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molecular pharmaceutics

Immunostimulation Mechanism of LPD Nanoparticle as a Vaccine Carrier

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Received October 6, 2004

Abstract: A novel and improved vaccine delivery system and/or adjuvant is actively sought to enhance the potency of vaccines. Previously, we reported that strong antitumor immunity could be generated when a peptide antigen was incorporated into LPD (cationic liposome−polycation−pDNA) nanoparticles. In this study, we found that both the cationic liposome and DNA are required for the full immunostimulation activity of LPD. The unique ability of LPD to readily move into local lymphoid tissues and to activate antigen-presenting cells might be responsible for its strong immunostimulatory activity. Moreover, cationic liposome stimulates the expression of CD80/CD86 on dendritic cells (DCs), but not the release of TNF-α from DCs, suggesting the existence of a NF-κB-independent immunostimulation pathway for cationic lipids such as DOTAP.

Keywords: Cationic lipid; plasmid DNA; costimulatory molecules; TNF-α; lymph nodes

Introduction

Traditionally, vaccines are comprised of either live attenuated or killed bacteria or viruses. Because of the risk associated with some of the traditional vaccines, new generation vaccines such as protein-, peptide-, and DNA-based vaccines have emerged. However, the potency of the new generation vaccine is often poor when administered alone; an adjuvant and/or a delivery system is often necessary.[1]

Recently, we have reported that when a MHC class I-restricted peptide (9 amino acids) epitope derived from the E7 protein of HPV 16, one of the cervical cancer-causing subtypes of HPV, was incorporated into LPD nanoparticles and then used to immunize mice, a strong antigen-specific antitumor response was observed.[2] The LPD/E7 induced an E7-specific CTL response and prevented the establishment of E7-expressing TC-1 tumor. Moreover, administration of LPD/E7 to TC-1 tumor-bearing mice caused complete tumor regression.[2]

LPD was originally designed as a liposome-based DNA delivery system for gene therapy.[3] It was engineered by combining cationic liposomes (composed of DOTAP and cholesterol) and polycation-condensed bacterial plasmid DNA. When they were mixed, the components spontaneously rearrange to form a virus-like structure with the condensed DNA located inside the lipid membranes.[4] When administered systemically in large doses, LPD rapidly initiates the production of several Th1 cytokines, most notably, TNF-α,

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10.1021/mp049907k CCC: $30.25
Published on Web 12/14/2004

IL-12, and IFN-γ.[5] Such cytokine production is associated with tumor static effects to some extent.[6] It was thought that NK cells play a major role in the nonspecific tumor-killing process.[6] NK cells activated by the proinflammatory cytokines may kill some tumor cells. The debris from the nonspecific killing may then be taken up by APC such as DCs and macrophages to initiate a specific CTL response for further tumor killing. The strongest evidence to support this hypothesis is that *in vivo* depletion of NK cells totally abolished the nonspecific tumor static effect from LPD.[6]

We hypothesized that the unmethylated CpG motifs from the bacterial plasmid DNA inside the LPD might be responsible for LPD's strong immunostimulation activity. Bacterial DNA, by interacting with TLR9, is known to be strongly immunolostimulatory.[7,8] However, more work still needs to be done to elucidate the immunostimulation mechanism of LPD and to understand which component(s) of the LPD is active and how the LPD stimulates immunity. In this study, formulations comprised of the original LPD, its individual components, combinations of the components, or LPD prepared with substituted components or its original components in varied proportions are examined for their ability to stimulate the expression of costimulatory molecules and the release of cytokines from DCs. A murine cell line, DC2.4, which has proven to be a good model for APC,[9,10] was used for these studies. Primary bone marrow-derived DCs (BMDCs) were also used to confirm some of the data from DC2.4 cells. In addition, when the HPV 16 E7 peptide was combined with LPD, the ability of LPD/E7 and selected formulations to inhibit the growth of a HPV positive tumor in mice was evaluated.

It has been shown that, as long as the administered antigens remain outside the lymphatic tissues such as local LN or spleen, they will be ignored by the immune system.[11,12] Being able to effectively bring antigen to local LN thus becomes one of the critical criteria for a successful vaccine adjuvant and/or delivery system. In the study presented here, the uptake and distribution of LPD and selected formulations by lymphocytes in mouse popliteal LN after subcutaneous footpad injection were also examined.

Experimental Section

Materials. DOTAP, cholesterol, and rhodamine-labeled DOPE were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Protamine sulfate (fraction X from salmon), poly-L-lysine (29 500 Da), and polyornithine (16 700 Da) were from Sigma-Aldrich (St. Louis, MO). The Cy5-labeled oligodeoxynucleotide (ODN) was from Invitrogen. Phycoerythrin (PE)- or fluorescein (FITC)-labeled antibodies were from BD Pharmingen (San Diego, CA). The plasmid (pNGVL3) containing the CMV promoter and no coding insert was obtained from the National Gene Vector Laboratory (Ann Arbor, MI). Plasmid DNA was purified using the Qiagen (Valencia, CA) EndoFree Giga-Prep kit. The MHC class I-restricted peptide from the HPV 16 E7 protein (amino acids 49−57, RAHYNIVTF) was synthesized in the University of Pittsburgh Peptide Synthesis Facility by solid-phase synthesis using an Advanced ChemTech model 200 peptide synthesizer and purified using HPLC.

DC2.4 cells were originally created by K. Rock. We obtained them from L. Falo, Jr., at the University of Pittsburgh Medical Center. TC-1 cells were from T. C. Wu at Johns Hopkins University (Baltimore, MD). TC-1 cells are C57BL/6 mouse lung endothelial cells transformed with the HPV 16 E6 and E7 oncogenes and activated H-ras. Cells were grown in RPMI medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin (Sigma), and 100 μg/mL streptomycin (Sigma).

Preparation of Liposome, LPD, and LPD-Derived Formulations. Liposomes and LPD were prepared as previously described.[3,4] Briefly, small unilamellar liposomes composed of DOTAP and cholesterol (1:1 or 1:0 molar ratio) were prepared by thin film hydration followed by membrane extrusion. The DOTAP concentration was fixed at 10 mg/mL. LPD was comprised of the DOTAP/cholesterol liposome, protamine, and plasmid DNA at a ratio of 9.0:0.6:1.0 (w/w/w), unless mentioned otherwise. To prepare LPD, required amounts of liposome (43 μL) and protamine (30 μg) were dispersed in 150 μL of an aqueous solution containing 10% dextrose (Sigma). Then, 150 μL of an aqueous solution containing pDNA (50 μg) with or without

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the E7 peptide was added dropwise into the mixture of liposome and protamine with gentle shaking. The complex was then allowed to remain at room temperature for at least 20 min prior to being used. Liposomes comprised of other cationic lipids were prepared similarly.

LPD-derived formulations were prepared on the basis that every 300 μL of solution contains 43 μL of the liposomes as described elsewhere with modification.[5] For individual components, 43 μL of liposome (L), 30 μg of protamine sulfate (P), or 50 μg of pNGVL3 (D) was prepared in a final volume of 300 μL of 5% dextrose. For the liposome/protamine sulfate (LP) combination, the appropriate quantities of liposomes and protamine sulfate were mixed in a final volume of 300 μL in 5% dextrose. For the liposome/DNA (LD) and DNA/protamine (PD) combinations, an equal volume of DNA in solution and either liposome or protamine sulfate in solutions were gently mixed together to a final volume of 300 μL. All formulations were prepared at least 20 min prior to use.

To further study the effect of liposome, DNA, and protamine on the immunostimulation activity of LPD, the following formulations were prepared. Some LPD particles were prepared with an amount of DNA equivalent to $^1/_2$, $^1/_5$, or $^1/_{10}$ of the original amount. LPD particles were also prepared from liposomes containing no cholesterol. Protamine in the LPD was replaced with either poly-L-lysine (25 μg in a final volume of 300 μL) or polyornithine (5 μg in a final volume of 300 μL) to form prepared liposome−lysine−DNA (LLD) particles and liposome−ornithine−DNA (LOD) particles, respectively. Finally, LPD particles were also prepared with an amount of liposome equivalent to $^5/_9$ and $^1/_9$ of the original amount.

The particle size and the ζ potential of the liposome, LPD, and LPD-derived formulations were measured following the manufacturer's suggestion using a Coulter N4 Plus particle sizer (Beckman Coulter, San Francisco, CA) and a Zetasizer 4 (Malven Instruments, Inc., Southborough, MA), respectively. Particle sizes were reported as the mean ± the standard deviation [polydispersity index (PI)]. ζ potentials were reported as the mean ± the standard deviation (n = 3).

Preparation of Primary DCs. Primary DCs were prepared from bone marrow as described with slight modifications.[13] Briefly, bone marrow cells were depleted of lymphocytes and cultured at a density of 5×10^5 cells/mL in 10% FBS-containing RPMI 1640 with granulocyte-macrophage-CSF and rIL-4 (10^3 units/mL each, provided by L. Falo, Jr.). Loosely adherent cells were collected on day 5. More than 90% of these cells were CD11c positive as confirmed by flow cytometry.

Expression of Costimulatory Molecules (CD80/86) on DCs after *in Vitro* Stimulation. DCs (1.5×10^6 cells/well) were seeded into six-well plates and then incubated with 75 μL of the formulations mentioned above at 37 °C in 5% CO_2. As a control, cells were also treated with 5% dextrose. Sixteen hours later, the cells were washed twice with BD Pharmingen staining buffer. One million cells were then stained with FITC-labeled anti-CD80 antibody and PE-labeled anti-CD86 antibody for 20 min at 4 °C. After being washed twice, the cells were analyzed with an EPICS-XL benchtop cytometer (Beckman Coulter) and using EXPO 32 software. Data were reported as the percent of DCs that were CD80 or CD86 positive.

Release of TNF-α from DCs after *in Vitro* Stimulation. DCs (3×10^5 cells/well, n = 3) were seeded into 24-well plates in 300 μL, cultured for 18 h, and then co-incubated with LPD and other selected formulations (24 μL) for 6 h. The TNF-α concentration in the culture supernatant was determined using an ELISA kit from R&D Systems Inc. (Minneapolis, MN).

Luciferase Expression in DC2.4 Cells after Stimulation. A luciferase reporter gene driven by a minimal promoter containing the NF-κB responsive element (pNF-κB-Luc, Stratagene, La Jolla, CA) was transiently transfected into DC2.4 cells using lipofectamine (Invitrogen). Twenty-four hours later, the cells (2×10^5, n = 3) were stimulated with LPD (6 μL), liposome comprised of DOTAP/Chol, or 5% dextrose as a control. The stimulation was stopped after 24 h, and luciferase activity was measured using Promega's Luciferase Assay System and an AutoLumat LB953 luminometer from EG&G Berthold Technologies (Oak Ridge, TN). Luciferase activity was normalized to protein concentration.

Animal Treatment Study. Six- to seven-week-old female C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were used in all animal studies. National Institutes of Health guidelines for the care and use of laboratory animals were observed. Subcutaneous tumors were established by injecting TC-1 cells (5×10^5) into the hair-trimmed flank of the mouse on day 0. On day 6, mice (n = 5−12) were then subcutaneously injected with 100 μL of selected formulations containing 10 μg of the E7 peptide. The size of the tumor was measured using a caliper two or three times a week. Tumor size was determined by multiplying the two largest dimensions of the tumor.

CTL Assay and Release of INF-γ from Splenocytes. CTL activity was measured using the CytoTox 96 NonRadioactive Cytotoxicity Assay Kit (Promega, Madison, WI). Mice were immunized subcutaneously on days 0 and 9 as mentioned above. On day 13, they were sacrificed, and splenocytes were prepared and cultured in RPMI medium with 10% FBS, 50 units/mL penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM nonessential amino acids, 40 units/mL IL-2, and 1 μg/mL E7 peptide for 4 days. Effector cells were plated into 96-well plates at various effector:target (E:T) ratios. Targets that were used were EL4 cells pulsed with the E7 peptide. Before being mixed with effectors, the targets were washed two times with medium and resuspended at a density of 2×10^5 cells/mL.

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The lysis reaction was carried out for 4 h at 37 °C, after which the plates were centrifuged, and 100 μL of medium was carefully removed from each well and assayed for lactate dehydrogenase activity. The extent of specific lysis was calculated with the absorbance at 490 nm as suggested by the manufacturer.

Also, splenocytes (1×10^6 cells in 300 μL, $n = 3$) were stimulated with 1 μg/mL E7 peptide for 48 h. The cells were spun down, and the IFN-γ level in the supernatant was measured using a mouse IFN-γ ELISA kit from Pierce (Rockford, IL).

Uptake of LPD and Selected Formulations by Cells in Popliteal Lymph Nodes after Footpad Injection. Briefly, 50 μL of DOTAP/cholesterol liposomes (L) prepared with 1% (m/m) rhodamine-labeled DOPE, LPD containing Cy5-labeled ODN (5%, w/w), or the liposome−DNA complex (LD) with Cy5-labeled ODN (5%) was subcutaneously injected into the footpads of both hind legs of C56BL/6 mice ($n = 4$). Sixteen hours after the injection, the popliteal lymph nodes were removed, pooled, and suspended in 5 mL of serum-free RPMI medium. Collagen was digested, and lymphocytes were prepared. One million cells were stained with FITC-, PE-, or Cy7-PE-labeled antibodies against CD11b, CD11c, CD19, NK1.1, and CD80 in appropriate combinations at 4 °C for 20 min. The cells were then washed twice with BD Pharmingen staining buffer and resuspended in 200 μL of buffer for flow cytometry analysis (CyAn XL, DakoCytomation Colorado, Inc., Fort Collins, CO). The percentage of cells that were Cy5 positive, the percentage of macrophages, DCs, B cells, or NK cells that were Cy5 positive, and the percentage of DCs that were CD80 positive were measured.

Statistical Analyses. Except where mentioned, statistical analyses were completed by performing one-way analysis of variance (ANOVA) followed by pairwise comparisons with Fisher's protected least significant difference procedure (PLSD). A P value of ≤ 0.05 was considered to be significant.

Results and Discussion

Over the past several years, it has become clear that the interaction between the pathogen-specific molecular pattern (PAMP) and TLR is crucial in inducing both innate and adaptive immunity.[14,15] Examples of PAMP include the unmethylated CpG motifs in bacterial DNA for TLR9 and LPS from Gram-negative bacteria for TLR4, to name a few. Interaction of PAMP with TLR on DC stimulates the maturation of DC, which then migrates to the local LN to present antigen to the T-cell as a complex of MHC and antigen.[16,17] In addition, TLR signaling also stimulates the expression of costimulatory molecules such as CD80 and CD86, which are required secondary signaling molecules for successful presentation of antigen and clonal T-cell expansion.[16,17] The mechanism of the TLR signal is very similar to that of the IL-1R family.[18] In the signal cascade, MyD88, a cytoplasmic adapter protein, associates with all the TLR identified so far (TLR1−11), although TLR4 also has a MyD88-independent pathway.[18,19] Binding of PAMP to TLR activates TLR, which forms a signaling complex with MyD88, IRAK, and tumor necrosis factor receptor-associated factor 6 (TRAF6). This is followed by the activation of the mitogen-activated protein kinase (MAPK) cascade and NF-κB. The downstream of this signal cascade includes the production of proinflammatory cytokines such as IL-1β and TNF-α by DC and the expression of costimulatory molecules on DC.[20,21] The involvement of TLR in many steps of the immunostimulation led to the concept of TLR as a general adjuvant receptor.[20,21] Thus, the expression of costimulatory molecules such as CD80 and CD86 on DC and the production of cytokines such as TNF-α by DC serve as good indications of which component(s) of the LPD nanoparticles is responsible for its strong immunostimulatory activity.

Figure 1A shows the expression of CD80 and CD86 on DC2.4 cells after *in vitro* stimulation. Clearly, DNA alone (D), protamine alone (P), and the combination of DNA and protamine [PD, particle size of 145 ± 41 (0.119) nm, ζ potential of −12 ± 1 mV] did not exhibit any activity. LPD [165 ± 54 (0.188) nm, 24 ± 2 mV] induced the highest level of expression of CD80 and CD86. This was also true for LPD prepared from liposomes without cholesterol [166 ± 30 (0.038) nm, 21 ± 1 mV], suggesting that cholesterol is not required for the activity. Lipoplex formed by combining the cationic liposome and DNA [LD, 217 ± 62 (0.120) nm, −6 ± 2 mV] exhibited activity similar to that of the LPD. This is not surprising in light of the fact that protamine itself is inert. However, it is surprising to find that the cationic liposome [139 ± 37 (0.097) nm, 27 ± 2 mV] alone exhibited activity that equaled ∼60% of that of the LPD. Taken together, these data show that both DNA and the cationic liposome are both required or responsible for stimulating the expression of CD80 and CD86 on DC2.4.

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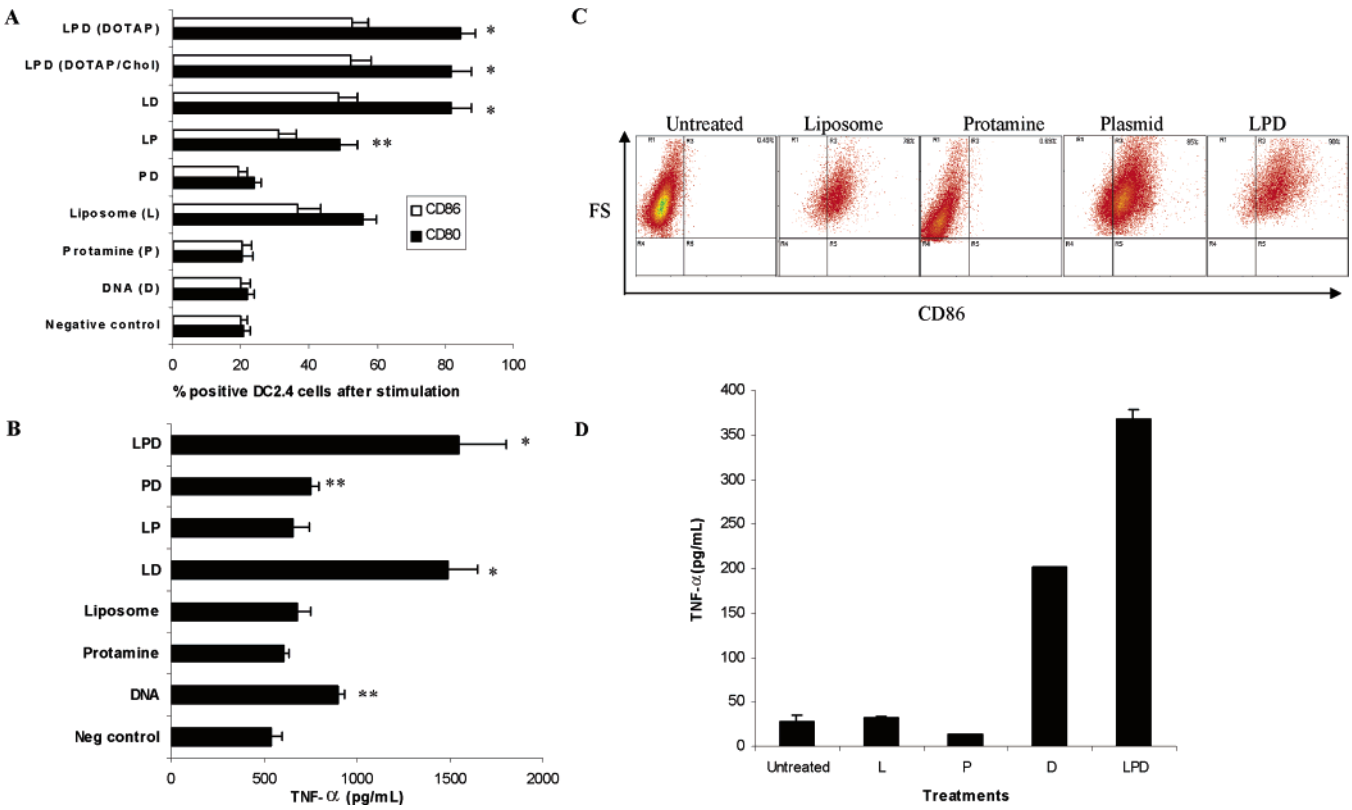

Figure 1. (A) Expression of costimulatory molecules (CD80 and CD86) on DC2.4 cells after incubation with LPD and other different formulations. PD is the protamine−DNA complex. LP is the liposome/protamine mixture. LD is the liposome−DNA complex. LPD (DOTAP) means LPD prepared from liposome comprised of only DOTAP. One asterisk indicates that the values for LD and LPDs are comparable to one another but significantly different from those of the others. Two asterisks indicate the values for L and LP are comparable to each other but significantly different from those of the others. Data reported are the mean ± the standard deviation ($n = 3$). (B) Release of TNF-α from DC2.4 cells after incubation with LPD and other different formulations. One asterisk indicates that the values for LD and LPD are comparable to each other but significantly different from those of the other formulations. Two asterisks indicate the values for DNA and PD are comparable to each other but significantly higher than that of the negative control. One representative of three separate experiments showing similar results is shown. Data reported are the mean ± the standard deviation ($n = 3$). (C) Expression of CD86 by BMDCs after stimulation with liposome, DNA, protamine, or LPD. The percentage of CD86 positive cells as shown in the upper right region is 0.49%, 78%, 0.69%, 85%, and 90% for untreated or liposome, protamine, plasmid, and LPD treated cells, respectively. (D) Release of TNF-α from BMDCs after incubation with liposome (L), DNA (D), protamine (P), or LPD. The results from D and LPD are different from each other, but both values are higher than that of the others. The experiments were all repeated two to three times. Reported is one representative.

It is known that CpG motif-containing bacterial DNA is the ligand for TLR9.[7] However, no information about how the cationic lipid (DOTAP) works is available.

To further investigate the effect of different components of the LPD on its ability to stimulate the expression of CD80 and CD86, LPD was prepared with its original material in varied proportions. Decreasing the amount of either the cationic liposome or DNA led to a decreased level of expression of CD80 and CD86, further supporting the idea that both DNA and cationic liposome are responsible for the immunostimulatory activity of LPD (data not shown). In addition, replacement of protamine with either poly-L-lysine or polyornithine did not have any significant effect on the expression of CD80 and CD86, indicating that the polycation acted as only a structural component of LPD in this *in vitro* model (data not shown).

Besides expression of CD80 and CD86, production of proinflammatory cytokines such as TNF-α by APC is another indication of the initiation of TLR signaling.[21] As shown in Figure 1B, again both DNA and cationic liposomes are required for the full activity of LPD. Also, protamine was inactive. However, it is noted that DNA alone induced a significantly enhanced level of TNF-α release over the unstimulated cells ($P < 0.05$), whereas the cationic liposome alone or the combination of the liposome with protamine (LP) was inactive ($P = 0.07$), in contrast to the expression of CD80 and CD86. Similar results were observed when BMDCs were used (Figure 1C,D), agreeing well with those previously reported.[22]

To further prove that both DNA and the cationic liposome are required for the full activity of LPD, an *in vivo* tumor therapy study was carried out. As shown in Figure 2A, 20 days after the treatment with E7 peptide-incorporated LPD (LPD/E7), the tumor (injected 6 days before the onset of treatment) almost totally regressed. Liposome/E7 and lipoplex/E7 (LD/E7) exhibited an effect to some extent but

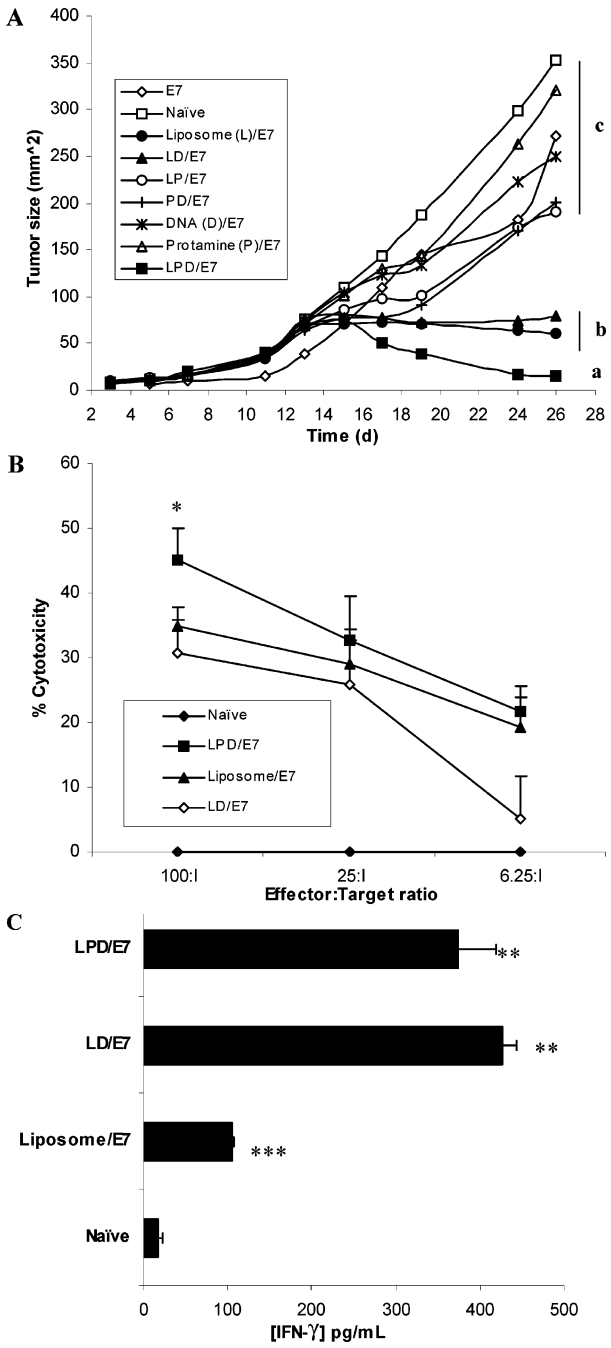

Figure 2. (A) Tumor growth kinetics on mice treated with LPD/E7 and other different formulations. Lowercase letters a−c indicate that the final mean tumor sizes were significantly different between each groups, but not different within each group. CTL response (B) and release of IFN-γ (C) from splenocytes isolated from mice immunized with LPD/E7, LD/E7, or liposome/E7. One asterisk indicates that at a target:effector ratio of 100:1, CTL from LPD is significantly different from that of the others. CTL activities from LD/E7 and liposome/E7 are not different, although they are significantly higher than that for the naïve mice. Two asterisks indicate that in panel C, the values for LD/E7 and LPD/E7 are both significantly higher than that of the naïve mice and the liposome/E7. Three asterisks indicate that the value for liposome/E7 is significantly higher than that for the naïve mice. Data reported are the mean ± the standard deviation ($n = 3$).

an effect significantly weaker than that of the LPD/E7. Tumors on mice treated with other formulations, including DNA/E7, protamine/E7, LP/E7, PD/E7, and E7 alone, kept growing rapidly with the final tumor size comparable to the size of those on the untreated naïve mice. Therefore, the result of this tumor therapy study again demonstrated that both DNA and the cationic liposome are required for the full immunostimulation activity of LPD. It is interesting to note that in the *in vivo* tumor model, protamine becomes functionally important, as the activity of LPD/E7 was significantly greater than that of LD/E7. There has been speculation that by condensing DNA, protamine helped to bring DNA inside the liposomes. The E7 peptide was thought to bind to DNA via electrostatic interaction.[2] In LPD/E7, the E7 peptide might be located inside the liposome and should be protected from enzymatic degradation after injection. On the other hand, E7 might be only loosely bound in the LD/E7 particles. In fact, the peptide incorporation efficiency of LPD/E7 was ~80%, whereas for LD/E7 and L/E7, it was ~65%. Panels B and C of Figure 2 show that the strong cell-mediated immune responses, including specific CTL activity and Th1-type cytokine (IFN-γ) release, might be responsible for the excellent antitumor activity of LPD/E7. It is not surprising to observe that the level of IFN-γ released from splenocytes isolated from mice immunized with lipoplex/E7 (LD/E7) was comparable to that of mice immunized with LPD/E7 (Figure 2B). Bacterial DNA is known to skew the cytokine release to be more Th1-biased.[23] The ability of cationic liposome incorporated with E7 peptide to induce immune response has been well documented.

Taken together, the experiments described above showed that both DNA and the cationic liposome are required for the full immunostimulation activity of LPD and that the polycation protamine is an important structural component of the LPD. It is expected that the bacterial DNA functions through TLR9. Both proinflammatory cytokine release and costimulatory molecule expression via the TLR9 are known to proceed through the MyD88-dependent signal cascade.[7,24] The observed unresponsiveness of DC2.4 cells to DNA alone (Figure 1A) is probably due to the fact that too little DNA was used. In fact, if more DNA is applied, it can also stimulate DC2.4 cells to express CD80 and CD86 (data not shown). Additionally, the cationic liposome stimulated the expression of CD80 and CD86 but not the release of TNF-α cytokine. This is further supported by the observation that

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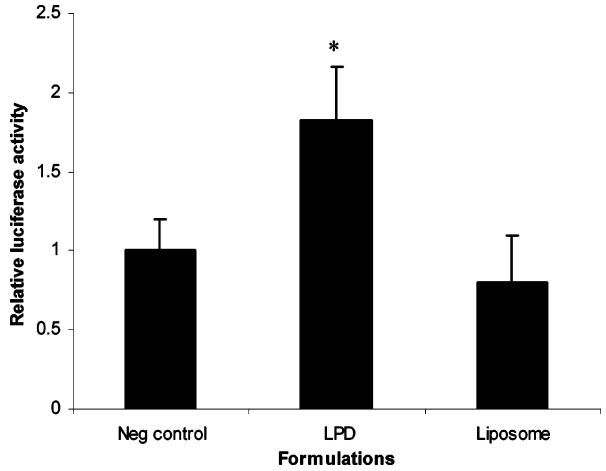

Figure 3. Relative luciferase activity in DC2.4 cells after stimulation with liposome (DOTAP/Chol) or LPD for 24 h. DC2.4 cells ($n = 3$) were transfected with the luciferase gene driven by a minimal promoter containing the NF-κB responsive element prior to stimulation. The reported value (mean $\pm$ the standard deviation) is one representative from three independent experiments. An asterisk indicates the value for LPD was significantly different from those of the others.

the liposome (DOTAP/cholesterol) alone did not stimulate any luciferase expression in DC2.4 cells transfected with a plasmid encoding the luciferase gene driven by a NF-κB responsive promoter, in contrast to the significantly higher level of luciferase expression induced by LPD (Figure 3). A similar situation exists in the TLR4 signal cascade. The MyD88-independent pathway leads only to costimulatory molecule (CD80 and CD86) expression, but not cytokine release.[20,25] TLR4 may not be the receptor for the cationic lipid (DOTAP) since LPS, the ligand for TLR4, has a chemical structure and charge content very different from those of the cationic lipids such as DOTAP.

As mentioned earlier, being able to activate DC alone is not enough to be an effective vaccine adjuvant and/or delivery system. The system must be able to successfully bring the antigen into local lymphatic tissues such as local LN for presentation of the antigen by mature DC to primary T-cells residing in the LN.[11,12,16] To study the distribution and uptake of LPD by cells in LN, LPD, lipoplex (LD), and liposome (L) particles were injected subcutaneously in the hind footpad of mice. Shown in Table 1 are the physical properties and the *in vivo* distribution of these three different particles among cells in the popliteal LN. Apparently, more cells in the popliteal LN took up LPD (~20%) than LD (~14%) and liposome alone (~1%). In case of LPD-treated mice, ~50% of the DC and NK and ~30% of macrophage and B-cells were LPD positive. These values are all higher than the corresponding ones in the lipoplex (LD)- and liposome (L)-treated mice (Table 1). Moreover, of all the DC in the popliteal LN, ~47, ~36, and ~36% were CD80 positive for LPD-, lipoplex-, and liposome-treated mice, respectively. These data indicate that not only could more LPD readily reach local LN they could also activate more DC in the LN than other lipid particles.

Table 1. Distribution and Uptake of LPD and Other Lipid Formulations among Cells in the Popliteal Lymph Nodes after Subcutaneous Footpad Injection[a]

	LPD	lipoplex (LD)	liposome (L)
particle size (nm)	165 ± 54 (0.188)	217 ± 62 (0.120)	139 ± 37 (0.097)
ζ potential (mV)	20 ± 1	-6 ± 2	27 ± 2
% particle$^+$ cells	20 ± 2	14 ± 2	1.1 ± 0.7
% particle$^+$ DC	53	32	17
% particle$^+$ MΦs	31	21	7
% particle$^+$ B-cells	27	19	1
% particle$^+$ NK-cells	48	34	6
% CD80$^+$ DC	47	36	36

[a] Four mice per group were used in this study. Data reported for the % particle$^+$ cells are the mean $\pm$ the standard deviation.

Particles may reach local LN either by direct draining through the efferent lymphatics or by the migration of DC, which picked up the particles at the injected site. The very small amount of liposome alone (~1%) recovered in the popliteal LN might be due to the fact that the liposome is highly positively charged, preventing its direct draining through the lymphatics. Apparently, indirect movement of the liposome via the migration of DC was also very limited. Interestingly, the very limited level of cationic liposomes that reached LN was enough to initiate antitumor activity (Figure 2A). As for LPD and lipoplex (LD), the differences in particle size and surface ζ potential certainly account, at least in part, for the observed differences in particle distribution and uptake in LN.

We conclude that both DNA and the cationic lipid (DOTAP) are required for the full immunostimulation activity of LPD. In addition, the LPD's unique ability to move to the local draining LN and to activate DC in the LN is responsible for its strong adjuvanticity.

Abbreviations Used

HPV, human papillomavirus; DCs, dendritic cells; NK, natural killer cells; APC, antigen-presenting cells; CTL, cytotoxic T lymphocyte; TLR, Toll-like receptor; LN, lymph nodes; MHC, major histocompatibility complex; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; Th1, T helper cell type 1; DOTAP, 1,2-dioleoyl-3-(trimethyl)-ammonium propane; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine.

Acknowledgment. We thank Erin McClelland for her assistance with the flow cytometry analysis. This work was supported by NIH Grants CA74918 and AI48851 to L.H.

MP049907K

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