, phosphate-buffered saline.

cells were scheduled so that the cytolytic activity of cells of all mice could be tested at the same time. At the times indicated, tumor size was measured and cells of the draining lymph node and spleen were tested for their capacity to lyse ^{51}Cr-labeled P815 target cells at an effector to target ratio of 50:1. The results in Fig. 1 show, in agreement with those in the companion paper (1), that the tumor that emerged from the tumor-*C. parvum* admixture grew for 9–10 d and then regressed, whereas the tumor without *C. parvum* continued to grow progressively. It can be seen, in addition, that rejection of the P815 mastocytoma in the presence of *C. parvum* was preceded by the generation in the draining lymph node and spleen of cells capable of lysing P815 tumor cells in vitro. The cytolytic response to the tumor growing in the presence of *C. parvum* was of much greater magnitude than the cytolytic response to the tumor growing in the absence of *C. parvum*. Moreover, because the lymph node draining the *C. parvum*-treated tumor contained twice as many cells as the node draining the untreated tumor (2.2×10^7 vs. 1.1×10^7), the augmented response was much greater than indicated in Fig. 1. It should be noted that the cytolytic response to both tumors peaked at about the same time on day 10.

Use of Irradiated Tumor Cells to Demonstrate the C. parvum-augmented Cytolytic Response. The problem with using replicating tumor cells for analyzing *C. parvum* potentiated anti-tumor immunity is that the quantity of tumor antigen increases progressively as the tumor grows. To avoid this variable, the production of cytolytic cells was followed in mice in which heavily irradiated, nonreplicating tumor cells were

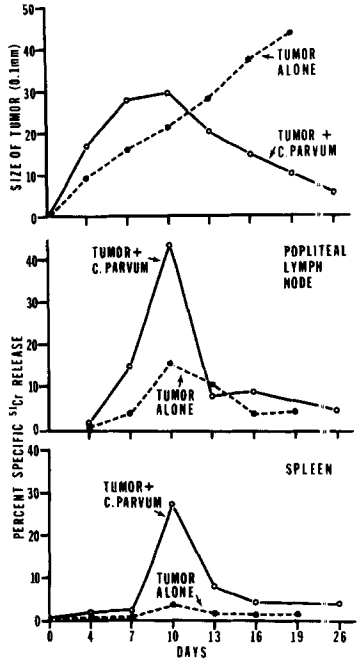

FIG. 1. Evidence that the onset of regression of a footpad tumor containing *C. parvum* was preceded by the substantial generation in the draining node and spleen of cells capable of lysing ^{51}Cr-labeled P815 target cells in vitro. The cytolytic response to the *C. parvum*-treated tumor was of much greater magnitude than the cytolytic response to the untreated control tumor. Cytolytic activity was measured with pooled lymph node and spleen cells from three mice at an effector to target ratio of 50:1.

substituted for living tumor cells in the admixture. It can be seen in Fig. 2 that injection of 10^7 irradiated tumor cells admixed with *C. parvum* resulted in the production of cytolytic cells in the draining node and spleen. In contrast, an injection of 10^7 irradiated tumor cells alone caused no response at all. Although the shape of the cytolytic curve suggests that the peak of the potentiated response to irradiated tumor cells was probably missed by sampling at the indicated times, it was almost certainly lower than that obtained with replicating tumor cells. This is consistent with the knowledge that an admixture of irradiated tumor cells and *C. parvum* gives a smaller therapeutic effect against a test tumor growing at a distant site (1).

Cytolytic Cells Are Tumor-specific T Cells. Because the ^{51}Cr release assay can measure the cytolytic activity of different types of effector cells, it was important to determine whether the cytolytic cells that are generated in response to the *C. parvum*-tumor admixture are T cells. Fig. 3 shows that this was the case, as it can be seen that cytolytic activity was ablated by pretreating lymph node cells harvested at peak response with anti-Thy-1.2 antibody and complement. This evidence is supported by the results of an additional experiment in which cytolytic activity was measured with lymph node cells enriched for T cells by depleting Ig-bearing B cells by "panning" on anti-Ig-coated dishes. It can be seen in Fig. 3 that enriched T cells were much more cytolytic on a cell for cell basis than whole lymph node cells.

Fig. 3 shows, in addition, that the amount of ^{51}Cr released from targets increased with time, and with larger effector to target cell ratios. Moreover, the cytolytic activity generated against the P815 mastocytoma was specific for this tumor, as evidenced by the finding that no cytolytic activity was expressed against three other DBA/2 tumors and a C57BL/6 tumor. However, a small amount of natural killer cell activity (anti-YAC-1) was expressed by draining lymph node cells (3.8% ^{51}Cr release). Cytolytic spleen cells harvested from the same animals showed the same characteristics except

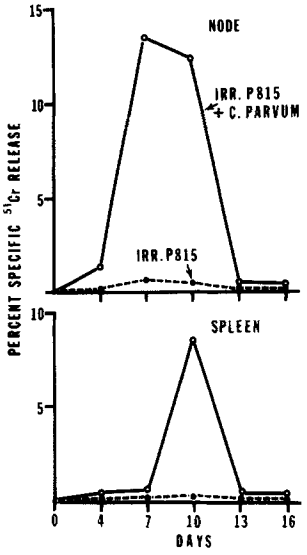

FIG. 2. Further evidence that *C. parvum* functions to augment the production of cytolytic cells. Footpad injection of 10^7 irradiated, nonreplicating tumor cells failed to evoke the production of cytolytic cells unless the tumor cells were injected admixed with *C. parvum*.

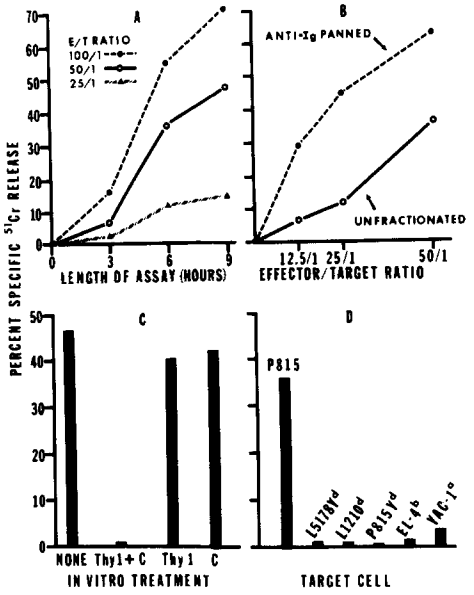

FIG. 3. Some properties of cytolytic cells harvested at peak response from the node draining a tumor growing in the presence of *C. parvum*. The degree of lysis of ^{51}Cr-labeled targets depended on the duration of the assay and the effector to target ratio (A). Cytolytic activity was substantially increased by removing Ig-bearing cells (B), and was totally eliminated by treating the lymph node cells with anti-Thy-1.2 antibody and complement (C). Cytolytic activity was directed against P815 targets, but not against three other DBA/2 (H-2^d) tumors or a B6 (H-2^b) tumor (D). A small amount of cytolytic activity was expressed against YAC-1 (H-2^a) cells (D).

that they expressed a greater amount of natural killer cell activity (9.8% ^{51}Cr release). Moreover, the same properties were displayed by peak response cells harvested from mice bearing untreated progressive tumors.

DBA/2 Mice Generate Less Cytolytic Activity than B6D2 F_1 Hybrids. All the foregoing results were obtained with semisyngeneic B6D2 F_1 mice. The possibility that some of the cytolytic activity was directed against parental antigens is discounted by the foregoing results, showing that no cytolytic activity was expressed against three other DBA/2 tumors. Even so, it was considered important to determine whether syngeneic DBA/2 mice would respond to the admixture of tumor cells and *C. parvum* in the same way as B6D2 mice. The results of an experiment designed to investigate this are shown in Fig. 4, where it can be seen that DBA/2 mice generated less peak cytolytic activity than B6D2 mice (25% ^{51}Cr release vs. 43.1%) in response to the admixture of *C. parvum* and 2×10^6 P815 tumor cells. It was not surprising to find, therefore, that tumors growing in the presence of *C. parvum* in DBA/2 mice did not completely regress. The reason for this quantitatively smaller response in syngeneic mice is the subject of an ongoing study.

Absence of a Cytolytic Response to the Therapeutic Admixture in Mice Bearing Established Tumors. It was shown in the companion paper (1) that the injection of a *C. parvum*-tumor cell admixture failed to give a significant therapeutic effect against a contralateral test tumor that had been growing for about 6 d. In view of the foregoing results, it seemed reasonable to predict that the absence of a therapeutic effect against a large test tumor would be associated with a failure of the admixture to evoke a

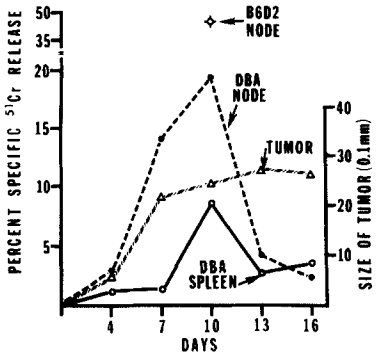

FIG. 4. The cytolytic response to the P815 tumor growing in the presence of *C. parvum* was of smaller magnitude in syngeneic DBA/2 mice than in semisyngeneic B6D2 F_1 mice. This smaller cytolytic response was associated with a substantial therapeutic effect against the tumors, but the tumors failed to regress completely.

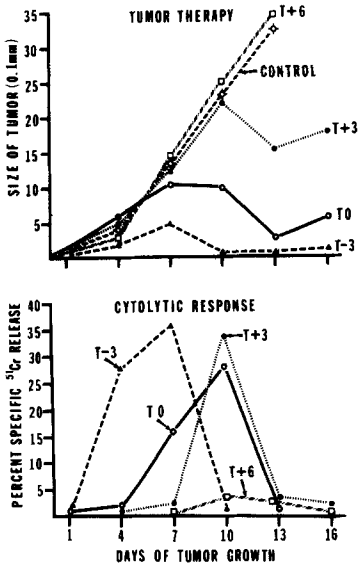

FIG. 5. Effect of injecting a therapeutic admixture of *C. parvum* and 10^7 irradiated tumor cells 3 d before, at the time of, or 3 or 6 d after implanting a test tumor in the contralateral footpad. The later the therapeutic admixture was given with respect to implanting the test tumor, the larger the test tumor grew and the less complete was its regression. In all cases, a substantial cytolytic response was generated in the node draining the site of injection of the admixture, except when the admixture was injected 6 d after implanting the test tumor. In this case, the admixture evoked no cytolytic response at all, and this was consistent with the absence of a therapeutic effect against the test tumor.

potentiated cytolytic response in the draining node and spleen. This possibility was tested by an experiment that measured the cytolytic response to the *C. parvum*-tumor cell admixture in the draining node and spleen when the admixture was injected 3 d before, at the time of, or 3 or 6 d after implanting the contralateral test tumor. In this experiment, irradiated tumor cells were used in the admixture. The results in Figs. 5 and 6 show that injecting the immunotherapeutic admixture 3 d before, at the time of, or 3 d after implanting the test tumor, engendered a significant cytolytic response

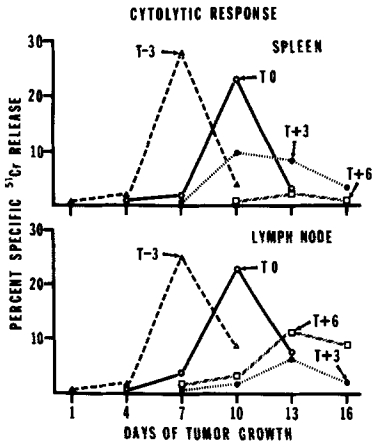

FIG. 6. Additional data from the experiment displayed in Fig. 5 showing concurrent measurements of the cytolytic response in the spleen and in the node draining the test tumor itself. The cytolytic responses in the spleen peaked at the same times as those in the node draining the site of injection of the admixture (Fig. 5). As with the admixture node, there was no cytolytic response at all in the spleen when the admixture was injected 6 d after implanting the test tumor, and there was a diminished response in the spleen when the admixture was given 3 d after implanting the test tumor. The bottom graph shows that injecting the admixture 3 d before, or at the time of implanting the test tumor primed the node draining the test tumor for an accelerated response of large magnitude to test tumor antigens.

in the draining node and spleen, and resulted in a significant therapeutic effect against the contralateral test tumor. However, in spite of the fact that the magnitude of the response was about the same in each case, the later the admixture was given, the later the cytolytic response occurred and the larger the test tumor grew before regression began. Consequently, giving the therapeutic admixture on the 3rd d of growth of the test tumor resulted in only partial regression. It can be seen that the cytolytic responses to irradiated tumor cells plus *C. parvum* were somewhat larger than that shown in Fig. 2. It should be noted, however, that in this experiment the mice were supporting the growth of an untreated test tumor in the contralateral footpad. The possibility must be considered, therefore, that the test tumor served to boost the response to the admixture. The most significant result from this experiment, however, was that no cytolytic response at all was evoked by the admixture when given on the 6th d of growth of the test tumor. It can be seen that this was associated with the total absence of a therapeutic effect against the test tumor.

Concurrent measurement of the cytolytic response in the node draining the test tumor showed that injecting the therapeutic admixture 3 d before, or at the time of implanting the test tumor, primed the host for an accelerated potentiated response in the lymph node draining the test tumor itself (Fig. 6). No such priming occurred, however, when the therapeutic admixture was given on the 3rd or 6th d of growth of the test tumor.

Discussion

This paper shows, in agreement with the companion paper (1), that implanting P815 tumor cells admixed with *C. parvum* results in the emergence of a tumor that grows progressively for 9–10 d and then regresses. It shows, in addition, that the onset

of tumor regression was preceded by a substantial generation in the draining lymph node and spleen of T cells capable of specifically lysing P815 target cells in vitro. Because the production of cytolytic T cells was much greater in response to the tumor growing in the presence of *C. parvum* than in response to a tumor growing alone, it is logical to conclude that the therapeutic action of *C. parvum* when admixed with tumor cells is based on its capacity to potentiate the production of cytolytic T cells. This adjuvant action was revealed by the additional finding that gamma-irradiated tumor cells failed to evoke the generation of cytolytic T cells unless they were injected admixed with *C. parvum*

The finding that progressive growth of the untreated P815 tumor evokes the generation of cytolytic T cells confirms the findings of others (4). It goes without saying that this cytolytic response was of insufficient magnitude to cause regression of the tumor. It is known, in this connection (R. J. North, E. S. Dye, and C. D. Mills, manuscript in preparation), that mice bearing a progressive P815 tumor acquire a state of concomitant anti-tumor immunity that is capable of inhibiting the growth of a P815 challenge implant. It is almost certain that this state of concomitant immunity is based on the cytolytic response described here. It is apparent, therefore, that intratumor *C. parvum* functions to augment this concomitant immune response to a level that is high enough to cause regression of a rapidly growing tumor. That *C. parvum* serves to augment concomitant immunity is supplied by the additional finding that the kinetics of the concomitant cytolytic immune response (Fig. 1) and the *C. parvum*-potentiated response were similar, in that both responses began on about day 4, peaked on day 10, and then decayed. However, the decay of the unpotentiated response occurred in the face of progressive tumor growth and increasing tumor antigen. In contrast, the decay of the *C. parvum*-potentiated response followed the onset of tumor regression and the progressive loss of tumor antigen. It would seem reasonable to conclude, therefore, that each response decayed for a different reason. Presumably, the *C. parvum*-potentiated response decayed because of the destruction of tumor antigen, which may have occurred much more rapidly than indicated by the curve for regression. In fact, the kinetics and magnitude of the *C. parvum*-potentiated response to the P815 mastocytoma are similar to the kinetics and magnitude of the cytolytic response to allografts (5, 6). The decay of the unpotentiated response to the P815 tumor, on the other hand, occurs with increasing tumor load and tumor antigen: a situation that is said to favor the generation of suppressor T cells (7–9). Evidence that the response decays because of the emergence of suppressor T cells is seen in recent findings from this laboratory (10, 11), which show that progressive growth of the P815 tumor, and of a syngeneic fibrosarcoma, results in the generation of a mechanism of T cell-mediated immunosuppression that prevents attempts to regress these tumors by the passive transfer of tumor-sensitized T cells. Recent passive transfer experiments (E. S. Dye, R. J. North, and C. D. Mills, manuscript in preparation) have shown, moreover, that the generation of suppressor T cells occurs on about day 9 of growth of the P815 mastocytoma, which is about the time that the cytolytic response begins to decay. It would seem reasonable to conclude, therefore, that concomitant immunity decays because of the tumor-induced generation of suppressor T cells.

It is too early to conclude, however, that suppressor T cells are responsible for the development of refractoriness of a progressive P815 tumor to the therapeutic action

of an admixture of *C. parvum* and irradiated tumor cells injected at a distant site. It was shown that although injecting a therapeutic admixture of *C. parvum* and irradiated tumor cells 3 d before, at the time of, or 3 d after implanting a test tumor in a contralateral site resulted in a potentiated cytolytic response and caused complete or partial regression of the test tumor, no such potentiated cytolytic response or therapeutic effect occurred when the admixture was injected 6 d after implanting the test tumor. Keeping in mind that the test tumor would have been growing for an additional 4 d before any cytolytic response to the therapeutic admixture would be expected to begin, it would seem reasonable to suggest that suppressor T cells would be present in large enough numbers to suppress a *C. parvum*-potentiated cytolytic response. The alternative possibility exists, however, that the response to the therapeutic admixture failed to occur because of the development of concomitant immunity to the test tumor. This could serve to abort the immunotherapeutic response by destroying the irradiated tumor cells in the admixture. In fact, there is evidence (12, 13) that this is the reason for the failure to obtain a cytolytic response to a secondary allograft that is given too soon after the primary graft is rejected. It is possible, therefore, that a paradoxical situation exists in which the progressive growth of a primary tumor evokes the generation of a state of concomitant immunity, which by virtue of its capacity to destroy a challenge implant of tumor cells is capable of destroying tumor cells injected as an admixture with an immunoadjuvant. Sooner or later, however, concomitant immunity decays, as suggested by the loss of cytolytic T cells shown in this study, and this decay is almost certainly caused by a mechanism of T cell-mediated immunosuppression, as shown by previous studies (10, 11). It is suggested, therefore, that immunosuppression eventually becomes the major obstacle to immunotherapy of established tumors with *C. parvum* and other immunoadjuvants. The loss of susceptibility of progressive tumors to intralesional therapy with immunoadjuvants is obviously not caused by an absence of tumor antigen at the site of adjuvant injection. It must be caused, instead, by the failure of the immunoadjuvant to potentiate a large enough anti-tumor immune response. The response to intralesional *C. parvum* therapy is currently under study in this laboratory.

Summary

It was shown that subcutaneous implantation of P815 tumor cells admixed with *Corynebacterium parvum* resulted in the emergence of a tumor that grew for 9–10 d and then regressed. The onset of tumor regression was preceded by the substantial generation in the draining lymph node and spleen of T cells capable of specifically lysing P815 target cells in vitro. The finding that the magnitude of this cytolytic response was much greater than the cytolytic response to a control tumor that grew progressively is consistent with the hypothesis that the anti-tumor action of *C. parvum* is based on its capacity to augment the production of T cells sensitized to tumor-specific transplantation antigens. This adjuvant action of *C. parvum* was revealed by additional experiments in which irradiated, nonreplicating tumor cells were substituted for living tumor cells in the admixture. The results support the conclusion that the potentiated cytolytic response to subcutaneous injection of an admixture of irradiated tumor cells and *C. parvum* is responsible for the ability of this admixture to cause the regression of a test tumor growing at a distant site. Finally, it was shown that the failure of the therapeutic admixture to cause the regression of distant test

tumors above a certain size was associated with a failure of the admixture to cause a potentiated, anti-tumor cytolytic response. We discussed the possibility that this failure was caused by the presence of a tumor-induced state of immunosuppression.

Received for publication 4 May 1981.

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