Combination Immunotherapy of Cancer in a Mouse Model: Synergism between Tumor Necrosis Factor and Other Defense Systems

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ABSTRACT

Bacterial lipopolysaccharide (LPS) induces the release of factors into the serum which enable mice to reject experimental tumors. One such factor is tumor necrosis factor which causes acute necrosis of syngeneic sarcoma transplants in mice. Effective therapeutic use of tumor necrosis factor is limited, however, by its toxicity. We show here that the efficacy of tumor necrosis factor can be substantially increased by combining its application with low doses of LPS. Our data suggest that LPS exerts its antitumor effects by engaging more than one defense mechanism. Characteristic for the activation of a biological system is a concomitant induction of negative feedback mechanisms which antagonize the initial stimulus. Interference with the negative feedback response may substantially increase biological reactions. We show here that the blocking of two negative feedback responses occurring as a consequence of treatment with LPS, namely the production of prostaglandin E and the generation of suppressor T-lymphocytes, increases dramatically the ability of mice to reject tumor transplants.

Thus, through appropriate combination of different factors one may reduce the dose of each below toxic levels and through interference with negative feedback responses increase the efficacy of antitumor reagents. We consider our findings in the context of formulating an effective immunotherapy of malignancies and as a promising step toward it.

INTRODUCTION

LPS, prepared from the outer membranes of gram negative bacteria has been shown to have strong antitumor effects in mice (1, 2). It causes acute necrosis of some (but not all) experimental tumor transplants in mice and, in many cases, complete regression of the tumor. The latter phase, tumor rejection, is in contrast to the former, tumor necrosis, an immunological phenomenon (3).

The application of LPS as a therapeutic has been hampered by its prohibitive toxicity. It was learned that LPS causes the release of several factors into the serum of treated animals and such serum, called TNS, caused tumor necrosis and frequent rejection of tumors without apparent toxicity to the tumor host (4, 5). The molecule in TNS responsible for tumor necrosis, TNF, is now produced in recombinant bacteria (6). TNF is a very efficient inducer of tumor necrosis but is also very toxic to animals. LPS-induced shock seems to be mediated by TNF (7). For the cure of a tumor a mouse seems to have to pay a smaller penalty on toxicity when TNF is administered within TNS than when administered in purified form. We have investigated this phenomenon in the study presented here. Our findings suggest that LPS acts on tumors through different pathways only one of which is mediated by TNF. In combination the different defense systems are more effective than each by itself.

MATERIALS AND METHODS

Tumors. Meth A tumors were maintained in BALB/c mice by weekly passages. For transplants, 10^6 cells/0.2 ml, were injected intradermally into two sites of the shaved abdominal skin of BALB/c × C57BL/6 F1 mice with a 30-gauge needle. The grade of tumor necrosis (progressive darkening in color of tumor portions) was scored 48 h after LPS treatment in a grading system consisting of grades 0, 1, 2, and 3 according to Carswell et al. (5). Grade 3 reflects total or near total necrosis of the tumor, grade 2 covers necrosis areas down to approximately one-half of the tumor, and grade 1 covers all necrosis areas smaller than one-half of the tumor. Tumor rejections, meaning complete disappearance of tumors for longer than 4 weeks, were scored from 4 weeks after LPS treatment.

Mice. BALB/c mice and BALB/c × C57BL/6 F1 mice were purchased from The Jackson Laboratory, Bar Harbor, ME. LPS (Escherichia coli 0111:B4) was from Ribi, Hamilton, MT; Indomethacin was from Sigma Chemical Co., St. Louis, MO; cyclophosphamide was from Johnson and Co., Evansville, IL.

Treatment. TNF or LPS was injected i.v. 7 days after tumor transplantation, after tumors had reached an average diameter of 10 mm. Indomethacin (100 μg/animal) was injected i.v. 1 day before and again together with LPS and/or TNF, and subsequently was added to the drinking water (20 μg/ml). Cyclophosphamide (30 mg/kg) was injected i.p. 2 days after LPS treatment. Freshly prepared BALB/c mouse serum was injected i.v. twice, 2 and 5 days after LPS injection, were performed into the intraorbital plexus.

RESULTS

Synergism between LPS and Tumor Necrosis Factor. We wished to know whether TNF was the exclusive mediator of the antitumor effects of LPS or whether other mediators identified in LPS-induced tumor necrosis serum also played a role. We investigated this question by combining TNF and LPS in the treatment of tumor-bearing mice. Syngeneic Meth A sarcomas were transplanted into the skin of BALB/c × C57BL/6 F1 mice and the animals were treated with TNF and/or LPS 7 days later when tumors had grown to the size of approximately 1 cm in diameter.

The results are shown in Table 1. TNF is an effective inducer of tumor necrosis but its therapeutic dose range is small. Substantial degrees of necrosis can be achieved with the injection of 10 μg TNF. There is a rapid decline of necrosis at lower doses. Higher doses were toxic and not administered here. LPS, in a nontoxic dose range, is a less potent inducer of tumor necrosis than purified TNF. Added together, however, the two biologics synergize. They cause a more effective tumor necrotization together than either one of the two substances alone.

A synergism between LPS and TNF becomes also apparent if one measures the proportion of tumor-bearing mice which reject the tumor after treatment (Table 1). By necrotizing the tumors so effectively, TNF produces initially the appearance of tumor rejection within 1 or 2 weeks of treatment. Some tumor cells seem nevertheless to survive TNF treatment and resume growth subsequently leading to sizable tumors 4 weeks after treatment. In order to obtain reliable data in terms of tumor rejection as a consequence of TNF treatment it is important to wait at least 4 weeks before treatment results are assessed. Our results shown in Table 1 are obtained in this way. Both LPS

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The abbreviations used are: LPS, lipopolysaccharide; TNS, tumor necrosis serum; TNF, tumor necrosis factor; NK, natural killer; IFN, interferon; PGE, prostaglandin E; INDO, indomethacin; IL-1, interleukin 1.
and TNF may cause tumor rejection alone, but both substances are substantially more active when added together. One μg of LPS, for example, has no effect alone but, injected together with TNF, it increases the rejection rate substantially above that induced by TNF alone.

Negative Feedback Effects in the LPS-Induced Antitumor Response. LPS, as mentioned above, induces the release of TNF and other mediators which mobilize the body's defense mechanisms. One such factor is interferon (8), which is a potent inducer of NK cell activity (9–11). Another factor is interleukin-1, a mediator that stimulates the antigen-dependent response of lymphocytes (12–14).

IFN activates NK cells. We have considered the possibility that NK cell activation contributes to the response of tumor-bearing mice to LPS, and studied the activation of NK cells in vitro. We reported that IFN has a dual effect on NK cells; it increases their cytotoxic activity and induces at the same time the production of PGE which blocks NK cell activity (9–11). Another factor is interleukin-1, a mediator that stimulates the antigen-dependent response of lymphocytes (12–14).

Table 1 Tumor necrosis factor and LPS synergism in their antitumor activities

<table>
<thead>
<tr>
<th>Treatment (μg/mouse)</th>
<th>Necrosis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rejection&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF (10)</td>
<td>2.0</td>
<td>1/10</td>
</tr>
<tr>
<td>TNF (3)</td>
<td>1.4</td>
<td>1/10&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>TNF (1)</td>
<td>0.6</td>
<td>0/20</td>
</tr>
<tr>
<td>LPS (20)</td>
<td>1.1</td>
<td>6/20</td>
</tr>
<tr>
<td>LPS (10)</td>
<td>0.4</td>
<td>0/20</td>
</tr>
<tr>
<td>LPS (1)</td>
<td>0.1</td>
<td>0/20&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>LPS (1) + TNF (3)</td>
<td>2.3</td>
<td>14/20&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>LPS (1) + TNF (1)</td>
<td>1.1</td>
<td>2/20</td>
</tr>
</tbody>
</table>

<sup>a</sup> Averaged degrees at 48 h after treatment.
<sup>b</sup> Number of rejections/tumors transplanted. Data combined from two experiments.
<sup>c</sup> Difference between e and d or e and d is significant at P < 0.005 by the χ<sup>2</sup> test.

Table 2 Indomethacin and cyclophosphamide enhancement of the antitumor effects of LPS

<table>
<thead>
<tr>
<th>Treatment (μg/mouse)</th>
<th>Necrosis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rejection&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS (20)</td>
<td>1.1</td>
<td>21/64 (33%)&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>LPS (20) + INDO&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.9</td>
<td>43/64 (67%)&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>LPS (20) + INDO + cyclophosphamide&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.9</td>
<td>20/24 (83%)&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>INDO</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Averaged degree of necrosis.
<sup>b</sup> Number of rejections/tumors transplanted. Data combined from all of the experiments.
<sup>c</sup><sup>d</sup> Difference between e and c or e and d is significant at P < 0.005. Difference between g and c is significant at P < 0.0005.
<sup>f</sup> See "Materials and Methods."
<sup>f</sup> 30 mg/kg/mouse, 2 days after LPS and INDO.

Fig. 1. Synergism between TNF and LPS causing necrosis and regression of tumor transplants in mice. Animals were treated in addition to TNF and LPS with INDO. A, TNF alone; A, TNF plus LPS (1 μg/mouse), A, degree of necrosis; B, percentage of regression.

Table 3 Antisuppressor cell factor in normal mouse serum enhancement of the rejection rate of tumor transplants in mice

<table>
<thead>
<tr>
<th>Treatment (μg/mouse)</th>
<th>Saline (50 μl/mouse)</th>
<th>Normal mouse serum (50 μl/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS (1) TNF (3)</td>
<td>11/16 (69%)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>15/16 (93%)&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>LPS (1) TNF (1)</td>
<td>3/16 (19%)&lt;sup&gt;h&lt;/sup&gt;</td>
<td>10/16 (63%)&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>LPS (1) TNF (0.3)</td>
<td>0/8 (0%)</td>
<td>0/8 (0%)</td>
</tr>
</tbody>
</table>

<sup>g</sup> Number of rejections/tumors transplanted. Data combined from two experiments performed in this fashion.
<sup>h</sup> Difference between e and d is significant at P < 0.05. Difference between e and d is significant at P < 0.025.

Table 4 Combination immunotherapy of cancer in mouse model

<table>
<thead>
<tr>
<th>Treatment (μg/mouse)</th>
<th>Necrosis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rejection&lt;sup&gt;b&lt;/sup&gt;</th>
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</tr>
<tr>
<td>LPS (1) + TNF (1)</td>
<td>1.1</td>
<td>2/20</td>
</tr>
</tbody>
</table>

<sup>a</sup> Averaged degree of necrosis.
<sup>b</sup> Number of rejections/tumors transplanted. Data combined from all of the experiments.
<sup>c</sup> Difference between e and d or e and d is significant at P < 0.005 by the χ<sup>2</sup> test.

with indomethacin. In this combination we observed toxicity and we lost some experimental animals.

LPS may also stimulate antigen-specific lymphocyte reactions through the release of IL-1 (12–14). An immune response of T-lymphocytes against tumor-associated antigens has been described by Berendt et al. (3, 18) as a consequence of LPS treatment. Not only immunologically specific effector T-cells are generated but also tumor antigen-related suppressor T-cells whose activity was shown to antagonize the function of effector T-cells and the ability of mice to reject tumors. The function of the suppressor cells was diminished by treatment of mice with low doses of cyclophosphamide (18, 19). We wished to know whether a reduction of suppressor cell function may further increase LPS-mediated tumor rejection above the level already achieved by combination treatment with INDO. Table 2 shows that this is the case. Cyclophosphamide injected 2 days after LPS and INDO allows almost all experimental animals to reject their tumors. We chose to delay injection of cyclophosphamide as compared to the injection of LPS and INDO because preliminary experiments had shown cyclophosphamide to interfere with the formation of tumor necrosis induced by LPS.

A factor which reverses the immune suppression in tumor-bearing mice has recently been discovered by Katz et al. (20) in normal serum. The factor does not occur in plasma or in the serum of platelet-deficient individuals and seems to be identical with platelet factor 4 (21). Fifty μl of normal mouse serum are reported to contain enough of this factor to reverse immune suppression in tumor-bearing mice. We have examined this serum factor in our tumor system and confirmed its activity (Table 3). The serum factor increased the ability of tumor-bearing mice to reject the malignancy in conjunction with LPS, TNF, and INDO.

Correlation between Tumor Necrosis and Tumor Regression. Inspecting tumor-bearing animals after treatment with either LPS or TNF we noted that TNF induces relatively consistent degrees of necrosis at a given dose. Plotting averaged degrees of necrosis against the percentage of tumor rejection associated
with that necrosis, we found a linear relationship (Fig. 2). Such a clear correlation cannot be seen with animals treated with LPS. Given injections of 20 μg of LPS, mice may exhibit all degrees of necrosis from 0 to 3. It is interesting that, while TNF-treated animals regressed only necrotized tumors, LPS-treated mice rejected tumors regardless of whether they were necrotized or not. Only a high degree of necrosis favored rejection.

DISCUSSION

Bacterial products and in particular LPS have long been known to cause damage to tumors in animals (1, 2). Their therapeutic use has been hampered, however, by the high degree of toxicity to the tumor host. The discovery that TNF, the serum of LPS-treated animals, mediates the antitumor effects of LPS without apparent toxicity to the treated animals seemed to offer a solution to the toxicity problem (4, 5). However, when the factor in TNS responsible for tumor necrosis, TNF, became available in purified form as a product of recombinant bacteria, it was noted that it exhibits considerable toxicity in mice. A single injection of 20 μg TNF may kill a mouse. Substantial antitumor activity on the other cannot be achieved with less than 10 μg/mouse. This shows that lethal and therapeutic doses of TNF are dangerously close together.

TNF is remarkably effective in causing necrosis of Meth A tumor transplants in mice, but we noted that in order to cause lasting tumor elimination TNF must completely necrotize tumors. This is in striking contrast to LPS which causes tumor elimination often with minimal or no necroptosis. The observation suggests that the action mechanism of LPS-TNS is different from the action mechanism of TNF, and that TNS may not exclusively act through TNF. In fact, in view of the long established notion that LPS-induced tumor necrosis and LPS-induced tumor regression are two distinct phases of LPS-mediated antitumor activities (3, 4), it would appear that TNF mediates only the early phase of tumor necrosis induction.

The experiments described here are based on the hypothesis that LPS-induced tumor necrosis serum contains a number of factors which engage different defense mechanisms with one of them involving TNF. Besides causing necrosis, TNS can also engage the immune system through IL-1 (12–14) and NK cells (9, 10). Each factor activates a defense mechanism which may have some antitumor activity alone and may cause tumor elimination but with the penalty of toxicity as we see in the case of TNF. Together they may be effective when applied at doses below toxicity levels.

The data presented here support this hypothesis in many ways. Both TNF and LPS cause tumor necrosis but at a given degree of necrosis LPS is by far more effective in causing the complete rejection of the tumor. In other words, LPS not only causes necrosis of tumors but also enhances the ability of the tumor host to eliminate those tumor cells which escaped necroptosis. If one considers the LPS-mediated antitumor reaction as consisting of two phases, the acute necrosis phase and the subsequent immunological rejection phase, it would appear that TNF is active only in the first phase and LPS in both phases.

TNF may not be the only factor in LPS-induced TNS to account for effective necrotization of tumors because TNF synergizes with LPS also in causing tumor necrosis. It is unlikely that this can be attributed to the release of additional TNF. LPS causes necrosis only in rather high concentrations but synergizes with TNF in low concentrations. This would suggest that in order to produce TNF, LPS is required in high doses, while in low doses it causes the release of factors that synergize with TNF (LPS itself does not act directly on tumor cells (5). One likely candidate for such a synergistic factor is interferon which occurs in the serum of LPS-treated mice (8, 10, 11) and whose ability to synergize with TNF has been demonstrated (22).

The LPS-mediated elimination of tumor cells which survive the attack of TNF requires an intact immune system and therefore seems to be an immunological process. Berendt et al. (3, 18) described the generation of T cells specifically reactive with tumor associated antigens and the concomitant increase in suppressor T-lymphocytes. We consider it likely that IL-1, of which LPS is such a strong inducer (12–14), is instrumental in the stimulation of tumor related T-cell responses.

In considering immunotherapy as a treatment of malignancies one must not overlook the fact that an immune response automatically induces its own negative feedback. The feedback response is important to keep the response from overshooting but, when initiated prematurely or when stimulated in a chronic fashion, as in patients with advanced cancer (23), it may completely block immune functions. Beneficial as feedback reactions may be for maintaining a balanced defense system, it may be desirable to block them at least temporarily when the task of eliminating malignancies demands it.

There are at least two negative feedback reactions whose prevention in animals facilitates the rejection of experimental tumors. One inhibitory pathway is mediated presumably by prostaglandin E, which we conclude from the fact that the pathway can be blocked by indomethacin. LPS, presumably via interferon, is a strong inducer of PGE synthesis (24). We have previously observed a strong synergism between LPS or IFN and INDO in the generation of NK cell in vitro (16). In that response IFN induces the activation of NK cells and simultaneously the production of PGE which inhibits NK cell activation. Blocking of PGE synthesis with INDO increases NK cell activation by IFN 5–50 times. In the tumor-bearing mouse INDO seems to block LPS-induced PGE production and it improves the animal’s ability to reject the tumor. Interference with suppressor T-cell-mediated negative feedback response has been shown previously to improve the ability of the animal to eliminate tumor transplants (18), and we show here that in concert with other measures to block negative feedback responses it increases the rejection rate to almost 100%. Cyclophosphamide has been shown to inhibit preferentially suppressor T-cells at the dose applied in the experiments here (19). In higher doses it affects also other lymphoid cell functions. Factors activated in serum during the clotting process and released...
by platelets have been shown to reverse cancer-related suppression of the humoral immune response (20, 21) and our data suggest that such factors also reduce suppressive effects on the host response against its tumor.

Applied in the right fashion LPS is thus an extremely effective therapeutic substance regarding Meth A tumor transplants in mice. Its main disadvantage, its toxicity, seems to be related to the production of TNF (7) whose sufficient production (judged by the degree of necrosis achieved) requires high doses of LPS. When TNF is used from an external source much lower doses of LPS are needed to complement TNF action. By apparently utilizing different defense systems of the body, LPS provides an example as to how these defense systems may interact to achieve an optimized attack against malignancies, and the study of LPS action may help in designing new strategies in the treatment of malignancies by activating the body’s means of defense in a concerted fashion.

Note Added in Proof

The synergistic antitumor effects of LPS and TNF are interpreted here as a therapeutic advantage allowing effective treatment with doses that are substantially below toxic levels. After this manuscript was submitted we have reexamined the toxicity of LPS and TNF when applied together and found that INF increases the LD50 of LPS 37.5 times in BALB/c mice.

REFERENCES

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