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Antitumor Mechanism of Intradermal Administration of Lipopolysaccharide

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Abstract. *We earlier demonstrated that 50 % of the lethal dose of lipopolysaccharide (LPS) from Pantoea agglomerans given by the intradermal (i.d.) route is about 300 times greater than that given by the intravenous (i.v.) route, and that 400 μg/kg of LPS administered i.d. significantly suppressed metastasis whereas administered iv. it did not. To learn the specific mechanism involved in this i.d. administration, the fate of LPS at the skin following administration and the concurrent production of endogenous tumor necrosis factor (TNF) in serum was examined. Histological observation following the i.d. administration of LPS (40 μg/kg) revealed neutrophiles in the skin 6 hours later. After 24 or 48 hours inflammatory cells were assembled at the site of injection. Endogenous TNF activity was found in the skin 24 hours after the injection and was significantly detectable even after 48 hours. Endogenous TNF was induced around tumor lesions of Meth A fibrosarcoma, MH134 hepatoma and Lewis lung carcinoma by treatment of LPS administered i.d. Taken together, these findings suggest that the antitumor activity of i.d. administered LPS results from the continuous supply of a small amount of this substance producing free TNF and activating inflammatory cells such as macrophages having membrane bound proTNF on their surface from the injected site to the tumor lesion for more than 48 hours.*

We reported that lipopolysaccharide (LPS) an endotoxin of Gram negative bacteria, when administered intradermally (i.d.) had an antitumor effect against an allogenic murine tumor model (1) and syngeneic murine tumors, Meth A fibrosarcoma, MH 134 hepatoma and Lewis lung (LL) carcinoma model (2,3), and cancer patients (4) without severe side effects. Intradermal (i.d.) or oral administration of LPS decreased its toxicity without lessening the curative effect on various intractable diseases (5,6).

In a comparison of the inhibitory effect of lung metastasis of Meth A to the mouse by LPS administered i.d. or intravenously (i.v.), a significant inhibition was observed in i.d. treated mice, whereas this was not true in mice treated i.v. (2). Although an effective dose of LPS by the i.d. route against the syngeneic tumor model was comparable to that by the i.v. route (3,7), the LD_{50} of i.d. LPS administration is about 300 times higher than that of i.v. administration (2). These results suggested that a different mode of action was involved in the antitumor effect by the two routes.

Three observations were noted in an attempt to understand the characteristics of the antitumor effect following i.d. administration of LPS. The first is that carbon clearance in blood was enhanced to the same level as by the i.v. route, and accumulation of inflammatory cells was noted in the healing lesions (1). The second is that the antitumor effect of the i.d. administration was neutralized by anti-TNF serum (2). The third is that we proved that i.d. administration of LPS efficiently induced endogenous TNF *in vivo* (8). These findings suggested that this administration route induced the activation of the inflammatory cells (macrophages, neutrophiles and others) that produce a moderate amount of free tumor necrosis factor (TNF) or the expression of membrane-bound TNF (precursor TNF) on activated macrophages (9,10), which are known to have a cytocidal effect (11,12) in the host body.

We then hypothesized that i.d. administered LPS was localized in the skin lesion and gradually released into the bloodstream from the skin, and exerting its antitumor effect by activating inflammatory cells.

We discuss here the putative mechanism of action of i.d. administration of *Pantoea agglomerans* LPS (LPSp).

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Materials and Methods

LPS. LPS from *Pantoea agglomerans* was purified by the conventional method (11); the purity was over 95 % (w/w) (protein contamination was less than 2 % (w/w), and nucleic acid contamination was less than 4 % (w/w)).

Kinetic and histological study of i.d. injected LPS. One μg of LPSp was administered into the mouse dermis of the abdomen to measure the residual LPSp. Skin was removed after injection and the skin tissues (dermis and tela subcutenea) of a 10 mm × 10 mm area were collected from the back side, except the cutis (epidermis) to prevent LPS contamination. After sonication of this tissue in saline, supernatant was obtained by centrifugation (2000 *g*), and the LPSp content and TNF activity of the sample were measured according to the described previously (10,12). For histological observation, each removed piece of skin was fixed by 10 % formalin solution and a paraffin slide was stained with hematoxylin-eosin using the conventional technique.

Induction of endogenous TNF in tumor lesion by LPSp administered i.d. Details of the procedure to obtain TNF-contained samples have been described previously (8,13,14). Briefly, Meth A fibrosarcoma (BALB/c), MH134 hepatoma (C3H/He) and Lewis lung carcinoma (C57Bl/6) bearing mice were treated i.d. with LPS. Ninety minutes after injection tumor tissues were taken, weighed, minced and homogenized. Supernatant of the homogenized sample obtained by centrifugation (7000*g* × 10minutes) was kept at -80°C until use. TNF assay was done by the method described (12,13).

Statistical analysis. Statistical evaluations of differences between groups were made by Student's t-test.

Results

Fate of LPS and TNF in the skin and histological change with time after i.d. administration of LPSp. To determine the kinetics in the skin after LPSp administration or the change in the amount of LPSp remaining in skin and also the amount of TNF activity, as an indicator of macrophage activation, were examined over time. Histological change with time in the skin was also observed. I.d. administered LPSp (40 μg/Kg) was found to exert an antitumor effect on Meth A tumor, was used for a kinetics and histological study.

The amount of LPSp was found to decrease in the skin with a half-life of 90 minutes during the first 5 hours and thereafter with a half-life of 12 hours (Figure 1). Even 48 hours after its injection, a significant amount of LPSp (more than 1 % of the dose) could be detected, presumably enough to induce free as well as precursor TNF.

As shown in Figure 1, free TNF was produced at the local site of skin by LPSp administration. A detectable amount of TNF was produced in the skin 3 hours after LPSp treatment and the maximum activity was observed after 24 hours. TNF activity decreased gradually, but was significantly detectable in the skin even at 48 hours.

Samples of removed skin as described in Materials and Methods with time after LPSp administration were analyzed histologically (Figure 2). A typical event observed was the accumlation of inflammatory cells. Neutrophiles were assembled around the blood vessels of the skin 6 hours after

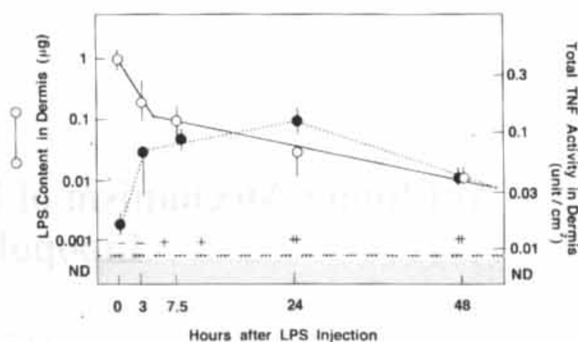

Figure 1. *Fate of LPS and TNF in the skin with over after LPSp administration i.d. LPSp (40 μg/kg) was administered i.d. into abdominal skin. Skin was removed after injection and the tissues of a 10 mm × 10 mm area were collected as described in Materials and Methods. LPS content (○) and TNF activity (●) of the samples were measured as described. (-), not detectable inflammatory cells; (+), detectable inflammatory cells; (++), relatively rich number of inflammatory cells. Symbols and bars represent mean values of three individual samples and their standard deviations, respectively. The shaded zone indicates the undetectable amount of LPS or TNF in the samples.*

LPSp injection. After 24-48 hours numerous inflammatory cells, mainly macrophages and neutrophiles were seen in the dermis and tela subcutenea. Relative amount of the assembled cells is indicated on the bottom line of Figure 1.

TNF amount in tumor lesion by i.d. administration of LPSp. The amount of TNF induced in tumor bearing-mice was examined in three strains: BALB/c mice-Meth A tumor, C3H/He mice-MH134 tumor and C57BL/6 mice-LL tumor. In all three, 200 to 350 U/g of TNF activity was observed in the tumor lesion (Figure 3). No differences due to strain or tumor model were recognized so far as the amount of TNF induced. The induction of TNF by i.v. LPS has been reported by Haranaka *et al.* to differ among mice strains (15).

Discussion

To determine the particular mechanism induced, the fate of LPS and histological analysis at the skin its i.d. administration was investigated. Endogenous TNF activity was also measured to assess the activation of assembling inflammatory cells.

As shown in Figure 1, i.d. administered LPSp was retained in skin for a long period (more than 1% of that injected was found in the skin 48 hours after injection) however, LPSp administered i.v. disappeared quickly (less than 1% of that injected was measurable in blood 1 hour after the administration (data not shown)). One μg of LPSp per mouse given i.d. continuously produced endogenous TNF at the site of injection for up to 48 hours thereafter, while i.v. injection of LPS only transiently induced endogenous TNF in serum for

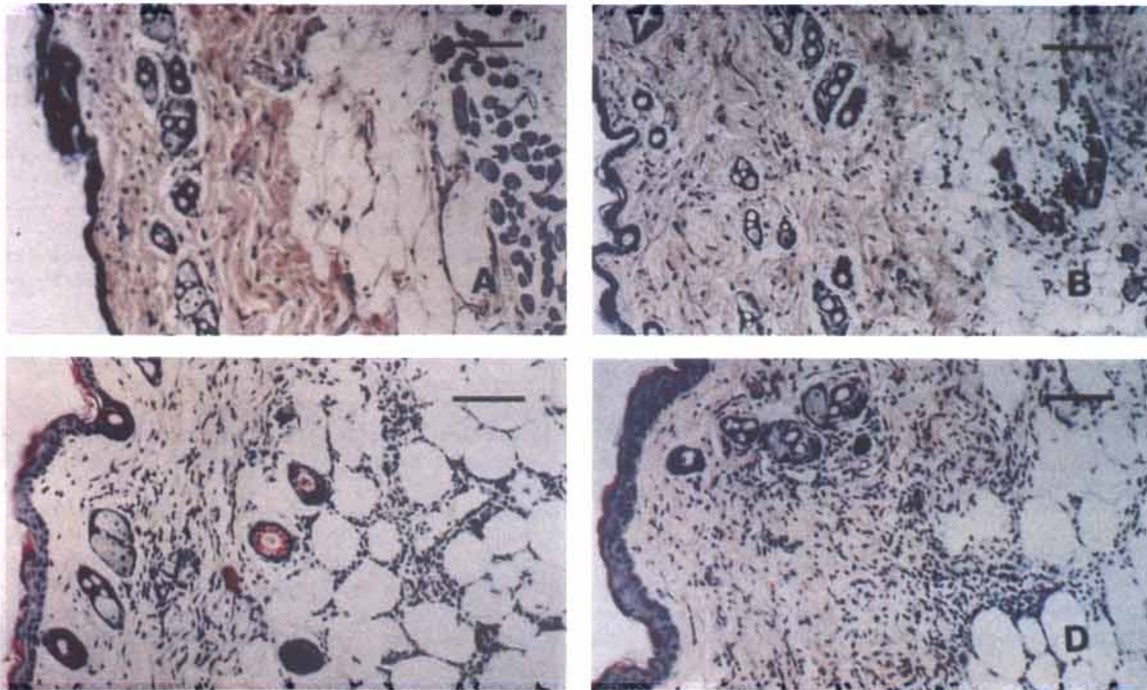

Figure 2. *Histological section of skin tissue injected with 1 µg of LPSp. Hematoxylin-eosin stain. a, control. b, 6 hours after LPS administration. c, 24 hours after LPSp administration d, 48 hours after LPSp administration. (Bar = 100 µm).*

from 30 to 120 minutes (7). These observations indicate that the substance administered i.d. remained a long time in the lesion resulting in continuous activation of migrating macrophages producing TNF in the skin whereas i.v. administration distributed LPSp quickly throughout the body producing a high but transient amount of TNF. Therefore, part of the reason for the different modes of action of LPS by the i.d. and i.v. routes can be ascribed to the difference in LPS fate and the kinetics of the TNF produced in the body. It is conceivable that the reduction in LPSp during the early stage might be due to its leaking into blood vessels, leading to the activation of inflammatory cells there in the same way as with i.v. administration. LPSp in the blood after i.v. injection rapidly dropped to less than 1 % of the injected amount after one hour (data not shown). The remaining amount of LPSp in the skin at a later stage may have other functions as discussed later.

As shown in Figures 1 and 2, the initial stage of inflammation was induced locally by i.d. administration of LPSp. In other words, LPS retained in this local region could initiate an inflammation which assembles and activates neutrophiles and macrophages to produce free TNF and/or precursor TNF. It is then expected that part of the inflammation containing those inflammatory cells is released into the bloodstream.

The gradual effect of i.d. LPSp administration can thus be postulated in at least two ways: one is the continuous assemblage of inflammatory cells (Figure 2) which are activated by LPSp in the area of injection and the continuous

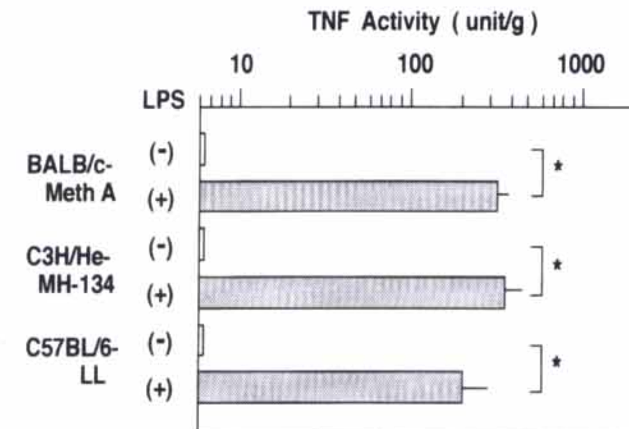

Figure 3. *TNF activity in the tumor lesion after LPSp i.d. injection. Meth A bearing-BALB/c mice, MH134 bearing-C3H/He mice or LL bearing-C57BL/6 mice were treated i.d. with saline (-) or 400 µg/kg of LPSp (+). Ninety minutes after the injection, tumor tissue was obtained and tested for TNF activity, as described in Materials and Methods. Columns and bars represent mean values and standard derivitions for individual samples. * Significant difference ($P<0.001$) between saline control and LPSp administration by Student's t-test.*

production and release of either free TNF or TNF-primed cells into the bloodstream, and the other is the continuous supply of LPSp from its injected site into the bloodstream and the continuous production of free TNF or TNF-primed inflammatory cells in the blood. Free TNF released into the

bloodstream over a long period (Figure 1) can prime inflammatory cells (16) in the bloodstream which can also participate in this event, though the amount is small. We previously reported that the essential factor in curing a tumor by biological response modifiers is the induction of TNF endogenously in the tumor lesion (7). I.d. administered LPS also induced a therapeutic dose of TNF in three kinds of murine tumor models (Figure 3).

An accompanying paper demonstrates that i.d. administration of LPSp has a more than 300 times greater advantage for use in antitumor therapy than does i.v. injection (2). This advantage means a more beneficial application of LPS via the i.d. route as a low toxicity Coley's vaccine. Thus, a new LPS delivery system, i.d. administration, seems to be hopeful for use in clinical situations, not only for antitumor therapy but for other intractable diseases as well (4,17,18).

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