Induction of Spleen Cell Growth and DNA Polymerase Activity by \textit{Corynebacterium parvum}\textsuperscript{1}

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ABSTRACT

\textit{Corynebacterium parvum} induces rapid proliferation of spleen cells with concomitant rapid increases in DNA synthesis as measured by \textsuperscript{3}H\textsuperscript{3}H]thymidine uptake and increase in DNA polymerase activity. Cell number increased exponentially over 10 days. DNA polymerase activity increased 8-fold after \textit{C. parvum} stimulation. The rapidity of response indicated a population response to the stimulus, and the data are consistent with a direct stimulation of spleen cell proliferation by \textit{C. parvum}.

INTRODUCTION

Mammalian cell systems can grow exponentially under certain in vivo conditions. Exponential cell growth in the intact animal in vivo has been established for some ascites lymphomas by Maruyama (16) and Maruyama and Brown (17) using survival time assays (17). Few normal tissues have been shown to exhibit exponential growth kinetics. We describe in this paper the growth of a normal tissue, in this case the spleen in response to the stimulus of the killed anaerobic bacterial vaccine \textit{Corynebacterium parvum}. This response is thought to correspond to a normal in vivo tissue response to challenge by a bacterial organism, in this case typical of host response to bacterial inflammation. \textit{C. parvum} has also been reported to stimulate antitumor reaction, presumably immunological, against some experimental tumors by Halpern et al. (11) and this is of potential tumor therapy importance (24). The study of host response and spleen cell growth provides insight into the growth and proliferation response for normal spleen tissue in the intact animal and is the subject of this report. The increase in spleen size following \textit{C. parvum} injection is well documented by Halpern et al. (11), Toujas et al. (27), Castro (6), Milas et al. (22), and Brozovic et al. (5). The present work describes the cellular kinetics and enzyme induction by \textit{C. parvum} as the spleen responds to the stimulus.

MATERIALS AND METHODS

Mice. C57BL/ym mice bred in this laboratory by a single line breeding system were used for this study. Male and female mice about 8 to 16 weeks of age and weighing 20 to 25 g were used; they were caged in clear Lucite cages with a regular natural light-dark cycle and were maintained on Purina laboratory chow and tap water \textit{ad libitum}. In any particular experiment, all mice were matched in age, sex, and size.

Vaccine. \textit{C. parvum} was kindly provided by Burroughs Wellcome Laboratory, Research Triangle Park, N. C. An alcohol-killed vaccine was used as a homogeneous suspension with the organisms (7 mg/ml, dry weight) in 0.9% NaCl solution. A dose of 0.05 ml or 0.35 mg was given i.p. Graded dose response data have been reported elsewhere (18). The dose used provides a strong stimulus to the C57BL mouse.

Spleen Cells. At varying time intervals after a single injection of \textit{C. parvum}, spleens were harvested from the stimulated animals. Animals were heparinized with 0.1 ml heparin i.p. and sacrificed by cervical dislocation after several min. The spleens were excised, weighed wet, and then individually dispersed in a tissue homogenizer at 0–4° after the spleens were first minced and washed in heparinized 0.9% NaCl solution. Dispersed cells in 0.9% NaCl solution with added 2% acetic acid for lysing erythrocytes were counted by phase microscopy to enumerate all nucleated cells. EDTA (10%) was used for enzyme assays.

DNA Synthesis. \textsuperscript{3}H\textsuperscript{3}H]dThd\textsuperscript{2} was obtained from New England Nuclear (Boston, Mass.), at a specific activity of 50 Ci/mmol. Randomly selected mice were given i.p. injections, on the appropriate day after stimulation with \textit{C. parvum}, of 5 µCi \textsuperscript{3}H\textsuperscript{3}H]dThd in 0.1 ml. After an uptake period of 30 min, each mouse was given an injection of unlabeled thymidine (10 µmol/ml) to terminate \textsuperscript{3}H\textsuperscript{3}H]dThd uptake (19). Spleens were harvested from sacrificed animals, the cells were dispersed and counted, and the label uptake was determined. Cells were pooled, washed 3 times with 0.9% NaCl solution, and counted, and a measured number of cells were collected in a tube. Cells were centrifuged and then were washed with cold 10% perchloroacetic acid 3 times and cold ethanol 1 time. Residues were suspended and then washed with methanol:ether (2:1, v/v) 1 time and ethyl ether 1 time. The acid-insoluble residues were placed in solution with the tissue solubilizer Aquasol (New England Nuclear). An aliquot of the extracts was counted in a scintillation cocktail in a liquid scintillation counter for \textsuperscript{3}H\textsuperscript{3}H]dThd uptake. Data were normalized to cpm/1 × 10\textsuperscript{8} cells.

Enzyme Assays. Radioactive deoxynucleoside triphosphates and nucleosides were obtained from New England Nuclear. Unlabeled deoxynucleoside triphosphates were purchased from P-L Biochemicals, Inc. (Milwaukee, Wis.), and were further purified by quaternary aminoethyl Sepha-

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\textsuperscript{2} The abbreviations used are: \textsuperscript{3}H\textsuperscript{3}H]dThd, tritiated thymidine; DNA-P-α, DNA polymerase α; CFU-S, colony-forming units-spleen.
dex chromatography. The template used for DNA-dependent DNA polymerases (7) was activated calf thymus DNA. All chemicals were reagent grade from commercial sources.

Spleen cells were suspended at a concentration of $1 \times 10^6$ cells/ml in 0.25 M potassium phosphate (pH 7.2) containing 1 mM mercaptoethanol. Cells were sonically dispersed for 15 sec and then were centrifuged for 1 hr at 100,000 x g; the supernatant solutions were used for all enzyme assays.

DNA polymerase activity was measured by incorporation of [3H]dTTP (50 cpm/pmol) into acid-insoluble material with activated DNA as substrate (7). Acid-insoluble material from the reactions was spotted on Whatman GF/C glass fiber discs at 4 time intervals during the reaction. Discs were processed as has been described elsewhere (7). DNA polymerases were assayed in crude extract with the use of different pH optima (3, 7, 8). The pH 7.2 enzyme represents the pH optimum for assay of DNA-P-a, which is the important enzyme for cellular proliferation (3, 8); the present data are concerned with the proliferative activity of cells. The DNA-P-a activity is inhibited by assay in the presence of N-ethylmaleimide and thus can be identified separately from the total DNA polymerase activity (3, 7, 8).

RESULTS

Spleen Weight Response to C. parvum. Spleens were excised, trimmed free of adjacent tissue, and weighed at intervals following the injection of 0.35 mg C. parvum i.p. into matched adult mice. Spleen weights began to increase shortly after C. parvum injection and, by serial determinations, were found to increase rapidly in size and weight. The weights and size reached a maximum by 10 days and then, over the subsequent 3 weeks, gradually returned to control levels. The increase in weight was plotted semilogarithmically, and the data of mean spleen weight over the initial 10 days of response fell on a curve, indicating an exponential increase in weight with time (Chart 1).

Spleen Cell Number Response to C. parvum. Spleens obtained after C. parvum stimulation were individually dispersed after heparinization of the animals, harvesting of the spleens, and gentle disruption in a tissue homogenizer with heparinized 0.9% NaCl solution (0.1 ml in 10 ml volume). An aliquot was counted, and the total nucleated cell population of the spleen was determined. Representative results from 2 experiments are shown for the first 14 days and over a 30-day period. The data are plotted semilogarithmically (Chart 2) with the cell number as a function of time. The cell number during the initial 10-day period increased exponentially and then declined gradually to control levels by 30 days. The data for the first 10 days can be fitted by the following relationship.

$$N = N_0 e^{kt}$$

where $N$ is the cell number, $N_0$ is the initial cell number, $e$ is the base of natural logarithms, $T_d$ is the cell-doubling rate and $t$ is the time after C. parvum injection. The doubling rate was constant over the initial 10-day response period. The cell-doubling time was calculated to be 73 hr, and the data showed excellent agreement (Chart 2) with the fitted growth curve ($r$, 0.97).

Spleen Cell DNA Synthesis Response to C. parvum. [3H]dThd was used to determine DNA synthesis activity of the spleen cells. The uptake of labeled thymidine changed
greatly after C. parvum injection. Within 6 hr after C. parvum injection, DNA-S activity was greatly increased and continued to show marked elevation for 1 week as measured by [3H]dThd uptake as determined in cpm per 10^6 cells (Chart 3). DNA-S activity was greatly increased during the early and midexponential period of spleen cell growth, began to fall with the early stationary phase (7 to 10 days), and gradually returned to control levels by about 30 days.

Spleen Cell DNA Polymerase Activity following C. parvum Injection. DNA polymerase activity was studied with time after C. parvum injection. The results are shown in Chart 4. DNA polymerase activity was low in control spleen cells, but it increased with time after C. parvum injection. As Chart 4 shows, DNA polymerase enzyme activity lagged behind [3H]dThd DNA synthesis activity, as has been shown before (20, 21), and gradually fell after 14 days as the proliferative activity of the spleen declined.

DISCUSSION

The results of these experiments show that an intense stimulation of spleen growth and cell proliferation takes place following a single injection of C. parvum i.p. The increase in spleen mass and cell number occurred immediately after C. parvum injection. Growth in cell numbers was prompt, with a cell-doubling time of 73 hr. Following a 10- to 14-day period of growth, the mass and cell number of the spleen returned to normal and control levels. C. parvum clearly induced proliferative activity; the growth curve was initially exponential and was similar to tumor growth curves described in a variety of in vivo tumor situations, especially those with ascites tumors (16, 17). The present data show that a normal tissue such as spleen, during response to an appropriate stimulus (in this case a bacterial antigen), may also show exponential growth kinetics. Clearly, however, after an appropriate level of response, cell loss (presumably death or migration) became a dominant feature with a rapid return of the spleen size and cell number to control levels. The latter feature is characteristically not displayed by neoplastic proliferation.

In the C. parvum-induced spleen proliferation model, certain characteristics resemble the transplantation-induced leukemic cell proliferation model (19). For the spleen, DNA synthesis activity increased, was greatly elevated within 6 hr after stimulation, and remained high during early and midexponential growth stages of the spleen cell. Activity declined with stationary phase and declined still further as the spleen returned to control size. The same features were observed with transplantation-induced tumor cell proliferation (19). The specific activity of DNA polymerases was found to increase after C. parvum-induced spleen cell stimulation. This result is consistent with other mammalian systems in which DNA polymerase activity has been studied (3). These systems include stimulated lymphocyte transformation as studied by Agarwal et al. (1), the system of Loeb et al. (14), cultured cells (8), regenerating liver (4), the system of Iwamura et al. (13), the system of Ove et al. (23), the system of Hunter et al. (12), the system of Lynch et al. (15), ascites tumor (20, 21), hepatic carcinogenesis (2), and the system of Chiù et al. (9). The specific activity of DNA polymerase β does not change with proliferation in mammalian cell systems (3, 8, 28) and was also found in our present experiment.

The temporal sequence of [3H]dThd uptake and DNA-P-α activity was found to increase with entry into DNA-S and DNA/synthetic activity by Spadari and Weissbach (25) and by Chiù and Baril (10). High levels of DNA polymerase activity persisted and remained elevated for at least 2 to 3 weeks after the peak DNA-S activity was reached in about 1 week in the present experiments. Essentially identical results were observed in earlier experiments with tumor (21) in which almost all cells were clonogenic. These results did not show a sharp drop in DNA polymerase activity with entry into a stationary growth phase, as has been reported for cultured mouse cells (8).

We have also reported that CFU-S increase in numbers in the spleen after C. parvum stimulation (18). The CFU-S represents a pleuripotent stem cell for the hemopoietic
system. However, it represents only a small fraction of the overall cell population as reported by Till and McCulloch (26). Our present results show that the total cell population was rapidly increasing and that the CFU-S was also increasing as a part of the overall cell population increase. The properties of the cells produced were not assessed in this study. Those experiments are currently underway. Toujas et al. (27) have found that bone marrow cells are necessary for the occurrence of splenomegaly following C. granulosum injection. Hematological changes also occurred (5). At the same time a decrease in the number of immunocompetent cells can be observed, as measured by the number of spontaneous sheep RBC rosette-forming cells, T-cells, and the spleen phytohemagglutinin-responsive cells that have been found (22, 27). Therefore, it appears that some other cell, presumably a macrophage or related mononuclear cell, was produced by the proliferative response of the spleen C. parvum.

The present studies show that a normal tissue and the normal spleen cell responded to an appropriate stimulus by a rapidly mobilized cell response and proliferation. Growth kinetics during the initial state was exponential. DNA synthesis and DNA polymerase assays showed that a strong proliferative stimulus was produced by the C. parvum on the spleen, beginning shortly after injection of the vaccine. DNA distribution analysis by FMF flow cytometer did not show a significant change during the cell growth period.

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REFERENCES


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