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# T-Cell-Independent Macrophage Activation in Mice Induced with rRNA from *Listeria monocytogenes* and Dimethyldioctadecylammonium Bromide

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**Purified rRNA from *Listeria monocytogenes* or *Pseudomonas aeruginosa* injected in combination with dimethyldioctadecylammonium bromide (DDA), protects mice nonspecifically against a lethal challenge of various extra- and intracellular bacteria. In the present study vaccination of BALB/c as well as C57BL/Ka mice with listerial RNA-DDA resulted in activation of fixed-tissue macrophages, as measured by an enhanced in vivo *L. monocytogenes* killing in spleen and liver. Evidence was found that macrophage activation by vaccination with rRNA-DDA occurred by a T-cell-independent mechanism. (i) Treatment of mice with cyclosporin A had no effect on the enhanced *L. monocytogenes* killing induced with RNA-DDA; (ii) in vitro exposure of RNA-DDA to spleen cell cultures did not give rise to any lymphocyte proliferation. No evidence could be found for a possible adjuvant activity for RNA-DDA in cellular responses; in fact, RNA-DDA had an inhibitory effect on lymphocyte proliferative responses to *Listeria* antigen and to concanavalin A.**

The concept of ribosomal vaccines was introduced by Youmans and Youmans (26), who showed that RNA-rich fractions from *Mycobacterium tuberculosis*, in combination with Freund incomplete adjuvant, protected mice against a lethal homologous challenge. The protective activity of this RNA-rich fraction decreased after treatment with RNase (27). Since these studies, protective ribosomal vaccines have been prepared from a wide variety of microorganisms. In most cases these ribosomal vaccines were more or less contaminated with cell surface components, whereas only in a limited number of studies the protective activities were shown to be RNase sensitive (for reviews, see references 7 and 10). A few reports showed that RNA also protected mice against heterologous challenge (1, 3, 12, 16, 17). The RNase sensitivity of some ribosomal vaccines, as well as the nonspecifically increased resistance induced with rRNA, point to RNA as an immunomodulating agent.

Recently we reported that highly purified rRNA from *Pseudomonas aeruginosa* (12) and from *Listeria monocytogenes* (1) protected outbred Swiss and inbred BALB/c mice against homologous and heterologous challenge, provided that the RNA was injected in combination with the adjuvant dimethyldioctadecylammonium bromide (DDA). Vaccination with RNA or DDA alone was ineffective. Since local protection induced with RNA-DDA appeared to be more effective than systemic protection, we suggested that the protection at least in part might be explained by nonspecific stimulation of inflammatory responses. The present study was undertaken to investigate the mechanism of increased resistance induced with rRNA. Vaccination with RNA-DDA resulted in the activation of fixed tissue macrophages in liver and spleen. Furthermore, evidence from several in vivo and in vitro experiments has led us to conclude that activation of macrophages by RNA-DDA was due to a T-cell-independent mechanism.

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## MATERIALS AND METHODS

**Bacterial strain.** *L. monocytogenes* L242, serotype 4b, was used for purification of RNA as well as for animal experiments. Virulence was increased by repeated mouse passage. Bacteria were cultured in medium as described previously (2). For stock suspensions, overnight-grown cultures were supplemented with 15% glycerol, and samples (1 ml) containing $2 \times 10^9$ CFU/ml were stored at −70°C. The *L. monocytogenes* stock suspensions were used immediately after thawing and dilution to the appropriate concentration in phosphate-buffered saline, pH 7.2 (PBS). The same batch of *L. monocytogenes* was used for all animal experiments, with a 50% lethal dose after intravenous (i.v.) injection of approximately $10^4$ CFU for BALB/c mice.

**Animals.** Male BALB/c mice were purchased from TNO, Zeist, The Netherlands, and used at an age of 7 to 8 weeks. Female C57BL/Ka nude (*nu/nu*) mice and their immunologically normal controls, heterozygous for the nude mutation (*nu/+*), were purchased from TNO, Rijswijk, and used at an age of 7 to 8 weeks. Nude mice and their controls were held in a separate isolated room; cages, bedding and food were autoclaved before use, and cages were covered with sterile filter caps or kept in a laminar-flow cabinet.

**Purification of RNA.** RNA was extracted from purified ribosomes as described previously (1, 11, 12). The purified RNA contained less than 1% protein. RNase treatment of RNA was carried out as described previously (1).

**Animal experiments.** Mice were injected i.v. with a sublethal dose of *L. monocytogenes* in 0.5 ml, and the in vivo killing or growth inhibition of *Listeria* was measured by the determination of the CFU in spleen and liver. At various time intervals upto 3 days after challenge mice were killed with ether. Spleens and livers were removed aseptically and homogenized separately in a total volume of 5 ml of distilled water in a tissue grinder. Serial 10-fold dilutions were plated out in duplicate on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.). The mean CFU per organ was

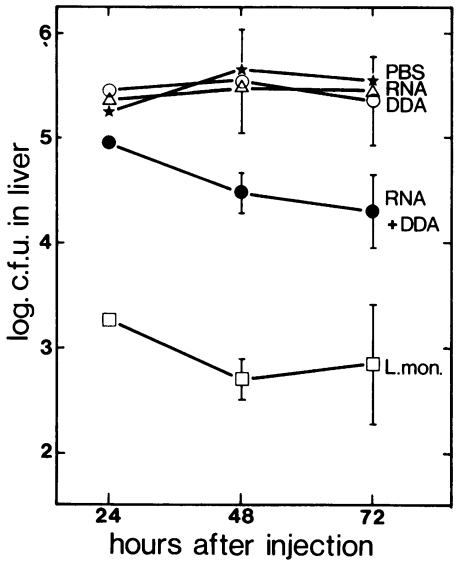


FIG. 1. Fate of *L. monocytogenes* in livers of mice after i.v. injection (log dose, 3.74 ± 0.03 CFU). Seven days before challenge the mice were vaccinated s.c. with live *L. monocytogenes* cells, rRNA-DDA, rRNA or DDA alone, or PBS (controls). Vertical bars indicate the standard deviations.

calculated for at least five mice from experiments carried out in at least 2 different weeks.

For vaccination experiments, mice were injected subcutaneously (s.c.) 7 days before challenge with 0.1 ml of PBS containing either $10^5$ listeriae or 100 μg of RNA plus 300 μg of DDA (Eastman Kodak Co., Rochester, N.Y.), 100 μg of RNA, or 300 μg of DDA. DDA was sonicated briefly before use. Injection of 0.1 ml of PBS served as a control.

**CyA.** Cyclosporin A (CyA; kindly provided by Sandoz, Basel, Switzerland) was dissolved in a concentration of 20 mg/ml in olive oil at 65°C. In animal experiments, 20 μl per mouse (20 mg/kg) was injected intraperitoneally (i.p.) on each day during the experiment, including the vaccination and the challenge period, starting 1 day before vaccination.

**Lymphocyte proliferation assay.** Spleen cells from BALB/c mice were prepared as usual and seeded into microtiter plates at a density of $5 \times 10^6$ cells per ml in 0.2 ml of Dulbecco minimal essential medium supplemented with 10% heat-inactivated fetal calf serum, 100 U of penicillin per ml and 100 μg of streptomycin per ml. The cells were incubated in the presence of various concentrations RNA and DDA for 72 h at 37°C in a humidified atmosphere of 7% $CO_2$ in air. During the last 4 h of the incubation 0.5 μCi of [$^3$H]thymidine (specific activity, 2 Ci/mmol) was added. The cells were harvested with a microharvester, and [$^3$H]thymidine uptake was measured in a liquid scintillation counter. Controls included spleen cells incubated with either 2.5 μg of concanavalin A (ConA) per ml or $10^7$ Formalin-killed *L. monocytogenes* cells (FKLM) per ml. Spleen cells were used from untreated mice and from mice vaccinated with RNA-DDA or live listeriae. The results are presented as the means of separate assays on cells from four individual mice.

**Statistical evaluation.** The data were analyzed for significant differences by the two-sample *t* test.

## RESULTS

**Influence of vaccination on the fate of listeriae in liver and spleen.** BALB/c mice were injected s.c. with the various preparations and 7 days later were challenged i.v. with *L. monocytogenes* organisms. The kinetics of listeriae in liver and spleen were followed up to 3 days after challenge. As shown in Fig. 1 (liver) and Fig. 2 (spleen), pretreatment of the mice with RNA or DDA alone had no effect on the viable counts of listeriae as compared with control mice pretreated with PBS. However, vaccination with the combination of RNA and DDA resulted in an enhanced in vivo killing of listeriae in both liver and spleen. In the liver the listeria counts in mice vaccinated with RNA-DDA were significantly lower compared with those in mice vaccinated with DDA alone, at 48 h ($P = 0.0001$) as well as at 72 h ($P = 0.0006$) after challenge. Also the spleen counts in RNA-DDA-treated mice were significantly lower than in DDA-treated mice, both at 48 h ($P < 0.0001$) and at 72 h ($P = 0.013$) after challenge.

This enhanced in vivo killing induced by vaccination with RNA-DDA was abolished after RNase treatment of the RNA. RNase treatment of RNA before vaccination with RNA-DDA resulted at 72 h after challenge in a log CFU of 5.42 ± 0.24 and 5.90 ± 0.28 in liver and spleen, respectively, both not significantly different from the listeria counts in mice pretreated with DDA alone. As expected, vaccination with live listeriae resulted in a very efficient killing of listeriae, especially in the spleen.

**Effect of T-cell deficiency on vaccination.** To find out whether the enhanced in vivo killing of listeriae induced with RNA-DDA was due to a T-lymphocyte-mediated activation of macrophages, vaccination experiments were carried out in two T-cell-deficient animal models. First, T cells were suppressed by treating mice with CyA, an inhibitor of T-cell functions. Second, athymic nude mice lacking T lymphocytes were used. BALB/c mice were treated i.p. with CyA, vaccinated s.c., and 7 days later challenged i.v. with *L. monocytogenes* organisms. Three days after challenge the

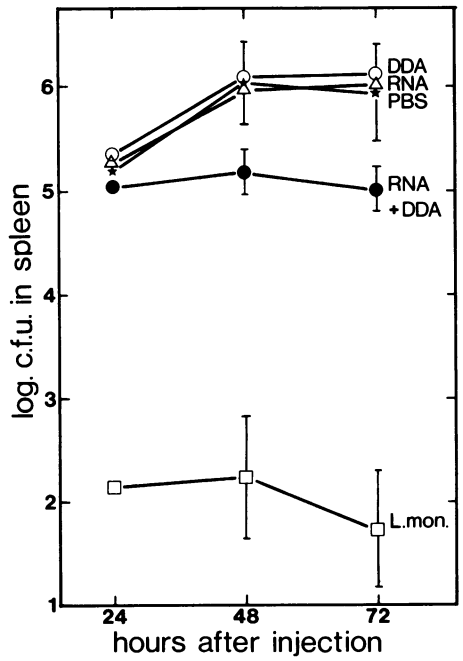


FIG. 2. Fate of *L. monocytogenes* in spleens of mice after i.v. injection (log dose, 3.74 ± 0.03 CFU). Seven days before challenge the mice were vaccinated s.c. with live *L. monocytogenes* cells, rRNA-DDA, rRNA or DDA alone, or PBS (controls). Vertical bars indicate the standard deviations.

TABLE 1. Effect of CyA on the viable counts in spleens and livers of mice[a]

| Organ | Treatment of mice[b] | Mean log CFU ± SD (P) after vaccination[c] of mice with: | | |
|---|---|---|---|---|
| | | PBS | RNA-DDA (P) | *L. monocytogenes* (P) |
| Liver | Olive oil | 5.62 ± 0.36 | 4.15 ± 0.52 (0.001) | 2.98 ± 0.42 (<0.0001) |
| | CyA | 5.68 ± 0.20 | 4.52 ± 0.27 (0.0001) | 6.55 ± 0.33 (0.003) |
| Spleen | Olive oil | 5.11 ± 0.35 | 4.14 ± 0.45 (0.0025) | 0.48 ± 1.07 (<0.0001) |
| | CyA | 5.44 ± 0.26 | 4.47 ± 0.25 (0.0004) | 5.03 ± 0.41 (NS) |

[a] Results were determined 3 days after i.v. injection of listeriae (log dose, 3.27 ± 0.13 CFU). The *P* values were calculated compared with PBS controls; NS, not significant.
[b] Mice were i.p. treated with CyA or olive oil during the vaccination and the challenge period.
[c] Mice were vaccinated s.c. with live *L. monocytogenes* cells, RNA-DDA, or PBS 7 days before challenge.

numbers of viable listeriae in spleen and liver were determined. Since CyA was dissolved in olive oil, control mice received olive oil i.p. CyA treatment of the mice had no effect on the enhanced in vivo killing of listeriae induced with RNA-DDA (Table 1). In contrast, the enhanced in vivo killing induced by vaccination with live listeriae was abolished completely when the mice were treated with CyA.

Athymic nude C57BL/Ka mice and their phenotypically normal controls were vaccinated s.c. and challenged i.v. 7 days later, and 3 days after challenge the viable count in spleen and liver was determined. The enhanced in vivo killing induced in control (*nu/+*) mice by vaccination with both live listeriae as well as RNA-DDA was in both cases no longer observed in nude (*nu/nu*) mice (Table 2).

**Lymphocyte proliferation in vitro.** The possible effect of RNA and DDA on T lymphocytes was also tested in a proliferation assay after in vitro exposure of spleen lymphocytes to RNA and DDA (Table 3). For positive controls, lymphocytes were treated in vitro with ConA. As expected, exposure of ConA to lymphocytes resulted into a marked proliferative response. In contrast, RNA-DDA failed to induce lymphocyte proliferation in the concentrations tested (1, 5, and 10 μg/ml). The concentrations RNA and DDA used did not affect cell viability, as judged by trypan blue exclusion.

To find out whether RNA-DDA possibly had adjuvant activity in cell-mediated immunity, lymphocyte proliferation was tested after in vitro exposure of FKLM to spleen cells from vaccinated mice (Table 4). As expected, vaccination of mice with live listeriae resulted into an increased proliferative response to FKLM compared with unvaccinated controls. Vaccination with RNA-DDA did not result into an increased response to FKLM. Surprisingly, after vaccination with live listeriae in combination with RNA-DDA, a decreased proliferative response of lymphocytes to FKLM was found, compared with vaccination with listeriae alone ($P = 0.001$). This inhibitory effect of RNA-DDA on lymphocyte proliferation was also observed in vitro, when spleen cells from unvaccinated mice were used (Table 5). When spleen cells were incubated with ConA in combination with RNA-DDA, the response decreased compared with that with ConA alone. The proliferative response to ConA also decreased when peritoneal macrophages from untreated mice were added during the exposure time, whereas an even further decrease was observed when peritoneal macrophages from RNA-DDA-vaccinated mice were added.

## DISCUSSION

In the present study we have shown that s.c. injection of purified rRNA in combination with the adjuvant DDA enhanced the in vivo killing of listeriae in liver and spleen after an i.v. challenge with a sublethal dose of *L. monocytogenes* cells. Since resistance against intracellular microorganisms like *L. monocytogenes* is generally believed to be mediated by activated macrophages with an enhanced bactericidal capacity (for review, see reference 13), our results strongly suggest that RNA-DDA activates macrophages. This is supported by previous findings that RNA purified from *L. monocytogenes* or *P. aeruginosa* injected together with DDA protected mice also nonspecifically against a lethal dose of heterologous bacteria (1, 12), and it is generally accepted that macrophages once activated exert their bactericidal activities nonspecifically.

Immunologically normal BALB/c as well as C57BL/Ka mice showed the enhanced in vivo listeria killing induced with RNA-DDA. This seems to be in conflict with the results of Medina et al. (16), who reported that only mice inherently resistant to *L. monocytogenes* (C57BL/67) and not inherently sensitive mice (A/J) could be protected against listeria challenge by immunization with *Listeria* RNA. Since BALB/c mice are inherently sensitive to listeriae (5), our results indicate that also in listeria-sensitive mice macrophage activation can be induced with *Listeria* RNA, provided that this RNA is administered in combination with DDA.

T-cell-mediated immunity plays a major role in protection

TABLE 2. Viable counts in spleens and livers of athymic nude (*nu/nu*) mice and immunologically normal controls (*nu/+*)[a]

| Organ | Type of mice | Mean log CFU ± SD (P) after vaccination[b] of mice with: | | |
|---|---|---|---|---|
| | | PBS | RNA-DDA | *L. monocytogenes* |
| Liver | *nu/+* | 4.99 ± 0.41 | 3.51 ± 0.34 (0.001) | 1.21 ± 0.48 (<0.0001) |
| | *nu/nu* | 4.64 ± 0.56 | 4.54 ± 0.39 (NS) | 4.85 ± 0.32 (NS) |
| Spleen | *nu/+* | 5.52 ± 0.26 | 4.10 ± 0.41 (0.0008) | 1.43 ± 0.22 (<0.0001) |
| | *nu/nu* | 4.65 ± 0.50 | 4.48 ± 0.43 (NS) | 4.36 ± 0.52 (NS) |

[a] Results were determined 3 days after i.v. injection of listeriae (log dose, 3.29 ± 0.18). The *P* values were calculated compared with PBS controls; NS, not significant.
[b] Mice were vaccinated s.c. with live *L. monocytogenes* cells, RNA-DDA, or PBS 7 days before challenge.

TABLE 3. Lymphocyte proliferation after in vitro exposure of spleen cells from untreated mice

| In vitro exposure with: | [$^3$H]thymidine uptake (cpm ± SD) | P |
|---|---|---|
| | 4,106 ± 944 | |
| RNA-DDA (1 μg/ml) | 4,813 ± 3,137 | NS[a] |
| RNA-DDA (5 μg/ml) | 2,558 ± 397 | NS |
| RNA-DDA (10 μg/ml) | 3,101 ± 762 | NS |
| ConA (2.5 μg/ml) | 79,086 ± 7,991 | <0.0001 |

[a] NS, Not significant ($P > 0.05$).

against listeriosis in mice. In listeria-immune mice, sensitized T lymphocytes act upon contact with *listeria* antigen by releasing lymphokines that activate macrophages to kill the bacteria (13, 14). Recently the lymphokine gamma interferon has been identified as the macrophage-activating factor (9, 18, 23). CyA, a specific inhibitor of T-cell functions (22, 25), has been found to inhibit lymphokine production, including production of gamma interferon (15, 21, 24). In the present study CyA indeed inhibited the in vivo macrophage activation induced by vaccination with live listeriae. In contrast, treatment of mice with CyA did not inhibit the enhanced in vivo killing of listeriae induced with RNA-DDA. Therefore we conclude that macrophage activation induced by vaccination with RNA-DDA was most likely not mediated by T lymphocytes. This conclusion is supported by the results of the in vitro lymphocyte proliferation experiments. As expected, lymphocyte proliferation was observed when spleen cells from untreated mice were cultured in the presence of ConA. Enhanced lymphocyte proliferation was also observed after exposure of FKLM to spleen cells from listeria-immune mice. However, lymphocyte proliferation was seen neither after in vitro exposure of RNA-DDA to normal spleen cells nor after exposure of FKLM to spleen cells from RNA-DDA-vaccinated mice.

Seemingly conflicting results were obtained in the experiments with athymic nude mice, since enhanced in vivo killing of listeriae induced by vaccination with RNA-DDA was not observed in nude mice. If macrophage activation by RNA-DDA were mediated by a T-cell-independent mechanism, one would expect macrophage activation to occur in athymic mice as well. However, several studies have shown that nude mice are more resistant to listeriosis in the initial phase of infection than normal mice, because of the presence of more highly activated fixed tissue macrophages (6, 8, 19, 20, 28), and it might be possible that macrophages once activated cannot become more activated by RNA-DDA.

It has been reported that rRNA has adjuvant activity in humoral responses, enhancing antibody production both in vitro (4) and in vivo (12). No such adjuvant activity could be observed in cellular response, as measured in the lymphocyte proliferation assay. Vaccination of mice with live

TABLE 4. Lymphocyte proliferation after in vitro exposure of spleen cells from vaccinated mice to FKLM

| Vaccination[a] | [$^3$H]thymidine uptake (cpm ± SD) | P |
|---|---|---|
| | 6,780 ± 141 | |
| Listeriae | 18,563 ± 1,931 | <0.0001 |
| RNA-DDA | 5,591 ± 1,558 | NS[b] |
| Listeria-RNA-DDA | 12,483 ± 1,326 | 0.001 |

[a] Vaccination s.c. with live listeriae or RNA-DDA or both 7 days before spleen cell harvesting.
[b] NS, Not significant ($P > 0.05$).

TABLE 5. Lymphocyte proliferation after in vitro exposure of spleen cells from untreated mice

| In vitro exposure with: | [$^3$H]thymidine uptake (cpm ± SD) | P |
|---|---|---|
| ConA (2.5 μg/ml) | 79,086 ± 7,991 | |
| ConA-RNA-DDA (5 μg/ml) | 45,793 ± 7,009 | 0.001[a] |
| ConA-Mϕ (control)[b] | 31,588 ± 1,815 | <0.0001[a] |
| ConA-Mϕ (RNA-DDA)[c] | 15,752 ± 3,475 | 0.0004[d] |

[a] Versus ConA control.
[b] Addition of $10^6$ peritoneal macrophages (Mϕ) per ml from untreated mice.
[c] Addition of $10^6$ peritoneal macrophages per ml harvested from mice 7 days after i.p. vaccination with RNA-DDA.
[d] Versus ConA-macrophage control.

listeriae together with RNA-DDA did not result in an enhanced in vitro proliferative response to FKLM as compared with vaccination with live listeriae alone; in fact the response to FKLM was decreased by the addition of RNA-DDA in the vaccination with live listeriae (Table 4). A similar inhibitory effect of RNA-DDA on lymphocyte proliferation was seen when spleen cells from normal mice were incubated with ConA together with RNA-DDA (Table 5). It is possible that RNA-DDA acts upon macrophages present in the spleen cell cultures to release substances that inhibit lymphocyte proliferation. This explanation is supported by the finding that the decreasing effect of RNA-DDA on lymphocyte proliferation to ConA could be simulated by the addition of macrophages harvested from RNA-DDA-vaccinated mice.

In the present study evidence was found that vaccination of mice with purified rRNA in combination with the adjuvant DDA induces macrophage activation by a T-cell-independent mechanism. Although the exact nature of this mechanism is still unclear, RNA-DDA might induce macrophage activation first by directly affecting macrophages or monocytes or second by triggering leukocytes other than T lymphocytes to release macrophage-activating factors.

## ACKNOWLEDGMENT

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