Cancer Immunol. Immunother. 8, 257–261 (1980)

Cancer Immunology and Immunotherapy

Systemic Administration of *Corynebacterium parvum* During Sensitization to Tumor Alloantigen-modified Response to Rechallenge

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Summary. *Intravenous administration of* Corynebacterium parvum *(*C. parvum*) to mice during a primary immune response against tumor alloantigens impairs their ability to generate memory cell-mediated cytotoxicity (CMC) in response to an intraperitoneal rechallenge with the same tumor alloantigens. Decreased CMC was observed in spleen and mesenteric lymph nodes, whereas CMC of lymphoid populations from the peritoneal cavity was merely delayed, reaching comparable levels to those found in control animals by day 5. Serum levels of cytotoxic antibody were unaffected, indicating that* C. parvum *administered during a primary immune response has selective effects on the cytotoxic memory response.*

Introduction

Administration of *C. parvum* to mice can cause pronounced changes in their T and B cell functions. Antibody responses to T-dependent and T-independent antigens are often enhanced [4, 5, 15] whereas T cell-mediated immunity is frequently decreased [1, 2, 9–14]. Administration of *C. parvum* to mice also increases resistance to syngeneic tumor inocula [8, 14, 16]. Pretreatment with *C. parvum* can inhibit sensitization to picryl chloride [1], generation of cell-mediated immunity to tumor alloantigens [2], and the expression of delayed-type hypersensitivity to sheep red blood cells [11, 13]. Furthermore, spleen cells from mice treated with *C. parvum* have decreased responses to polyclonal T cell mitogens [14] and tumor alloantigens in vitro [9].

We have reported previously that systemic administration of *C. parvum* following SC immunization with tumor alloantigens resulted in an impaired expression of primary cell-mediated cytotoxicity in the spleen [10]. Spleen cells from these mice have, in addition, markedly reduced ability to generate memory cytotoxicity when cultured in vitro with the same alloantigens [9].

This investigation was undertaken to determine whether IV administration of *C. parvum* during a primary response to a tumor alloantigen affects the in vivo development of a cellular cytotoxic response upon rechallenge.

Materials and Methods

Animals

Male C57BL/6 mice (Strong, San Diego, CA) were used in most experiments. For some experiments, male BALB/c mice (Jackson, Laboratory, Bar Harbor, ME) were used. Mice were obtained at 6–8 weeks of age and rested for at least 1 week before the initiation of an experiment.

Immunization

The P815 mastocytoma was maintained in ascites form by weekly passage in DBA/2 mice, the strain of origin (Microbiological Associates, Walkersville, MD). C57BL/6 mice were alloimmunized by a single SC injection of 2×10^7 P815 in the right flank. BALB/c mice were similarly immunized with EL-4 leukemia (of C57BL/6 origin). The mice were divided into two groups. In one group each mouse received an IV injection of 700 μg *C. parvum* (lot 102-V, Burroughs Wellcome, Research Triangle Park, NC) 9 days after primary immunization. The other group served as immune control. Both groups were rechallenged IP with 2×10^7 cells of the tumor used for primary sensitization 14 days after primary immunization (5 days after *C. parvum*). Mice were sacrificed for collection of sera and for assay of CMC at various times after rechallenge.

Assay of CMC

Mice were sacrificed by cervical dislocation. Peritoneal cells were obtained by lavaging the peritoneal cavity with 10 ml cold minimal essential medium (MEM, Microbiological Associates) containing 5 IU heparin/ml. The spleen, mesenteric lymph nodes, and thymus were

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0340-7004/80/0008/0257/$ 01.00

removed aseptically, and single cell suspensions prepared by expression of the organ through a stainless steel mesh. Cytotoxic activity was measured, the immunizing cell being used as the target cell in a ^{51}Cr-release assay, as described by Brunner et al. [3]. Mixtures of effector cells and target cells at several ratios were incubated for 4 h at 37° C before centrifugation. One-half of the supernatant was removed and both samples were counted. Specific release was calculated according to the following formula:

$$\% \text{ specific release} = \frac{\% \text{ release exptl. group} - \% \text{ background release}}{\% \text{ maximal release}} \times 100 .$$

Maximal release was determined by addition of HCl to a final concentration of approximately 1 *M* and resulted in the release of 80%–90% of total incorporated counts. Medium alone was used to determine background release, which was between 7% and 15% of total incorporated counts. Addition of cells from naive mice or from mice receiving *C. parvum* without alloimmunization did not result in release of counts above that of medium alone. The results were fitted by linear regression to a plot of log (effector cell number : target cell number) versus percentage specific release. One lytic unit was defined as the number of effector cells required to cause a specific release of 25% of the ^{51}Cr incorporated by the target cells.

Assay of Cytotoxic Antibody Activity

Mice were bled from the jugular vein. The blood was kept at room temperature for 30 min and overnight at 4° C before centrifugation to separate the serum from the clot. Sera were decomplemented by heating to 56° C for 30 min. ^{51}Cr-labeled target cells (0.1 ml; 10^6/ml) were added to 0.1 ml serial two-fold dilutions of serum and incubated at 37° for 30 min. Normal rabbit serum (0.1 ml) was added as a source of complement, and the incubation was continued for a further 30 min. Two milliliters of MEM was added, the samples were centrifuged, and the percentage release was determined. The titer of a serum was defined as the reciprocal of the highest dilution resulting in a release of more than 10% above complement control.

Antithymocyte Serum Treatment of Peritoneal Exudate Cells

T cells were removed from peritoneal exudate cell (PEC) populations by incubation of 10^7 cells/ml in a 1 : 45 dilution of absorbed rabbit anti-mouse thymocyte serum (Microbiological Associates) for 30 min at 4° C. The cells were centrifuged and incubated at the same concentration in a 1 : 15 dilution of agarose-absorbed guinea-pig complement for 30 min at 37° C. This procedure killed 45%–60% of PEC, 100% of thymocytes, less than 10% of bone marrow cells, and approximately 50% of spleen cells. The spleen cell cytotoxicity to allogeneic target cells was abrogated as the proliferative response to the T-cell mitogen, phytohemagglutinin.

Results

Groups of C57Bl/6 mice immunized SC with P815 with or without subsequent IV administration of *C. parvum* were rechallenged IP with 2×10^7 P815 tumor cells 14 days after primary alloimmunization. The levels of cytotoxicity activity (residual CMC from the primary immunization) found in spleen, peritoneal cells, and pooled mesenteric lymph nodes at the time of rechallenge are shown in Table 1. The IP challenge was given at a time when cytotoxic activity in alloimmune controls was rapidly decreasing, while in *C. parvum*-treated mice the activity was hardly detectable.

Table 1. Cytotoxic activity on day of rechallenge

Effector cell source[a]	*C. parvum*[b]	% Specific release[c] $\bar{x} \pm SD$	% Inhibition
Spleen	–	15.7 ± 3.3	–
Spleen	+	7.1 ± 1.8	56*
Lymph nodes	–	7.9 ± 0.8	–
Lymph nodes	+	6.7 ± 0.1	15
Peritoneal cavity	–	34.9 ± 5.4	–
Peritoneal cavity	+	21.6 ± 3.6	38*

[a] All mice were immunized with P815 SC 14 days before assay
[b] Mice receiving *C. parvum* were injected IV with 700 μg 9 days after immunization with P815 (5 days before assay)
[c] E:T = 100:1 for spleen cells; 20:1 for lymph node cells; 50:1 for peritoneal cells
* Significantly different from control ($P < 0.05$)

Memory CMC Response

Following IP rechallenge the appearance of cytotoxic lymphocytes was measured in spleen, lymph nodes, and peritoneal cavity. We reasoned that such an approach would allow us to measure the appearance and distribution of cytotoxic lymphocytes in the whole animal, avoiding distortions that might develop from examining a single lymphoid organ. As shown in Fig. 1, memory cytotoxicity in spleen was minimal on day 2 and peaked on day 7, declining slowly thereafter. By contrast, spleen cells from *C. parvum*-treated animals exhibited a markedly decreased cytotoxic activity at all time points tested, ruling out a depression of CMC caused solely by an alteration in the kinetics of response. Decreased CMC was observed at all effector-to-target cell ratios (E : T) tested, and when compared at the peak of the response revealed a more than ten-fold decrease in lytic units. Kinetics of generation of memory cytotoxicity in lymph nodes was similar to that of spleen, the lymphocytes obtained from *C. parvum*-treated alloimmunized mice showing significantly decreased cytotoxic capacity. As with spleen cells, depressed cytolytic activity was observed at all E : T ratios tested (100 : 1–4 : 1) (Table 2).

The impaired ability of *C. parvum*-treated alloimmunized mice to generate measurable CMC in spleen and lymph nodes following an IP rechallenge persists for at least 1 month following *C. parvum* injection (Fig. 2). As shown, rechallenge 35 days after *C. parvum* inoculation (44 days after primary immunization) still resulted in de-

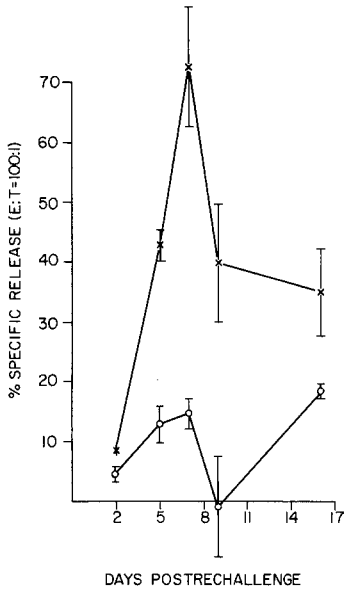

Fig. 1. Kinetics of development of memory spleen cell cytotoxicity following IP rechallenge with 2×10^7 P815 cells. All mice were immunized by SC injection of 2×10^7 P815. Those receiving *C. parvum* were injected IV with 700 μg 9 days after alloimmunization. All mice were rechallenged 14 days after primary immunization. (x), alloimmune controls; (0), *C. parvum*-treated alloimmune mice. Values shown are means ± 1 SD; E : T = 100 : 1

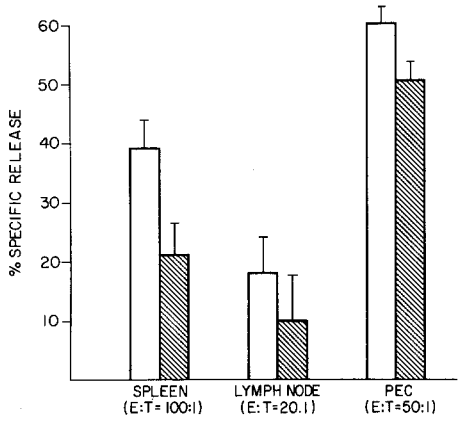

Fig. 2. Persistence of decreased reactivity as a result of *C. parvum* inoculation. C57Bl/6 mice were alloimmunized by SC injection of 2×10^7 P815. Half were injected IV with 700 μg *C. parvum* 9 days later. All mice were rechallenged IP with 2×10^7 P815 44 days after primary immunization and sacrificed for assay 5 days after rechallenge. Values shown are means ± 1 SD

Table 2. Kinetics of memory cell-mediated cytotoxic response in lymph nodes

Spleen cell source	% Specific release ($\bar{x}$ ± SD; E:T = 20:1) Days after challenge[c]			
	2	6	7	16
Alloimmune[a]	9.2 ± 3.2	17.4 ± 3.3	21.2 ± 3.2	12.6 ± 1.5
Alloimmune + CP[b]	3.2 ± 0.7*	− 0.2 ± 3.2*	− 1.7 ± 2.0**	6.5 ± 0.4*

[a] C57BL/6 mice alloimmunized SC with P815 14 days before challenge
[b] C57BL/6 mice alloimmunized SC with P815 14 days before challenge and injected IV with *C. parvum* 5 days before challenge
[c] Intraperitoneal inoculation of 2×10^7 P815
* Significantly different from controls ($P < 0.05$)
** Significantly different from controls ($P < 0.01$)

Table 3. Kinetics of memory cell-mediated cytotoxic response in peritoneal cavity

Peritoneal cell source	% Specific release ($\bar{x}$ ± SD; E:T = 50:1) Days after challenge[c]				
	2	5	7	9	16
Alloimmune[a]	25.5 ± 1.7	56.7 ± 9.8	66.9 ± 7.9	58.8 ± 1.6	36.2 ± 2.0
Alloimmune + CP[b]	3.8 ± 0.7*	56.6 ± 1.5	53.6 ± 3.2	60.1 ± 2.7	27.4 ± 1.5**

[a] C57BL/6 mice alloimmunized SC with P815 14 days before challenge
[b] C57BL/6 mice alloimmunized SC with P815 14 days before challenge and injected IV with *C. parvum* 5 days before challenge
[c] Intraperitoneal inoculation of 2×10^7 P815
* Significantly different from controls ($P < 0.01$)
** Significantly different from controls ($P < 0.05$)

creased cytotoxic activity in these two organs when measured 5 days later.

Kinetics of the secondary cytotoxic response measured in PEC differed from that seen in other organs tested (Table 3). In controls, CMC was already detectable 2 days after rechallenge, peaked around day 7, and remained elevated for at least 16 days. On day 2, PEC from *C. parvum*-treated alloimmune mice showed minimal CMC compared with controls, whereas at all other times the level of cytotoxic activity was comparable. The decrease in CMC detected on day 2 correlated with the presence of a large number of tumor cells in the peritoneal cavity of these mice (162×10^6 P815 compared with 32×10^6 in controls). Evidently *C. parvum*-treated alloimmunized mice experience a delay in the development of secondary CMC in PEC, allowing the tumor cells to grow for a longer period of time. In fact, when these mice were rechallenged with a larger tumor inoculum the delay in the cytotoxic response allowed the tumor to increase sufficiently in volume to cause appreciable ascites, which persisted for 5–7 days. It is important to note, however, that all mice survived disease-free.

The isolated demonstration of CMC in PEC raised the question as to whether these cytotoxic cells were indeed T cells. As shown in Table 4, treatment with anti-thymocyte serum and complement in vitro completely abrogated their activity, clearly demonstrating that the cytotoxic cells in PEC were T cells. The observations reported here are not peculiar for C57BL/6 (H-2b) mice immunized with allogeneic P815 mastocytoma cells (H-2d). An identical effect was observed when BALB/C (H-2d) mice were immunized with EL4 (H-2b) allogeneic tumor cells (data not shown). Clearly, the effects of *C. parvum* on memory CMC are not restricted to a particular mouse strain or alloantigen.

Table 4. Effect of treatment with ATS and complement on PEC cytotoxicity

PEC source	ATS + C′	% Specific release[a] $\bar{x} \pm SD$	
		E:T = 50:1	E:T = 10:1
Alloimmune[b]	–	58.6 ± 3.8	16.0 ± 2.8
Alloimmune	+	− 0.4 ± 0.8	− 0.7 ± 0.6
Alloimmune + CP[c]	–	58.1 ± 6.9	16.5 ± 2.6
Alloimmune + CP	+	− 0.5 ± 1.0	− 0.9 ± 0.7

[a] Obtained 9 days after IP challenge with 2×10^7 P815
[b] C57BL/6 mice alloimmunized SC with P815 14 days before challenge
[c] C57BL/6 mice alloimmunized SC with P815 14 days before challenge and injected IV with *C. parvum* 5 days before challenge

Effects of C. parvum on Secondary Cytotoxic Antibody Response

In contrast to the marked alteration reported in CMC, cytotoxic antibody titers were not affected by *C. parvum* administration (Table 5). Neither the magnitude nor the kinetics of the antibody response were significantly different from those seen in control alloimmune mice.

Discussion

Generation and expression of memory CMC after rechallenge in vivo with P815 is markedly inhibited when *C. parvum* is given during primary immunization to the same alloantigen. By contrast, the development of the cytotoxic antibody response was unaffected. The preferential inhibition of cell-mediated immunity is consonant with the report by Scott [14] of unchanged antibody titers to sheep red blood cells in mice showing depressed delayed cutanous hypersensitivity to the same antigen.

In a previous report we demonstrated the *C. parvum* can significantly decrease the ability of spleen cells to generate a secondary response in vitro [9]. This study clearly demonstrates that a similar impairment is present following in vivo restimulation. The kinetics of memory CMC was similar to that seen in spleens from control immune mice, except that the *C. parvum*-treated mice exhibited a significant decrease in the magnitute of the response at all times tested. Woodruff [17] reported that resistance to a syngeneic fibrosarcoma induced by administration of x-irradiated tumor cells was abolished

Table 5. Cytotoxic antisera titers following challenge

Serum source	Log_2 titer ($\bar{x} \pm SD$) Days after challenge[c]				
	0	2	5	9	16
Alloimmune[a]	4.0 ± 0.0	5.5 ± 0.8	6.0 ± 1.1	4.2 ± 2.0	5.3 ± 0.6
Alloimmune + CP[b]	4.0 ± 1.0	4.3 ± 1.0	5.8 ± 0.6	5.5 ± 0.5	4.4 ± 0.7

[a] C57BL/6 mice alloimmunized SC with P815 14 days before challenge
[b] C57BL/6 mice alloimmunized SC with P815 14 days before challenge and injected IV with *C. parvum* 5 days before challenge
[c] Intraperitoneal inoculation of 2×10^7 P815

when *C. parvum* was injected in the interim between initial inoculation and rechallenge. The effect of *C. parvum* on memory CMC reported here may, in part, explain these observations. This association is merely speculative at this time. The biological relevance of a decreased memory CMC in the rejection of syngeneic tumor remains to be established.

It is interesting to note that the suppressed animals were able to mount a response of normal magnitude at the site of rechallenge, in spite of the depressed activity in other lymphoid organs. These observations raise interesting questions in relation to the origin of the memory cells detected in PEC. As previously shown, memory cells are functionally absent or very deficient in the spleens of these mice [9], and therefore the possibility of these cells deriving from spleen is highly unlikely. Preliminary studies in our laboratory indicate that splenectomy following alloimmunization does not impair the mouse's ability to respond to an IP rechallenge. It is possible therefore that the peritoneal cavity itself is a site through which sensitized lymphocytes traffic and home. The appearance of cytotoxic cells in the peritoneal cavity following the primary immunization is consistent with this hypothesis.

The question of the source of cytotoxic and primed memory cells in the spleen remains to be answered. The cytotoxic and primed memory cells in the spleen may well be the result of migration of cytotoxic cells to that organ from the site of sensitization. These cells would home and proliferate in the spleen. The organ distribution and site of priming of memory cells remains an open question to this date. It appears, however, that spleen represents an important source of cells for in vivo priming. Of particular relevance is the recent work by Hall [6, 7], who reported that cells that mediate rejection of primary heart allografts in rats (i.e., cells with potential for immediate cytotoxic activity) are derived from the recirculating small lymphocyte pool found in lymph nodes and thoracic duct though only small numbers are found in the spleen. Cells able to mediate rejection after in vivo priming (memory), however, were found in all lymphoid organs and were not of the recirculating subpopulation. It is possible that *C. parvum* injection during an on-going primary CMC response causes redistribution of primed memory cells. The ability of *C. parvum* to affect lymphocyte trapping and traffic is well known [4], and some of the effects reported here could well be due to altered traffic patterns. Further investigations carried out in a mouse model with *C. parvum* may serve to elucidate the functions or patterns of recirculation of subpopulations of lymphocytes during the course of a normal immune response.

Acknowledgements. This investigation was supported in part by grant T32 CA 09120, National Cancer Institute, DHEW, and grant CA 12800, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014.

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Received September 28, 1979/Accepted January 31, 1980