Effect of Corynebacterium parvum on Human T-Lymphocyte Interferon Production and T-Lymphocyte Proliferation in Vitro

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ABSTRACT

Heat-killed, preservative-free preparations of strain CN 6134 Corynebacterium parvum have been demonstrated to induce interferon in cultures of normal adult human T-lymphocytes. C. parvum was also shown to induce human T-lymphocytes to proliferate in vitro. The maximum interferon and proliferative response was observed 7 days after initiation of culture at a final C. parvum concentration of 140 µg/ml. Human monocyte-derived macrophages did not produce interferon in response to C. parvum or in response to products of C. parvum-stimulated T-lymphocytes. The addition of macrophages to cultures of T-lymphocytes did not significantly enhance the production of interferon by the T-lymphocytes; however, it did significantly enhance the proliferative response to C. parvum. C. parvum can also enhance the production of interferon by T-lymphocytes stimulated with phytohemagglutinin in the presence of macrophages, and the amount of interferon produced in the presence of both agents was greater than the sum of the amounts of interferon stimulated by each agent acting alone. The type of interferon produced by T-lymphocytes in response to C. parvum is similar to type II or immune interferon, because it was labile to low pH and heat. Thus, a link has been found between two agents, C. parvum and interferon, both of which have in common antitumor properties and the ability to modulate the immune response.

INTRODUCTION

Interferon has numerous and diverse biological effects beyond its ability to confer protection on cells against viruses (15). Furthermore, it can be induced by a wide variety of substances in addition to viruses, including bacteria (23). Although it was originally described solely as an antiviral protein (24, 25), interferon is now known to have a multiplicity of effects on the immune response (4) and to have antiproliferative effects on both normal and malignant cells (18). In animal models it is effective in inhibiting the growth of spontaneous (18), transplantable (17), and virus-induced tumors (16).

Corynebacterium parvum has also been shown to have antitumor effects in numerous animal models (20, 30). Currently, it also is being used with chemotherapeutic agents as an immunomodulating agent in the treatment of human cancers. Although the mechanism of its antitumor effect is complex and not completely understood, it appears to be mediated by the multiplicity of effects of the organism on the immune response (29).

Thus, interferon and C. parvum share in common a capacity to influence the immune response and effect tumor growth. Taking this information into consideration, we have developed the hypothesis that the antitumor effect of C. parvum is mediated in part by interferon produced during the stimulation of the immune system. Implicit in this hypothesis is the supposition that C. parvum can, in fact, induce interferon in cells of the lymphoid or reticuloendothelial systems. Therefore, the present studies were performed to explore this issue by determining: (a) whether human peripheral blood T-lymphocytes or monocyte-derived macrophages can produce interferon in response to C. parvum; (b) if so, whether human macrophages can enhance the production of C. parvum-stimulated interferon by lymphocytes, just as they do with interferon induced by mitogens and bacterial and viral antigens (3); (c) whether C. parvum can stimulate a proliferative response in T-lymphocytes; (d) if so, whether macrophages can enhance the proliferative response of the T-lymphocytes; and (e) whether C. parvum can augment interferon production by T-lymphocytes in response to mitogens.

MATERIALS AND METHODS

C. parvum. Preparations of heat-killed, preservative-free C. parvum strain CN 6134 were provided through the courtesy of Dr. John Whisnant of the Burroughs Wellcome Company, Research Triangle Park, N. C. Each vial contained 20 ml with 7 mg dry weight per ml. The final concentration used in cultures ranged from 35 to 210 µg/ml.

PHA. PHA was obtained from Difco Laboratories, Inc., Detroit, Mich. Samples were diluted in Dulbecco's calcium- and magnesium-free phosphate-buffered saline. The final concentration of PHA in culture was 34 µg/ml.

Preparation of Human Macrophage Cultures. Leukocyte-rich plasma was obtained from the blood of normal adult donors by dextran sedimentation according to the technique of Goldblatt et al. (14). Pure cultures of macrophages were prepared in Leighton tubes by the growth and differentiation of monocytes over a 7-day period according to the procedure of Epstein and Cline (6).

The macrophages were cultured in 1 ml McCoy's Medium

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5A supplemented with 30% human AB serum. After 7 days the number of macrophages adherent to the glass coverslips was 2 to 5 \times 10^4. Prior to use in experiments, coverslips in each Leighton tube were graded for extent of differentiation and cell number so that comparable cultures could be included in each experimental group.

Isolation of Purified Human T-Lymphocytes. Blood for the isolation of lymphocytes was obtained from the same normal adult donors who had previously donated a sample of blood for the preparation of macrophage cultures 1 week earlier. Leukocyte-rich plasma was obtained by dextran sedimentation (14), and T-lymphocytes were isolated by their passage through a sterile nylon fiber column as described by Epstein et al. (9). Such nylon fiber column effluents prepared from normal donors are known to contain >99% lymphocytes of which >94% are T-cells as determined by fluorescence microscopy with fluorescein-labeled anti-Cohn Fraction II serum (9). Such T-cell preparations contain less than 0.3% monocytes as assessed by the peroxidase staining technique of Kaplow (26).

Preparation of Combined T-Lymphocyte-Macrophage Cultures. One \times 10^6 nylon fiber column-isolated lymphocytes in 1 ml of McCoy's Medium 5A supplemented with 30% human AB serum were added to Leighton tube macrophage cultures from which the media had been decanted. Cultures were incubated at 37° in a 5% CO_2 atmosphere for 1 to 10 days. Parallel cultures were prepared with either macrophages or T-lymphocytes. Unless otherwise stated, C. parvum, PHA, or the 2 agents together were added at the initiation of culture and were present for the entire duration of culture. Control cultures were prepared without C. parvum or PHA. Viability of the cells in culture was determined by trypan blue dye exclusion. In some experiments only the supernatants of T-lymphocytes previously stimulated with C. parvum were added to macrophage cultures, and these cultures were examined 3 to 6 days later for the presence of interferon.

Harvesting Techniques. A 10 \muCi/ml solution of \[^{3}H\]thymidine (50 \muCi/ml; New England Nuclear, Boston, Mass.) was added to each culture during the final hr of incubation. At the time of harvest, C. parvum was removed from the supernatants by their passage through a Hoefer multiport filtration apparatus (Hoefer Scientific Instruments, Palo Alto, Calif.). The supernatants were stored in sterile tubes at 5° prior to assay for interferon. The cell pellet was assessed for the extent of incorporation of \[^{3}H\]thymidine into DNA as a measure of the proliferative response of the lymphocytes to C. parvum or PHA. For this the procedure of Epstein et al. was used (10). The stimulation index is defined as

\[
\frac{\text{cpm (stimulated)} - \text{cpm (unstimulated)}}{\text{cpm (unstimulated)}}
\]

with geometric means.

Interferon Assay. A virus plaque reduction assay for interferon was performed as described previously (2) with human foreskin fibroblasts and bovine vesicular stomatitis virus as the challenge virus.

Characterization of Interferon. Supernatants were characterized for their species specificity by performing the assay for interferon on mouse L-cells instead of on human fibroblasts. The protein nature of the interferon was evaluated by studying its response to the action of trypsin (2). Also, to classify the interferon as a classical (type I) or immune interferon (type II), we examined the response of the interferon to heat and low pH. Aliquots of the supernatants were kept for 1 hr in a 56° water bath prior to their assay for interferon. Other aliquots of the same samples were brought to pH 2 with 1 N HCl for 18 hr. The samples were then readjusted to pH 7.0 with 1 N NaOH and assayed for interferon. The final titer was corrected for the dilution of the samples with HCl and NaOH.

**RESULTS**

Effect of C. parvum on Interferon Production. Our first experiments were performed to determine whether C. parvum could induce interferon in human T-lymphocytes and, if so, to define the optimum dose of C. parvum and duration of culture for maximum production of interferon. Using trypan blue dye exclusion, we found that the presence of the C. parvum did not affect the viability of the lymphocytes or macrophages. Our previous experience with mitogens (8, 9) and antigens (7, 10) had indicated that the greatest amount of interferon was produced in cultures containing both T-lymphocytes and macrophages. Consequently, in our initial experiment combined T-lymphocyte-macrophage cultures were prepared with C. parvum in concentration ranging from 35 to 210 \mu g/ml and harvested at 3 or 7 days. The results of one of 2 representative experiments are shown in Chart 1. No interferon was noted in the absence of C. parvum; in those cultures containing C. parvum, maximum levels of interferon were observed after 7 days of culture with a final C. parvum concentration of 140 \mu g/ml. In 2 additional experiments in which time course studies were performed with only the final C. parvum concentration of 140 \mu g/ml, maximum interferon levels were again observed after 7 days of culture.

Using these optimal conditions (final concentrations, 140 \mu g/ml; 7-day culture period), we then studied the blood of 14 normal donors and compared the results obtained in cultures of T-lymphocytes alone with that observed in combined T-lymphocyte-macrophage cultures prepared in either the presence or the absence of C. parvum. The data shown in Chart 2 indicate that, in the absence of C. parvum, no interferon was detected in the supernatants of cultures of either T-lymphocytes or T-lymphocytes and macrophages. In the presence of C. parvum, T-lymphocytes of 11 of the 14 donors produced interferon, with an observed geometric mean titer of 59 with a range of 25 to 141 (mean \pm 2 S.E.). In the presence of macrophages and C. parvum, the geometric mean titer and range was 156 (range, 95 to 256). Statistical analysis of the data by the Wilcoxon signed-rank test demonstrated a significant difference between the interferon titers observed in C. parvum-stimulated T-lymphocyte cultures and the nonstimulated controls (p < 0.005). Thus, C. parvum is an effective inducer of interferon in T-lymphocytes. A similar level of statistical significance was observed when C. parvum-stimulated T-lymphocyte-macrophage cultures were compared with their nonstimu-
C. parvum-stimulated T-Lymphocyte Interferon

Chart 2. Semilogarithmic plot of the effect of C. parvum on interferon production in 7-day cultures that contained human T-lymphocytes or T-lymphocytes plus macrophages. Each symbol represents the mean of 2 samples. Square symbols were included only for ease of identification of the 3 donors whose T-lymphocytes alone did not respond to C. parvum. Inasmuch as the data are plotted on a logarithmic scale, the overall mean depicted is the geometric mean, calculated on the basis of logarithmic values.

Effect of C. parvum on T-Lymphocyte Proliferation. In the case of other agents that induce T-lymphocytes to produce interferon, such as mitogens and antigens, lymphocyte blastogenesis and DNA synthesis are often concomitant events (7, 8). To determine whether C. parvum had the same effect, we assessed the proliferation of T-lymphocytes of the 14 donors by the incorporation of [3H]thymidine into DNA. Data were obtained at 7 days and at a final concentration of 140 μg/ml because 4 preliminary experiments had indicated this as the time and dose at which maximum response occurred. The results (Chart 3) indicated that the geometric mean value ± 2 S.E. for T-
lymphocytes alone was 270 (range, 180 to 404). In the presence of C. parvum, there was a statistically significant increase in [3H]thymidine incorporation (p < 0.01) to a mean value of 892 (range, 497 to 1601) cpm. This increase represented a stimulation index of 2.3. Similarly, in the T-lymphocyte-macrophage cultures, the addition of C. parvum resulted in a statistically significant (p < 0.01) increase from 393 (range, 244 to 633) to 2178 (range, 1039 to 4564) cpm, reflecting a stimulation index of 4.5. In contrast with these experiments, the presence of 2 to 5 × 10⁴ macrophages in the cultures significantly enhanced the degree of lymphocyte proliferation in response to C. parvum (p < 0.01).

Effect of C. parvum on Mitogen-induced Interferon Production and Lymphocyte Proliferation. Another agent known to stimulate lymphocytes to produce interferon is PHA (8). It was therefore of interest to determine whether C. parvum and PHA would interact in the stimulation of interferon production. Cultures containing T-lymphocytes and macrophages were prepared, and either C. parvum (140 µg/ml), PHA (34 µg/ml), or the 2 agents together were added at the initiation of culture. These cultures were harvested after 3 days because PHA is known to have its maximum effect on interferon production at this time. The data from 6 experiments are depicted in Chart 4. In 5 of the 6 experiments, the addition of C. parvum with PHA resulted in a more than additive effect or potentiation of interferon production by the lymphocytes. The difference between the values expected and those observed was statistically significant (p < 0.005). In 2 additional experiments, we demonstrated that C. parvum over a range of concentrations from 35 to 175 µg/ml was effective in potentiating the PHA interferon response, but the maximum effect was observed with 140 µg/ml.

Maximum titers of interferon were found when C. parvum was added at the same time as PHA, at the initiation of the cultures. However, potentiation could occur if C. parvum were added up to 24 hr after PHA, and the data from 2 such experiments are shown in Chart 5.

Potentiation by C. parvum of T-lymphocyte proliferation in the presence of PHA also occurred in the T-lymphocyte-macrophage cultures (p < 0.05). Of interest was our observation that macrophages were necessary for the potentiating effect on interferon production and proliferation, and this was the case because optimum interferon production by T-lymphocytes in response to PHA requires the presence of macrophages (3, 8). In 14 experiments on T-lymphocytes alone, the addition of C. parvum simultaneously with PHA did not result in potentiation of the interferon produced or the enhancement of lymphocyte proliferation over that which was expected from the sum of the effects of the 2 agents.

Characterization of the Interferon Produced in Response to C. parvum. Several studies were performed to characterize the interferon produced in response to C. parvum. The material had definite antiviral effects, inasmuch as it conferred protection on human foreskin fibroblasts against the challenge virus. Like most interferons it was species specific; consequently, since it was of human origin, it did not protect mouse L-cells against the same challenge virus. The protein nature of the antiviral material was confirmed by its susceptibility to inactivation by treatment with trypsin.

Finally, we compared the interferon induced in T-lymphocytes by C. parvum with that induced in human buffy coat leukocyte preparations by Sendai virus with regard to their sensitivity to heat and low pH. The data are summarized in Table 1. Although there is some variability from sample to sample, the C. parvum-stimulated interferon is, by both criteria, clearly different (by the Wilcoxon signed-rank test) from that produced when buffy coat leukocytes are stimu-
C. parvum-stimulated T-Lymphocyte Interferon

lymphocytes stimulated with mitogens or antigens are known to produce another type of interferon known as type II or immune interferon, which is considerably less stable to heat and low pH than is the type I interferon (5). Although the production of some type I interferon cannot be completely ruled out, the sensitivity to heat and low pH exhibited by the C. parvum-induced interferon in the present studies was similar to that of immune interferon produced by human T-lymphocytes in response to mitogens or antigens (33). The interferon produced in macrophage-T-lymphocyte cultures in response to both C. parvum and PHA also showed less stability to heat and low pH, when compared with type I interferon, and thus could also be considered as an immune interferon.

DISCUSSION

It was observed that the peritoneal exudate cells of mice treated with C. parvum produced a substance capable of inhibiting the multiplication of bovine vesicular stomatitis virus and encephalomyocarditis virus (1). The author rejected the notion that the antiviral factor was interferon on the basis of 2 observations: (a) that the inhibitor was not destroyed by heating; and (b) that its action was not blocked by treatment of the cells to be tested with actinomycin D. At present it is known that these objections were invalid, since not all mouse interferons are heat labile (19, 23), and the physical properties of interferon produced in response to various stimuli can be affected by the method of preparation of the cells, the nature of the cells present in addition to lymphocytes, or the immune status of the donor (5). Furthermore, assays for interferon have now been developed that capitalize on the fact that interferon can act to inhibit actinomycin-resistant viral RNA synthesis (32).

Additional suggestive evidence that C. parvum might be effective in inducing interferon came from studies in which administration of C. parvum to mice protected the animals against lethal Herpesvirus infections (12, 28). Recently, the production of interferon by C. parvum-treated murine spleen cells was observed (21, 27), as was the production of interferon in vitro by human leukocytes prepared on Hypaque-Ficoll gradients (22).

Our studies demonstrate that C. parvum can stimulate human T-lymphocytes to produce interferon with the maximum response occurring after 7 days of culture and with a final C. parvum concentration of 140 μg/ml. At the present time, we do not know whether the interferon produced by normal T-cells in response to C. parvum occurs as a result of immune specific induction. Because of the widespread prevalence of antibody to C. parvum in normal sera (35) and the considerable likelihood of exposure to similar organisms with possible antigenic determinants in common with C. parvum (Corynebacterium acnes, Corynebacterium diphtheroides), immune specific induction of interferon seems quite likely. However, considerably more interferon was produced by the purified T-cells in response to C. parvum than had been previously observed with sensitized T-cells treated with specific bacterial (7) or viral antigens (10), and even more than that was observed when nonspecific mitogens, such as PHA and pokeweed mitogen, served as the inducers in nonsensitized cells (9).

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In the situation for mitogens and antigens, the presence of monocyte-derived macrophages in the in vitro system has been shown to enhance the production by lymphocytes of interferon as well as several other mediators of cellular immunity (3). The reason for such enhancement is not well understood, but it is thought that the macrophage facilitates the presentation of the mitogen or antigen to the lymphocyte. The amount of interferon induced by C. parvum was not enhanced by the addition of 2 to 5 x 10^4 macrophages, whereas interferon stimulated by mitogens and antigens is enhanced when the same experimental conditions are used. It is possible that a stimulant like C. parvum, already particulate, might not require the benefits of macrophage processing for presentation to the lymphocytes. However, the exact reasons for the difference in the macrophage dependence of the C. parvum and mitogen or antigen systems are not yet known.

Our studies indicated that C. parvum also triggered T-lymphocyte proliferation, as measured by the incorporation of [3H]thymidine into DNA. The mitogenic effect of C. parvum on human T-lymphocytes has also recently been described by Godal et al. (13), but the exact concentrations of C. parvum used were not stated. In our studies the maximum proliferative response was observed with a final C. parvum concentration of 140 µg/ml, whereas Hirt et al. (22) found a maximum response with a final concentration of 14 µg/ml. Such differences might be attributable to differences in the cell populations used. In our studies purified T-cells were used; in those by Hirt et al. (22) combined T- and B-lymphocytes from Ficoll gradients were used. Hirt et al. (21) recently found that B-lymphocytes are the source of interferon in the mouse and that such interferon production in the murine system was macrophage dependent. Studies on the response of purified B-lymphocytes of human derivation have not yet been performed; therefore the reasons for differences between the murine and human systems are not yet apparent.

In our studies macrophages were found to enhance significantly the proliferative response of the T-lymphocytes to C. parvum. This observation was of interest because it demonstrates that the 2 aspects of the cellular immune response that we are able to quantify, the extent of lymphocyte proliferation and the extent of mediator (interferon) production, can have different degrees of macrophage dependence. In our studies the proliferative response to C. parvum was highly macrophage dependent, whereas mediator production was not. This may be a reflection of the fact that separate, distinct subpopulations of T-lymphocytes are responsible for interferon production and the proliferative response. Such studies on human T-cell subpopulations stimulated with C. parvum are now in progress in our laboratory. In animal studies (34), interferon-producing capacity and proliferative capacity to mitogens and antigens were found to reside in different lymphocyte subpopulations.

Macrophages were necessary, however, for maximum expression of the more than additive effects on interferon production observed when T-lymphocytes were cultured for 3 days in the presence of both C. parvum and PHA; this was due to the fact that optimum interferon production by T-lymphocytes in response to PHA requires the presence of macrophages. Our observations on interferon parallel those made on migration-inhibitory factor in a canine model in vivo (31). In those experiments migration-inhibitory factor production by lymphocytes in response to streptolysin O was enhanced by prior treatment with C. parvum. The fact that a more than additive effect was observed in our studies suggests that the mechanism of induction of interferon by each agent is different, but how the potentiation occurs is not yet known. It is possible that one agent might enhance the sensitivity of the receptor for the other agent or that the 2 agents together combine in some way to become a more effective inducer.

Attempts to purify human immune interferon have been limited by the lack of availability of large quantities of high-titered material. Thus, the use of PHA in combination with C. parvum in T-lymphocytes-macrophage cultures might prove to be a valuable source of such high-titered preparations. Also, it might be possible in the future to use the ability of C. parvum to enhance PHA-induced interferon production in T-lymphocyte-macrophage cultures or the ability of C. parvum to induce interferon in human T-lymphocytes as a predictive test for the efficacy of C. parvum in the treatment of patients with cancer.

Thus our studies have provided a link between C. parvum and interferon, both of which have in common antitumor properties and the ability to modulate the immune response. Further studies are necessary, however, to implicate directly interferon in the multiplicity of effects that C. parvum has on the immune response.

ACKNOWLEDGMENTS

The authors wish to thank Della Goldblatt, Nancy McManus, and Constance Reese for excellent technical assistance; David Epstein for assistance with statistical calculations; and Diana Zawacki for typing the manuscript. They also wish to thank Dr. John Whisnant for his helpful discussions and enthusiastic support of this project.

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Effect of *Corynebacterium parvum* on Human T-Lymphocyte Interferon Production and T-Lymphocyte Proliferation *in Vitro*

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*Cancer Res* 1978;38:4467-4473.

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