Role of Lipopolysaccharide in the Production of Plasma Cell Tumors in Mice Given Mineral Oil Injections

Micsunica Platica and Vincent P. Hollander

Research Institute of the Hospital for Joint Diseases, Mount Sinai School of Medicine, New York, New York 10035

ABSTRACT

The lipopolysaccharide (LPS) content of peritoneal fluids of BALB/c mice given mineral oil injections and of normal mice was measured. Peritoneal fluids were passed through DEAE-Bio-Gel columns to remove an inhibitor to the Limulus amebocyte lysate reaction and then were assayed for LPS by a spectrophotometric Limulus amebocyte lysate test. A highly significant difference between control animals and animals given mineral oil injections was found. A clear correlation between LPS concentration and time after first oil injection was shown. P-200 gel chromatography and heat stability of the active material were consistent with the behavior of LPS. The possible role of LPS in the pathogenesis of plasma cell tumor is discussed.

INTRODUCTION

Previous work from our laboratory showed that chronic administration of ng doses of Escherichia coli LPS stimulates the rate and extent of plasma cell tumorigenesis in BALB/c mice given mineral oil injections (5). This paper demonstrates that administration of i.p. oil or pristane to BALB/c mice in the usual regimen to produce PCT’s causes an increase in the peritoneal fluid LPS.

MATERIALS AND METHODS

Female BALB/c mice (Texas Inbred Mice Co., Houston, Texas, and Mammalian Genetics and Animal Production Section, Drug Research and Development, National Cancer Institute, Bethesda, Md.) were used throughout the experiment. The mice were maintained on Purina laboratory chow and water ad libitum. For oil treatment, 0.5 ml of either Primol D (Exxon Corp., New York, N. Y.) or pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemical Co., Milwaukee, Wisc.) was injected i.p. at 2, 4, and 6 months of age.

Measurement of LPS. All glassware was soaked overnight in aqua regia, washed with distilled water until acid-free, and heated in an oven at 180° overnight. Whenever possible, disposable plastic ware was used. LPS-free distilled water was prepared by distilling water over KMnO₄ in an all-glass apparatus or was purchased from Abbott Laboratories, North Chicago, Ill. Lyophilized LAL was purchased from Sigma Chemical Co., St. Louis, Mo., or from Associates of Cape Cod, Woods Hole, Mass., and was reconstituted with LPS-free water according to the supplier’s directions.

Collections of Peritoneal Exudates. Peritoneal fluid was collected only from mice in good condition. All bloody ascites were discarded because such animals might harbor gross tumor difficult to find. No animal had dilated bowel suggestive of intestinal obstruction. None but 2 animals had gross tumor. The peritoneal fluid of these 2 mice, 22 and 23 months after the first injection of mineral oil, was not bloody. Animals were anesthetized with ether. The abdomen of each mouse was shaved, and the skin was sterilized with Betadine scrub. The skin was incised, and the peritoneum was opened with separate instruments. The peritoneal cavity was irrigated with 2 ml of LPS-free 0.9% NaCl solution by means of an LPS-free Pasteur pipet, and the peritoneal washing was removed and tested for LPS activity. All animals analyzed 1 to 7 months after the beginning of the treatment were given pristane injections. The rest of the mice were given mineral oil injections. Peritoneal washings and fluids were checked for sterility. No contamination was recorded.

LAL Test. A mixture of 20 μl of LAL and 120 μl of sample was incubated for 1 hr at 37°. Absorbance at 360 nm was determined before and after incubation. The procedure was a more rapid adaptation of a sensitive method published from this laboratory (9), which did not lend itself to analysis of the multiple samples required in the present work. LPS concentration was determined in duplicate from an E. coli LPS standard curve. The coefficient of variation was 8.5 to 10.8% within assays and 15.5 to 19% between assays. Samples showing less than 0.01 ng of LPS per ml were called negative in these experiments.

P-200 Chromatography. Gel chromatography demonstrated that LAL-active material was macromolecular; 50- x 0.6-cm Bio-Rad columns (737-1250) (Bio-Rad, Rockville Center, N. Y.) containing Bio-Gel P-200, 50 to 100 mesh, equilibrated with LPS-free 0.5 M NaCl were used. LPS was found in the first ml of effluent after the void volume. Void volume was determined with blue dextran (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.).

Separation of LPS from Peritoneal Fluid Inhibitor. LPS peritoneal fluids and washings were chromatographed on DEAE-Bio-Gel columns. This procedure separates LPS from an inhibitor to the LAL reaction found in plasma (11). LPS-free disposable Pasteur pipets were used throughout as columns. The columns were filled to a height of 2.5 cm with DEAE-Bio-Gel (Bio-Gel A, 100 to 200 mesh, wet; Bio-Rad, Richmond, Calif.) equilibrated with 0.1 M phosphate buffer.
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pH 7.4, or 0.1 M triethanolamine hydrochloride buffer, pH 7.4. The effluent was tested for LPS, and only negative columns were used. Samples (0.1 ml) consisting of LPS standards and peritoneal fluids or washings were applied to the columns, and 5 aliquots of 0.5 ml each were collected with the same buffer eluent and analyzed for the presence of LPS.

RESULTS

LPS added to mouse peritoneal fluid could not be detected by addition to the LAL system directly. Apparently, peritoneal fluid contains an inhibitor to the lysate reaction similar to that found in plasma (11, 15). Levin et al. (11) removed the inhibitor by treating plasma with chloroform. Reinhold and Fine (15) used an acidification method for the same purpose. However, these procedures did not lend themselves to measurement of small samples of peritoneal fluid, so that the DEAE-Bio-Gel chromatographic procedure described above was employed. When 1 ng of E. coli LPS was adsorbed on the column, it would be eluted in the first ml of effluent with a recovery of 65 to 75%. Addition of LPS in buffer, plasma, or control peritoneal fluid gave similar results.

We summarize our analyses of 17 peritoneal washings from control mice and 26 peritoneal fluids from mice given mineral oil injections (Chart 1). No peritoneal fluids were free of LPS among mice given oil injections. Of the control fluids 71% had less than 0.1 ng of LPS per ml, while only 11% from animals given oil injections contained less than 0.1 ng of LPS per ml. These latter samples were from mice given oil injections less than 2 months before sampling. The concentration of LPS correlated with the time after the first oil injection. There was no significant difference between peritoneal fluids from control mice and from mice given pristane less than 2 months before sacrifice. The LPS concentrations in peritoneal fluid collected from animals 2 to 5 months and 2 to 7 months after pristane injections were significantly higher than they were in controls (p < 0.001 by Student’s t test). The mean LPS for control was 0.09 ng/ml, and for all oil-treated mice (oil-treated refers to mineral oil injections) it was 1.2 ng/ml (p < 0.001). Mineral oil and pristane were checked for endotoxin contamination and were negative. Neither mineral oil nor pristane interfered with the LAL test.

Further Characterization of LPS. The LAL test for LPS is very sensitive, but a number of proteolytic enzymes, nucleotides, and peptidoglycans give a positive test (7, 20). Recently, we found that dithiols, such as diithiothreitol, also gave the reaction. Chromatography on DEAE-Bio-Gel was done on all samples to free LPS of the inhibitor to LAL and substances giving false-positive reactions. In addition, heat stability and behavior on Bio-Gel P-200 was evaluated. Seven samples of peritoneal fluid positive for LPS were shown to be unchanged in LPS content by heating at 100° for 20 min. Such heat stability is characteristic for LPS and unusual for proteolytic enzymes. Three samples positive for LPS were chromatographed on Bio-Gel P-200 with elution

# 4 M. Platica, W. Harding, and V. P. Hollander. Dithiols Stimulate Endotoxin in the Limulus Reaction, submitted for publication.

DISCUSSION

The working hypothesis motivating this study is that i.p. oil treatment of BALB/c mice produces PCT's because chronic oil-induced peritonitis allows LPS to leak from the gut into the peritoneal cavity. Much evidence favors this hypothesis. PCT's do not form in germ-free BALB/c mice given mineral oil injections, in spite of the development of the expected granulomatous peritoneal reaction (12, 14). When PCT's are induced in conventional mice, tumors are restricted to the peritoneal cavity and, in fact, form within the lipid granulomatous lesion produced by oil injection. Potter (13) has found many PCT lines that produce immunoglobulins that cross-react with antigens of enteric organisms. We have shown that i.p. administration of nanogram doses of LPS enhanced the PCT development (5). If endogenous LPS stimulates plasma cell tumorigenesis, we should expect to find it in the peritoneal fluid of the mice given oil injections.

The LAL test is the most sensitive method for the detection of LPS (6, 16, 21). This sensitivity is required because the concentrations found in this study cannot be measured with any other known procedure. The method has drawbacks. Extreme care to avoid contamination of reagents and glassware with the ubiquitous LPS is required. Plasma and peritoneal fluid contain an inhibitor to the Limulus reaction, which must be removed (11). Moreover, the test may give false-positive reactions. Trypsin, synthetic polynucleotides, and peptidoglycans (7, 17, 20) may react in the LAL test and must also be removed. In this study all samples have gone through DEAE-Bio-Gel chromatography, which removed the inhibitor to the LAL reaction and presumably other proteins and allowed LPS to pass through the column. We have examined representative positive samples for heat stability and for exclusion of the active substances
by Bio-Gel P-200. These criteria do not absolutely identify the active fraction as LPS but represent highly probable identification. We are currently attempting to develop a radioimmunoassay for Lipid A, which may lead to absolute identification of LPS in peritoneal fluids. The method used in this work is extremely laborious, and chromatographic separation of LPS from the inhibitor makes the study of large numbers of mice impractical. The number of mice used in this study was restricted to the number required for demonstration of highly significant differences. The radioimmunoassay procedure should make possible the detection of much smaller differences. The analysis for LPS demonstrated highly significant differences. The rationale for the separation of LPS from the inhibitor makes the study of peritoneal fluids from control animals and from animals given oil injections.

Our working hypothesis is that oil granulomata cause LPS to leak into the peritoneal cavity and stimulate plasma cell tumor development in a manner similar to that reported by us for i.p. administration of LPS. Accordingly, it was necessary to eliminate mice with plasmacytomomas from the study, since PCT's might produce more LPS leak from the gut than did granulomas and would invalidate any claim for tumorigenicity on the part of LPS. Almost all plasmacytomomas are accompanied by bloody ascites, and so peritoneal fluids were not analyzed. Only 2 mice shown in Chart 1 had gross tumor (22 and 23 months after mineral oil injection); the high i.p. LPS in these mice was not obviously different from the greatly elevated values found in mice long after mineral oil treatment. Since this report is concerned only with mice that do not have plasmacytomomas, we were aware that the most important part of our argument was concerned with the period after oil treatment and before the time that PCT's could be expected. Peritoneal fluid collected 2 to 5 and 2 to 7 months after mineral oil treatment shows a highly significant increase in LPS over controls. PCT formation at 5 months after pristane treatment is very rare. At this time it is very difficult to distinguish PCT from plasma cell hyperplasia by microscopic examination, and nothing would be gained by serial sections of the countless granulomata present. At 7 months after pristane few PCT's would be expected (14), and of those most would be eliminated by discarding bloody ascites and by gross examination for PCT.

It is not clear why mice do not show significantly increased LPS for the first 2 months after oil injection. However, at this time there is much less granulomatous reaction than there is at 5 months after oil treatment. We interpret these data to show that increased i.p. LPS results from i.p. oil treatment and precedes tumor development. The LPS found in the control peritoneal washings may be the result of contamination from animal skin or LAL blank, which we cannot characterize, or of LPS absorbed from normal gut. The low concentrations of LPS found in the peritoneal fluids from mice given oil injections less than 2 months before sampling suggest that LPS is not simply a contaminant from injection but arises from the chronic peritonitis that develops after oil treatment. These results are consistent with but do not prove our hypothesis that LPS, a well-known B-cell mitogen (1-4, 8) plays an intermediate role in the pathogenesis of PCT in the BALB/c mouse given oil injections. We have described the prevention of PCT by administration of cortisol (18) and certain glycoprotein pituitary hormones (19) and the enhancement of PCT by administration of growth hormone and testosterone (10) to the mice given oil injections. Current studies attempt to correlate these modulating factors with i.p. LPS.

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