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MODULATION OF ANTIBACTERIAL RESISTANCE BY SUBCELLULAR PREPARATIONS
FROM LISTERIA MONOCYTOGENES

MODULATION OF ANTIBACTERIAL RESISTANCE BY SUBCELLULAR PREPARATIONS
FROM LISTERIA MONOCYTOGENES

(MODULATIE VAN DE WEERSTAND TEGEN BACTERIELE INFECTIES DOOR
SUBCELLULAIRE PREPARATEN GEISOLEERD UIT LISTERIA MONOCYTOGENES)

PROEFSCHRIFT

ter verkrijging van de graad van doctor in de Geneeskunde
aan de Rijksuniversiteit Limburg te Maastricht, op gezag van de
Rector Magnificus, Prof. Dr. F.I.M. Bonke, volgens het besluit van
het College van Dekanen, in het openbaar te verdedigen op vrijdag,
28 november 1986 om 16.00 uur

door

ADRIANUS C.J.M. ANTONISSEN

geboren te Oudenbosch in 1953

PROMOTOR: Prof.Dr. C.P.A. van Boven
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Prof.Dr. J.M.N. Willers

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CHAPTER 1

INTRODUCTION

Listeria monocytogenes is a Gram-positive bacterium which behaves, when it has entered the body, as a facultative intracellular parasite. Its natural host cell is the macrophage, in which the organism is able to survive and even to multiply (WHO Scientific Group, 1973). However, a few days after infection macrophages acquire the capacity to kill Listeria, a phenomenon which is known as "macrophage-activation" (Mackaness, 1962, 1969). The activation of macrophages is due to the action of Listeria-specific T (thymus-derived)-cells, which recognize macrophage-associated Listeria-antigens. The interaction between Listeria-specific T-cells and macrophage-associated Listeria-antigens induces T-cell-proliferation as well as the secretion of soluble mediators (lymphokines) by T-cells which promote the accumulation and immobilization of macrophages at sites of Listeria-infection and activate these cells to kill Listeria (Hahn and Kaufmann, 1981).

Opsonizing antibodies are only of minor importance (if at all relevant) for the elimination of Listeria monocytogenes. Immunization of mice with a sublethal dose of viable Listeria induces a high level of protection against lethal infection, but only a minimal production of opsonizing antibodies. Conversely, immunization with killed bacteria induces high titres of opsonizing antibodies, but does not induce protection (Mackaness, 1962).

The ineffectiveness of killed-Listeria vaccines has greatly hampered identification of the immunogenic components of this bacterium. However, in 1979 Van der Meer *et al.* reported that mice could be protected against lethal Listeria-infection by immunization with killed bacteria in combination with the adjuvant dimethyldioctadecylammonium bromide (DDA). Subsequently it was shown that protection could also be induced with subcellular

preparations from Listeria monocytogenes combined with DDA (Willers et al., 1979). In the latter study bacteria were mechanically disrupted and separated into a cell-wall fraction and a cell-contents fraction by centrifugation. The cell-contents fraction was found to be more effective than the cell-wall fraction and therefore selected for further purification. The immunogenic components present in the cell-contents fraction could be extracted with phenol. The phenol-extract, which contained 60% nucleic acids, protected mice against listeriosis in a dose as low as 1 µg. Its protective properties were destroyed by RNase-treatment, indicating that RNA was the major protective component of this preparation. The latter observation created a problem of an interpretive nature, as immunity to micro-organisms in general is assumed to be based on recognition of cell-surface components or exotoxins.

Using a modification of the fractionation procedure of Willers et al. (1979), Gonggrijp et al. (1980) showed that RNase-sensitive as well as RNase-insensitive vaccines could be prepared from the Gram-negative bacterium Pseudomonas aeruginosa.

In the procedure of Gonggrijp et al. bacteria were mechanically disrupted using a cold Hughes pressure cell. After treatment of the broken-cell suspension with DNase to reduce viscosity, unbroken cells and large cell-envelope fragments were removed by centrifugation and filtration. Subsequently a crude ribosomal preparation was prepared by ultracentrifugation. The crude ribosomal preparation was separated into cell-envelope fragments (fraction I) and purified ribosomes (fraction II) by gel-filtration through a Sepharose-CL-6B column in the presence of 1 M NH₄Cl.

Fraction I induced serotype-specific protection against P. aeruginosa infection without the use of adjuvants. The protective properties of fraction I were not affected by RNase-treatment or treatment with proteolytic enzymes. Fraction II induced some protection without adjuvants, but was considerably more effective when tested in combination with DDA. The protective properties of fraction II (tested in combination with DDA) were destroyed by

RNase-treatment, but not by treatment with proteolytic enzymes. Subsequently it was shown that protection could also be induced with purified ribosomal RNA extracted from fraction II, combined with DDA (Gonggrijp et al., 1981 a).

Whereas fraction I induced serotype-specific protection - which was most likely due to the induction of lipopolysaccharide (LPS)-specific antibodies (Gonggrijp et al., 1981 b) - protection induced with RNA plus DDA was serotype-nonspecific as well as species-nonspecific (Gonggrijp et al., 1981 a, 1983). Thus, two completely different protective components could be isolated from a single micro-organism: fraction I, which induced serotype-specific immunity, and ribosomal RNA, which induced a general increase of antibacterial resistance when combined with the adjuvant DDA.

Purpose of this study

The purpose of investigations presented in this thesis is to establish whether cell-envelope fragments (fraction I), purified ribosomes (fraction II), and purified rRNA isolated from L. monocytogenes using the fractionation procedure of Gonggrijp et al. (1980) induce protection against Listeria-infection, and to elucidate the mechanism of protection induced by these preparations.

Following chapter 2 in which a review of the literature on Listeria monocytogenes is presented, the chemical composition and the protective properties of fraction I and fraction II from L. monocytogenes serotype 4b are described in chapter 3. Both preparations protected mice against homologous challenge, provided that they were combined with the adjuvant DDA. Ribosomal RNA was identified as the major protective component of fraction II, because the protective properties of this preparation were destroyed by RNase-treatment, but not by treatment with proteinase K. Furthermore, protection could also be induced with purified RNA extracted from fraction II. The protective properties of fraction I were not affected by

RNAse-treatment, or treatment with proteinase K. Otherwise, the protective properties of fraction I were highly similar to those of fraction II and/or purified RNA. For instance, fraction I plus DDA as well as RNA plus DDA also protected mice against L. monocytogenes serotype 3, Streptococcus pneumoniae, and P. aeruginosa. Therefore it was decided to investigate whether fraction I and RNA might differ with respect to the ability to induce or elicit a delayed hypersensitivity (DH) response, which is assumed to be a reflection of the capacity of experimental animals to mount a T-cell-mediated mobilization of macrophages to sites of Listeria-antigen deposition.

As shown in chapter 4, fraction I plus DDA induced DH against killed Listeria, but RNA plus DDA did not. Furthermore, DH induced by immunization of mice with viable Listeria could be elicited with fraction I, but not with RNA.

The DH-inducing components present in fraction I were found to be of a proteinaceous nature, as proteinase K-treatment destroyed the ability of fraction I to induce DH against killed Listeria. As relatively little is known with respect to the immunogenic components of Listeria, including DH-inducing components, it was felt of interest to further characterize the DH-inducing components present in fraction I.

Experiments with protein-fractions isolated from fraction I described in chapter 5 suggested that several proteins contributed to the induction of DH with fraction I. However, DH could also be induced with a purified 20,400 MW protein.

Further experiments were dedicated to elucidation of the mechanism of protection against listeriosis induced with RNA plus DDA.

In chapter 6, it is shown that mice treated with RNA plus DDA have an increased capacity to mobilize polymorphonuclear leukocytes (PMNs) and macrophages to the inflamed peritoneal cavity as compared to mice treated with DDA alone. Results from transfer

experiments with resident and inflammatory peritoneal cells suggested that inflammatory PMNs as well as inflammatory macrophages from mice treated with RNA plus DDA were activated as compared to the analogous cell-populations from mice treated with DDA alone. Experiments with polyanionic analogues of rRNA are described in chapter 7. From the substances tested, only the synthetic double-stranded RNA poly A:U combined with DDA approached the protective capacities of Listeria-rRNA plus DDA. Protection against listeriosis induced with poly A:U was associated with enhanced inflammatory responsiveness, in particular of PMNs.

Some results of the experiments described in the previous chapters suggested that protection against listeriosis induced with RNA plus DDA is not a pure local phenomenon but is at least partially due to systemic effects. For instance, protection against intraperitoneal infection could also be induced by subcutaneous instead of intraperitoneal immunization (chapter 3). Furthermore, inflammatory PMNs and inflammatory macrophages appeared to be more important than resident peritoneal macrophages for the expression of protection induced with RNA plus DDA (chapter 6). For a more detailed analysis of the systemic effects of RNA plus DDA another experimental model was used than the ones previously employed, i.e., mice were vaccinated subcutaneously (s.c.) and protection was measured as the capacity of mice to eliminate Listeria from spleen and liver after intravenous (i.v.) challenge with a sublethal dose of live Listeria. This method was used because it allows a more accurate comparison of various vaccination protocols. Experiments in which the s.c./i.v. model is employed are described in chapter 8.

Immunization of mice with RNA plus DDA induced an accelerated elimination of Listeria from spleen and liver, but was found to be considerably less effective than immunization with viable Listeria. However, this situation was completely reversed when mice were treated with the T-cell-suppressive drug cyclosporin A before and during the immunization period. Cyclosporin A completely inhibited the induction of protection with viable Listeria, but

did not affect the induction of protection with RNA plus DDA. Therefore protection induced with RNA plus DDA was apparently due to a T-cell-independent mechanism, as already suggested by the ability of poly A:U to mimic the protective properties of RNA (chapter 7), and the inability of RNA to induce DH against Listeria (chapter 4).

In chapter 9, in which a summary and general discussion are presented, two major conclusions are drawn:

- i. Protection against Listeria monocytogenes induced with RNA plus DDA is due to a T-cell-independent mechanism.
- ii. Whereas the elimination of intracellular parasites such as Listeria monocytogenes is generally assumed to be the privilege of activated macrophages, both activated macrophages and activated PMNs contribute to the expression of protection against listeriosis induced with RNA plus DDA.

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CHAPTER 2

LISTERIA MONOCYTOGENES: REVIEW OF LITERATURE

Listeria monocytogenes is a Gram-positive bacterium which behaves, when it has entered the body, as a facultative intracellular parasite. Its natural host cell is the macrophage in which the organism is able to survive and even to multiply (WHO Scientific Group, 1973). The bacterium has been isolated from sewage, water, mud, fodder, silage, earth and a variety of animals including mammals, birds, fish, crustaceans and insects (Kampelmacher and Van Noorle Jansen, 1975; Weis, 1975; Gregorio and Eveland, 1975; Hyslop, 1975; Kampelmacher and Van Noorle Jansen, 1979). In addition, Listeria has been detected in the stools of upto 77% of healthy workers and 44% of healthy pregnant women (Kampelmacher and Van Noorle Jansen, 1972; Kampelmacher, Huysinga and Van Noorle Jansen, 1972).

Despite the ubiquitous presence of the bacterium, listeriosis is a relatively rare disease, which primarily affects the fetus and the neonate (Seeliger and Finger, 1976; Kampelmacher and Van Noorle Jansen, 1979; Relier, 1979). In 55 to 74% of cases, neonatal listeriosis has been found to be associated with maternal disease during pregnancy which generally presents itself with influenza-like symptoms (Relier, 1979; editorial, 1980). According to Relier (1979) predominant clinical features of neonatal listeriosis were respiratory problems, enlargement of spleen and liver and neurological disorders. Meningitis was only found in a minority of cases. The overall mortality rate in 75 cases reviewed by Relier (1979) was 22.6%.

In a recent review (Nieman and Lorber, 1980) meningitis was found to be the most commonly reported form of adult listeriosis, accounting for 55% of all cases, whereas non-meningitic CNS infection accounted for an additional 6.5%. Primary bacteremia was reported in 24.5% of cases and endocarditis in 7.5%. The remaining 6.5% included cases of localised infection. Meningitis primarily affected males (70% of cases) and 62% of patients with meningitis

were > 50 years of age. In contrast, primary bacteremia was approximately equally divided over both sexes (53% males) and 59% of patients were < 50 years of age.

Underlying disease was present in approximately 70% of cases of adult listeriosis. In 46% of all cases patients had received immunosuppressive therapy, 48% of whom were renal transplant patients. Cancer patients accounted for 22% of all cases, and 75% of patients with underlying malignancies had received immunosuppressive therapy. The overall mortality was 25%, but considerable differences were noted between different groups of patients. In patients with listerial meningitis and underlying malignancies, it was 60% despite antibiotic therapy, whereas in otherwise healthy individuals it was only 13%. In immunosuppressed patients without malignancies, mortality from listerial meningitis was 28%. In patients with primary bacteremia, the overall mortality was 11%. In this group mortality was restricted to patients with underlying malignancies, 33% of whom died.

The propensity of Listeria monocytogenes to infect the fetus and the neonate and adults with underlying disease was corroborated by a recent outbreak of listeriosis which was transmitted by pasteurized milk (Fleming et al., 1985).

Ampicillin (in combination with gentamicin) has been suggested as the drug of choice for the treatment of listeriosis (Relier, 1979; Traub, 1981). Also other antibiotics have been shown to be effective against Listeria in vitro (Wiggins, Albritton and Feeley, 1978); but in vitro activity does not always correlate with in vivo therapeutic effect. In a murine model of experimental listeriosis, ampicillin was found to be effective both in vitro and in vivo, whereas the cephalosporins cefasdone and cephalotin were only active in vitro (Kawaler and Hof, 1984). However, also with ampicillin discrepancies between in vitro and in vivo activity have been observed. In contrast to normal mice, athymic (nude) mice with impaired T cell-mediated immunity could not be cured from listeriosis with ampicillin (Bakker-Woudenberg et al., 1981; Hof, Emmerling and Seeliger, 1981). Also impairment of macrophage functions by treatment of mice with dextran sulfate

reduced the efficacy of ampicillin treatment (Hof et al., 1981). Encapsulation of ampicillin within liposomes to increase intracellular delivery of the antibiotic enhanced its efficacy in normal mice, but did not cure athymic mice from listeriosis (Bakker-Woudenberg et al., 1985). These observations stress the importance of the host's immune system in the eradication of infections due to Listeria monocytogenes.

It has been suggested that part of the mortality associated with listeriosis might be due to a delay in diagnosis of the disease (editorial, 1980; Howard et al., 1980). Indeed, both blood and cerebrospinal fluid (CSF) can be sterile for several days after the onset of severe clinical symptoms (Howard et al., 1980). In addition, Listeria can be mistaken for a diptheroid contaminant or a streptococcal species (Nieman and Lorber, 1980). Furthermore, the infection may proceed without a rise in antibody titres (Kampelmacher and Van Noorle Jansen, 1979; Mackenness, 1962), whereas positive serological findings may be due to cross reactions with other organisms (Seeliger and Hühne, 1979). Therefore, serological tests are considered to be useful only in combination with cultural and biochemical reactions (Seeliger and Hühne, 1979). The predominant serotypes isolated from pathological sources are 1/2a and 4b (Seeliger and Hühne, 1979).

Biochemical pathology and virulence factors

The pathogenesis of listeriosis and the mechanisms of protection against the infection have been studied extensively in experimental animals.

In lethally infected mice a progressive decrease in total body weight and in the intake of food and water has been observed. Absolute and relative liver weight, however, is increased. Biochemical alterations in the liver include a decrease in overall nitrogen and protein, increased lipid content and increased xanthine dehydrogenase activity. Blood urea levels remain constant for the first 24 hours of infection but then increase continuously to the acute state of infection (Wilder and Sword, 1967a). Blood and liver glucose and liver glycogen show a progressive decrease

after infection but pyruvate and lactate levels are increased (Wilder and Sword, 1967b; McCallum and Sword, 1970). The hepatic ATP/ADP ratio is decreased with a concomitant increase in the NAD^+/NADH ratio (McCallum and Sword, 1972a). Studies on mouse liver mitochondria during experimental listeriosis indicated derangement of the first site in oxidative phosphorylation (McCallum and Sword, 1972b). The pattern of lysosomal enzymes appearing in plasma during listeriosis suggested the occurrence of tissue damage in the liver (Sword and Wilder, 1967).

Virulent strains of Listeria monocytogenes generate an oxygen-labile, cholesterol-inhibitable hemolysin capable of killing phagocytic cells and also displaying cardiotoxic and lethal properties (Kampelmacher and Van Noorle Jansen, 1972; Kingdon and Sword, 1970, a, b, c). Hemolysin production might increase the amount of iron available to the micro-organism, which is restricted by several iron-binding proteins (Weinberg, 1971). The in vitro production of hemolysin was found to be inversely related to the medium iron concentration (Cowart and Foster, 1981). Iron stimulated both the in vivo and in vitro growth of Listeria monocytogenes and experimental iron overload caused a reduction of LD50 values with more than 3 log units. Conversely, an approximately 30-fold increase of LD50 values was observed in mice treated with the iron chelator Desferal (Sword, 1966).

The monocytosis seen in Listeria monocytogenes infections could be reproduced by injection of a lipid-extract (designated "monocytosis-producing agent") (MPA) from the bacterium. MPA might contribute to the pathogenesis of listeriosis, as it was found to cause a derangement of steroid- and carbohydrate metabolism (Holder and Sword, 1969). A purified cell-wall fraction isolated from Listeria induced a decreased resistance to infection when injected five days before until one day after challenge with live organisms (Baker and Campbell, 1978). Furthermore, Listeria-peptidoglycan was reported to be resistant to lysozyme (Kamisango et al., 1982) which might contribute to the persistence of the infection.

Host response to experimental listeriosis

Response to primary infection: According to Mitsuyama et al. (1978) the host response to experimental listeriosis can be divided into three phases:

- First phase

When normal mice are injected intravenously with a relatively small inoculum of Listeria monocytogenes, most of the bacteria are trapped in the liver within 10 min after administration. By 6 h after infection, the number of viable bacteria in the liver is decreased approximately tenfold. This phase of host resistance is attributed to fixed macrophages. It is inhibited by carrageenan treatment, but not by X-irradiation. The initial reduction of the number of bacteria is not seen when the inoculum is given intramuscularly, probably because of the small number of fixed macrophages in the muscle.

- Second phase

From 6 to 72 h after infection the number of bacteria shows a progressive increase, but appears to be limited due to accumulation of free phagocytes at the site of infection. During this period bacterial growth is markedly enhanced by X-irradiation or carrageenan treatment. Involvement of free phagocytes was inferred from the observation that the effect of irradiation could not be inhibited by shielding of the liver during irradiation. These free phagocytes could be polymorphonuclear granulocytes (PMNs) because this was the predominant cell type in Listeria-induced lesions in the liver for the first 48 h after infection (Mackness, 1970). However, Mitsuyama et al. (1978) suggested that the second phase of host resistance was most likely due to circulating immature macrophages (monocytes), because carrageenan primarily affects macrophages and because blood monocytes or their precursors are radio-sensitive unlike fixed macrophages. These conflicting results could be reconciled, if restriction of the growth of Listeria during the second phase of host resistance as defined by Mitsuyama et al. (1978), is assumed to be due to some form of co-operation

between PMNs and macrophages. Such a co-operation would be expected to be inhibited by treatments which affect either PMNs (X-irradiation) or macrophages (carrageenan treatment). In this respect it should be noted that PMN-mediated macrophage activation has been observed (Chapes and Haskill, 1983).

- Third phase

From day 4 after infection the number of bacteria in spleen and liver begins to decrease to become undetectable by day 9. This last phase of host resistance is attributed to immunologically activated macrophages. It is inhibited by X-irradiation and carrageenan treatment.

Response to secondary infection: In mice immunized with viable Listeria 14 days previously, the number of bacteria in the liver shows a continuous decrease after rechallenge. The course of infection during the first six hours, however, is almost identical to that in non-immunized controls. Therefore, it was suggested that in immunized animals the first phase of host resistance is followed immediately by the third phase (Mitsuyama et al., 1978). Similar results were reported by Mackaness (1962) who studied the outgrowth of Listeria in spleens of mice reinfected at varying intervals after a primary infection. Enhanced elimination of Listeria was observed even 13 weeks after immunization. Enhanced elimination without preceding outgrowth, however, was only seen when mice were reinfected 1 or 2 weeks after primary infection.

Several other systems have been used to analyze the course of secondary infection: In the procedure used by Miyata et al. (1982) mice were immunized intravenously with living bacteria and challenged via the intraperitoneal route. Elimination of the challenge inoculum from the peritoneal cavity was taken as a measure of host response and the involvement of peritoneal macrophages in this process was studied. Enhanced elimination of the inoculum was observed as early as 5 days after immunization but was more pronounced after 15 days. Macrophages obtained by lavage of the peritoneal cavity 5 days after immunization did not show enhanced phagocytosis or killing of Listeria in vitro. However, a marked

increase in the number of peritoneal macrophages was observed 24 h after challenge. In contrast, macrophages obtained 15 days after immunization were found to be activated, i.e. their capacity to kill Listeria in vitro was increased when compared with normal macrophages or macrophages obtained 5 days after immunization. The increase in the number of peritoneal macrophages observed in mice challenged 5 days after immunization was absent when the challenge was given after 15 days.

Thus, the generation of acquired cellular resistance was divided in two phases: a first phase characterized by the accumulation of normal macrophages at the site of re-infection, and a second phase, characterized by the appearance of so-called activated macrophages (Miyata et al., 1982).

Absence of increased bactericidal properties has also been observed in murine peritoneal macrophages 7 days after intraperitoneal immunization with live bacteria. However, peritoneal macrophages obtained from immunized mice 18 h after intraperitoneal injection of heat-killed Listeria were found to be activated (Harrington-Fowler, Henson, and Wilder, 1981). Both the enhanced accumulation of normal macrophages as described by Miyata et al. (1982) and the accumulation of activated macrophages as described by Harrington-Fowler et al. (1981) were apparently due to some form of recognition of Listeria-antigens, because neither phenomenon occurred when mice were injected with substances unrelated to Listeria monocytogenes.

Mechanism of immunity against Listeria monocytogenes

Elucidation of the mechanism of immunity against Listeria was initiated by Mackaness, who observed that resistance could be transferred to normal mice with spleen cells from animals immunized with a sublethal dose of living bacteria (Mackaness, 1969). Protection could not be transferred with serum from mice immunized with either viable or killed Listeria. Serum from mice immunized with killed Listeria contained high titres of agglutinating antibodies, but immunization with killed bacteria did not protect mice against listeriosis (Mackaness, 1962).

The transfer of protection with spleen cells was found to be specific, as spleen cells from mice immunized with BCG did not protect recipient mice against Listeria. However, when mice were injected with BCG-immune spleen cells in combination with viable BCG, their resistance against Listeria was found to be enhanced. Furthermore, listericidal macrophages could be isolated from mice injected 48 h previously with BCG-immune spleen cells in combination with BCG, but not from animals which had received normal spleen cells in combination with BCG or BCG-immune spleen cells alone. As immune-spleen cells by themselves were not bactericidal, it was concluded firstly, that the expression of immunity against intracellular bacteria is based upon a specific interaction between sensitised lymphoid cells and the immunizing organism, and secondly, that this specific interaction generates a nonspecific signal which activates macrophages to kill intracellular bacteria (Mackanness, 1969).

The lymphoid cells involved in the transfer of protection against Listeria were found to be thymus-derived (T-)cells, as immune spleen cells treated with antiserum against a thymocytic surface antigen (anti- θ serum) plus complement no longer protected recipient mice against listeriosis (North, 1973). Subsequently, Listeria-specific T-cells were shown to accumulate in sterile peritoneal exudates, induced by the injection of casein. On a per cell basis, exudate cells were approximately six times more efficient than spleen cells in protecting recipient mice against infection (North and Spitalny, 1974).

The interactions between Listeria monocytogenes, Listeria-specific T-cells and macrophages have been analysed in detail. The results of most of these studies have been reviewed by Hahn and Kaufmann (1981) and Unanue (1981).

In vitro activation of Listeria-specific T-cells (measured as T-cell proliferation) by killed Listeria was found to require the presence in the incubation mixture of macrophages expressing Ia-antigens (Weinberg and Unanue, 1981). Ia-antigens (immune response region associated antigens) are products of the I locus of the major histocompatibility complex (MHC) of the mouse (H-2). H-2I is

involved in the genetic control of specific immune responses to certain polypeptide antigens and also codes for cell surface structures that are expressed on cells of the immune system (B-cells, macrophages and some T-cells) (Hahn and Kaufmann, 1981). Ingestion of killed Listeria by macrophages was found to be essential for the induction of T-cell proliferation. Heterologous bacteria could not substitute for Listeria (Weinberg and Unanue, 1981). Typically, the interaction between Listeria-specific T-cells and Listeria-pulsed macrophages could not be blocked by anti-Listeria antibodies. Furthermore, this interaction was genetically restricted, i.e., both T-cells and macrophages had to be derived from donors which were identical at the I-A subregion of the I locus of H-2 (Farr, Kiely and Unanue, 1979).

These and other observations have led to the concepts of antigen-processing and antigen-presentation by macrophages, which means - in this context - that T-cells will not respond to antigens of intracellular bacteria unless these antigens are modified by macrophages and presented to T-cells in combination or close association with MHC-products (dual recognition model). In an alternative model, it is proposed that MHC-products are modified by the antigen and that the T-cell recognizes a new antigenic determinant (altered self-model) (Hahn and Kaufmann, 1981).

Culture of Listeria-immune T-cells with Listeria-pulsed macrophages also resulted in the acquisition of tumoricidal activity by the macrophages. The generation of tumoricidal macrophages required homology at the I region between macrophages and T-cells, as well as the presence of macrophages bearing Ia molecules. Macrophage activation was found to be mediated by a soluble factor which activated syngeneic as well as allogeneic macrophages, and macrophages lacking detectable Ia molecules. Thus, only the induction, but not the expression of tumoricidal activity was genetically restricted (Farr et al., 1979). H-2 restricted binding of T-cells to macrophages was essential for the generation of tumoricidal and mitogenic activity (Ziegler and Unanue, 1979).

Zinkernagel et al. (1977) reported that the transfer of enhanced resistance against Listeria with Listeria-immune spleen cells

was H-2I restricted. In contrast, Cheers and Sandrin (1983) concluded that the adoptive transfer of resistance was H2-K restricted, and suggested that the results of Zinkernagel *et al.* (1977) might have been confounded by restriction at non-H-2 loci (Cheers and McKenzie, 1983).

In transfer experiments with Listeria-immune peritoneal exudate T-cells, only a low level of resistance could be transferred to H-2K incompatible recipient mice. I-A incompatibility did not inhibit the transfer of protection, indicating that H-2K restriction was more important than restriction at H-2I-A (Hahn and Näher, 1984; Näher, Sperling and Hahn, 1985). The crucial factor in H-2K restricted transfer of protection appeared to be an accelerated formation of granulomas (circular lesions composed of a compact and orderly arranged cellular infiltration of predominantly mononuclear cells) upon infection of recipient mice with Listeria, which was not observed when acceptor mice had received H-2I-A compatible/H2-K incompatible T-cells (Hahn and Näher, 1984; Näher *et al.*, 1985). Furthermore, viable bacteria had to be injected to promote granuloma formation (Näher *et al.*, 1985), whereas killed Listeria were sufficient for the H-2I restricted generation of T-cell mitogenic and macrophage tumoricidal activity in vitro (Farr *et al.*, 1979 a, b).

Also in the rat discrepancies between in vitro and in vivo studies have been observed. In vitro proliferation of Listeria-specific T-cells upon stimulation with Listeria-antigen was found to be restricted by RT1-B (which is the analogue of murine H2-I-A) whereas the transfer of protection was more effective in the case of RT1-A (analogue of murine H-2K) homology than in the case of homology at RT1-B (Jungi and Jungi, 1982; Jungi *et al.*, 1982).

Using antisera against T-lymphocyte cell surface antigens (Lyt-antigens; Ledbetter *et al.*, 1980), Kaufmann, Simon and Hahn (1979) concluded that the systemic transfer of protection and delayed-type hypersensitivity (DTH) against Listeria was mediated by T-cells expressing Lyt1, Lyt2 and Lyt3 antigens (Lyt 1,2,3⁺ T-cells).

Systemic transfer of protection and DTH could also be achieved

with Listeria-specific cloned T-cells, but required relatively high cell numbers. Cloned T-cells were obtained by in vitro propagation of Listeria-immune peritoneal exudate T-cells in the presence of syngeneic accessory cells (irradiated spleen cells) and killed Listeria. In vitro proliferation as well as the transfer of protection with cloned T-cells was H-2I-A restricted (Kaufmann and Hahn, 1982). Cloned T-cells were less effective than uncloned cells in the systemic transfer of protection, which was probably due to the trapping of cloned T-cells in the lungs of recipient mice. On the other hand, cloned T-cells were more effective than uncloned cells in the local transfer of protection, which was assessed by combined injection of T-cells and live Listeria into the footpads of recipient mice (Kaufmann, 1983).

The T-cell clone studied by Kaufmann (1983) was derived from a T-cell line of $\text{Ly}1^+2^-$ phenotype. Näher, Sperling and Hahn (1984) reported that maturation of $\text{Ly}1,2,3$ cells into cells of $\text{Ly}1^+2^-$ phenotype also occurs in vivo. Furthermore, also $\text{Ly}1^+2^-$ cells generated in vivo had a reduced capacity to transfer systemic protection, but were quite effective in the local transfer of resistance towards Listeria. Local transfer of protection with cloned T-cells was genetically restricted and specific for L. monocytogenes. On the other hand, both systemic and local protection could be transferred to syngeneic as well as allogeneic mice with supernatants from cloned T-cells stimulated in vitro with killed Listeria in the presence of accessory cells. T-cell supernatants also protected mice against Salmonella typhimurium (Kaufmann, 1983), confirming the nonspecific nature of the effector phase of resistance against intracellular bacteria (Mackness, 1969).

Subsequently, it was shown that Listeria-immune $\text{Ly}1$ T-cells produced γ -interferon ($\text{IFN-}\gamma$) in vitro upon stimulation with killed Listeria in the presence of antigen presenting cells (proteose peptone-induced peritoneal exudate cells from normal syngeneic mice). Killed Listeria could be substituted by the T-cell mitogens concanavalin A (ConA) or phytohemagglutinin (PHA). However, PHA-induced as well as killed Listeria-induced IFN pro-

duction was several fold higher with Listeria-immune T-cells than with normal T-cells. Killed Listeria alone were not mitogenic and an unrelated antigen (sheep red blood cells) did not induced IFN production.

Also Listeria-specific cloned T-cells produced IFN- γ in vitro (Kaufmann et al., 1983). Recombinant IFN- γ (rIFN- γ) protected recipient mice against local as well as systemic infection with Listeria monocytogenes. Furthermore, it protected mouse L-cells against the cytopathic effect of vesicular stomatitis virus and it also activated macrophages to kill tumor cells in vitro. Whether in vitro incubation of macrophages with rIFN- γ also resulted in enhanced listericidal activity was not reported (Kiderlen, Kaufmann, and Lohmann-Matthes, 1984). However, both human and murine recombinant IFN- γ (rIFN- γ) were shown to activate macrophages to kill Toxoplasma gondii in vitro (Nathan et al., 1983; Murray, Spitalny and Nathan, 1985), indicating that IFN- γ can enhance macrophage microbicidal activity.

Besides IFN- γ , Listeria-immune Lyt1 T-cells also produced interleukin 2 (IL2; formerly TCGF (T-cell growth factor)) and migration inhibition factor (MIF) which is thought to promote the immobilisation of macrophages at sites of infection (Kaufmann et al., 1982; Sperling, Kaufmann, and Hahn, 1984).

In vitro proliferation of Listeria-immune T-cells upon incubation with macrophages and killed Listeria was mainly due to expansion of the Lyt1⁺23⁻ subset. Proliferation was suppressed by activated macrophages from mice immunized with viable Listeria or treated with Corynebacterium parvum. Suppression of proliferative responses was probably due to prostaglandin secretion by macrophages. Lyt1⁺23⁺ cells contributed to the proliferation of the Lyt1⁺23⁻ subset by counteracting macrophage-mediated suppression (Kaufmann, Simon, and Hahn, 1982).

Using a different set of markers, Kaufmann et al. (1985) found that the systemic transfer of protection and DTH against Listeria depended on a co-operation between T-cells carrying the L3T4 and the Lyt2 marker, respectively. L3T4 and Lyt2 are reciprocally expressed by different subsets of T-cells. Therefore L3T4⁺ cells

could be obtained by incubating unselected T-cells with anti-Lyt2 antiserum plus complement and vice versa. Protection and DTH could be transferred with a combination of both subsets, but not with either subset alone. Previously it was shown that systemic protection and DTH could not be transferred with Lyt1^+23^- cells, Lyt1^-23^+ cells, or a combination of both subsets, which prompted the conclusion that T-cells carrying all three surface markers ($\text{Lyt1}23$ cells) were essential for the systemic transfer of protection and DTH (Kaufmann *et al.*, 1979). However, in the latter study anti-Lyt1 antiserum was used to obtain the Lyt1^-23^+ subset. Because Lyt1 is expressed on all T-cells (albeit at differing densities) (Ledbetter *et al.*, 1980) treatment with anti-Lyt1 antiserum plus complement may have eliminated part of the Lyt2^+ population.

As L3T4 and Lyt2 antigens appear to be involved in the interaction of T-cells with class 2 (murine H-2I) and class I (murine H-2K, D) encoded products, respectively (Swain *et al.*, 1984), Kaufmann *et al.* (1985) noted that their findings would indicate that both class I restricted Lyt2^+ T-cells and class 2 restricted L3T4^+ T-cells contribute to the transfer of protection and DTH.

At first sight, this concept is incompatible with the observations of Cheers and Sandrin (1983) who showed that H-2K homology between donor and acceptor mice is necessary and sufficient for the adoptive transfer of protection. However, as noted by Näher *et al.* (1985), the possibility that Lyt2^- (and thus L3T4^+) T-cells might contribute to the transfer of protection in a manner which is not restricted by MHC products, i.e. which is not based upon recognition of Listeria-antigens in combination with Ia-antigens, has not been formally excluded. The H-2K restriction observed by Cheers and Sandrin (1983) and Näher *et al.* (1985) is rather surprising, as class I restriction is a typical feature of the cytotoxic response against virus-infected cells (Ada, Leung and Ertl, 1981). Whether Listeria-specific T-cells kill Listeria-infected cells is under investigation (Kaufmann *et al.*, 1985). However, it is difficult to imagine how killing of infected cells could contribute to the elimination of Listeria, unless ingested bacteria

are also killed in the process.

In the studies of Cheers and Sandrin (1983) and Näher et al. (1985) partially MHC-incompatible T-cells were transferred to define the MHC locus involved in the restriction of the adoptive transfer of protection. As a consequence of allogeneic stimulation, transferred T-cells of Lyt2^- phenotype may have produced IL2, which is a helper factor for cytotoxic responses (Farrar et al., 1982). In the study of Kaufmann et al. (1985) coculture of a combination of L3T4^+ and Lyt2^+ cells with allogeneic irradiated spleen cells promoted the generation of T-cells which were cytotoxic for P815 tumor cells. L3T4^+ but not Lyt2^+ cells could be substituted by IL2 containing supernatants from con A stimulated spleen cells, indicating that L3T4^+ cells provided help for the generation of cytotoxic effector cells from the Lyt2^+ subset.

Thus, *in vivo* studies with congenic mouse strains and serologically selected T-cells point to a helper function for L3T4^+ cells and an effector function for Lyt2^+ cells, whereas the reverse relation emerges from experiments which rely in whole or in part upon *in vitro* stimulation of Listeria-specific T-cells with killed Listeria. Furthermore, it should be noted that injection of a killed Listeria-preparation into Listeria-immunized mice appeared to be much more effective for the elicitation of $\text{IFN-}\gamma$ production than reinfection with viable bacteria (Nakane and Minagawa, 1984). In addition, rather high amounts of $\text{rIFN-}\gamma$ were required to protect mice against systemic infection with Listeria (Kiderlen et al., 1984). These observations raise the question whether $\text{IFN-}\gamma$ might not function as a helper factor which increases the sensitivity of T-cells for stimulation by IL2 (Farrar et al., 1982), rather than as a factor which activates macrophages to kill Listeria.

Czuprynski, Henson and Campbell (1984) found that mice immunized with viable Listeria responded with an increased influx of PMNs and macrophages upon intraperitoneal injection of killed Listeria or proteose peptone. Inflammatory PMNs and macrophages killed Listeria *in vitro* more efficiently than resident macrophages, but on a per cell basis, cells from control mice were as

effective as cells from immunized animals. Therefore, Czuprynski et al. (1984) suggested that an increased ability to mobilize phagocytes to sites of infection, rather than macrophage activation, might be of prime importance in resistance to listeriosis. Also human PMNs have been shown to kill Listeria in vitro (MacGowan et al., 1983). Otherwise, the contribution of PMNs to resistance against listeriosis has received little attention. Tatsukawa et al. (1979) reported that murine PMNs did not kill Listeria in vitro, but in their assay also macrophage-mediated killing of Listeria was hardly if at all detectable.

Increased inflammatory responsiveness as reported by Czuprynski et al. (1984) could be transferred to normal recipient mice with Listeria-immune T-cells (Czuprynski, Henson and Campbell, 1985).

In summary, collective data from studies dedicated to the function of T-cells in anti-Listeria immunity indicate that Listeria-immune T-cells migrate to inflammatory foci provoked by Listeria-infection where they promote the accumulation of phagocytes and granuloma formation, and activate macrophages to kill Listeria. Matters to be resolved are, among others, the contribution of PMNs to anti-Listeria immunity, the interactions between PMNs and Listeria-specific T-cells, and the contribution of cytotoxic T-cells to protection against Listeria.

Non-specific enhancement of host resistance against Listeria monocytogenes

From the literature reviewed in the previous section, it has become clear that the induction and expression of T-cell dependent protection against Listeria is based upon a specific recognition of Listeria-antigens by T-cells. Nevertheless, enhanced resistance against Listeria has also been induced with substances which are structurally unrelated (as far as can be judged) to Listeria antigens. Therefore, enhanced resistance induced with such substances is termed "non-specific". The expression "non-specific resistance" is also used to indicate that a substance induces enhanced resistance against several unrelated micro-organisms. Non-specific resistance against Listeria has been induced with

viable and killed heterologous bacteria (Bloksma, De Reuver, and Willers, 1983), lipopeptides (Floc'h et al., 1981; Parant et al., 1980), polyacrylamide beads (Fontan and Fauve, 1983), synthetic and natural RNA (Fauve, 1971; Medina, Vas and Robson, 1975), an acellular extract (AEP) from Pseudomonas aeruginosa (Fauchère, Simonet and Véron, 1982), β -glucan from Pichia fermentans (Hadibi, Bastide and Bastide, 1981), zymosan (Bloksma et al., 1983), talc (Fontan et al., 1983), and tumor necrosis serum (TNS) (Parant, Parant, and Chedid, 1980).

AEP-treatment of mice resulted in an increased accumulation of phagocytes at the site of infection. In comparison with cells from control-treated animals, macrophages from AEP-treated mice responded with an enhanced initial rate and a higher peak production of chemiluminescence upon in vitro stimulation with Listeria, suggesting that AEP-treatment stimulated phagocytosis and oxygen-dependent bactericidal systems. However, total chemiluminescence was essentially identical for macrophages from AEP-treated and control-treated mice. Furthermore, enhanced clearance of an intravenous inoculum of Listeria from the livers and spleens of AEP-treated mice was not detectable until 2-3 days after infection (Fauchère et al., 1982). At approximately the same time a specific immune response towards Listeria begins to develop (North, 1973). Therefore, it was suggested that AEP probably only increased the efficiency of phagocytes as effectors of the specific immune response. AEP also protected mice against Salmonella typhimurium, Serratia marcescens, Klebsiella pneumoniae and Pseudomonas aeruginosa (homologous as well as heterologous), but the mechanism of protection against these organisms was not analysed in detail (Fauchère et al., 1982).

Several biological effects of β -glucans might be relevant for the induction of enhanced resistance against Listeria with zymosan and glucan from Pichia fermentans (Bloksma et al., 1983; Hadibi et al., 1981). β -Glucans were shown to enhance the generation of cytotoxic T-cells, which might be due to an effect of β -glucans on macrophages. Glucan-activated macrophages had a decreased capacity to secrete prostaglandin E₁, which suppresses the proliferation

and cytotoxic effector function of cytotoxic T-cells (Hamuro, Röllinghof, and Wagner, 1978). Prostaglandin E₁ has been shown to promote the induction of suppressor T-cells (Fulton and Levy, 1981). Conversely, inhibitors of prostaglandin synthesis (aspirin and indomethacin) inhibited the generation of T-suppressor cells (Nicklin and Shand, 1982). Therefore, glucan-treatment might aid in the generation of a specific immune response towards Listeria by inhibiting T-suppressor cell functions. Studies with zymosan-derived glucan indicated that glucan-administration resulted in increased granulocyte and macrophage production (Burgaleta and Golde, 1977). Thus, also increased numbers of available effector cells might contribute to enhanced resistance against Listeria induced with zymosan, or glucan from Pichia fermentans.

Enhanced resistance against Listeria induced with polyacrylamide beads or talc (Fontan and Fauve, 1983; Fontan et al., 1983) could be transferred to normal mice with protein fractions isolated from granulomas which develop upon subcutaneous injection of these materials. From talc-induced granuloma a protein was purified (MW 55000 + 2000; pI 5.0 + 0.1) which enhanced the resistance of mice towards Listeria when given in a dose of 5 µg (Fontan et al., 1983).

Medina et al. (1975) reported that inherently resistant (C57BL/6J) but not inherently sensitive (A/J) mice could be protected against Listeria with RNA. The inherent resistance of C57BL/6J mice was found to be linked with increased macrophage and PMN inflammatory responses (Stevenson, Kongshavn, and Skamene, 1981; Gervais, Stevenson, and Skamene, 1984). The inherent susceptibility of A/J mice appeared to be partially due to the C5 deficiency of these mice, which prevents the generation of the chemotactic complement fragments C5a and C5a des arg (Gervais et al., 1984; Czuprynski et al., 1985). Transfer of C5-sufficient plasma to A/J mice enhanced PMN inflammatory responses (Czuprynski et al., 1985), and transfer of C5-sufficient serum enhanced the resistance of A/J mice towards Listeria infection (Gervais et al., 1984). Whether complement factors were involved in the induction of enhanced resistance with RNA has not been established. It

should be noted that there are also C5-sufficient strains of mice (for instance BALB/c) which are nonetheless sensitive to Listeria-infection (Cheers and McKenzie, 1978). However, also differences in inherent resistance towards Listeria between C5-sufficient mice were found to be associated with differential inflammatory responses upon infection with Listeria (Mandel and Cheers, 1980).

Inflammatory agents were reported to promote the production and/or release of IL1 (Gery et al., 1981), and IL1-like (Goto et al., 1984) and IL2-like (Jin Sheng et al., 1982) factors. Inflammatory rat PMNs were shown to produce low MW (< 10,000) factors which promoted the proliferation of macrophages and stimulated macrophage chemiluminescent responses (Bird, Jin Sheng, and Giroud, 1984).

Apparently, non-specific stimuli can induce the production of factors which are at least functionally similar to mediators produced as a consequence of interactions between Listeria, Listeria-immune T-cells, and macrophages, which might explain why even rather exotic substances can enhance the resistance of experimental animals towards Listeria.

Killing of Listeria monocytogenes.

Reactive oxygen metabolites.

Several studies have been dedicated to the possible involvement of reactive oxygen metabolites (Pick and Keisari, 1981; Halliwell, 1982) in the elimination of Listeria. Virulent strains of Listeria monocytogenes were found to be catalase-positive and contained relatively high levels of superoxide dismutase (SOD). Strains containing only a high level of SOD and a catalase-positive strain containing a relatively low level of SOD were avirulent. Furthermore, in vivo virulence appeared to be associated with resistance towards a superoxide generating system in vitro (Welch et al., 1979). These observations suggest that the contribution of reactive oxygen products to the elimination of Listeria might be restricted to relatively avirulent strains.

Listeria-immune antigen-elicited (LIAE) macrophages (obtained

by immunization of mice with viable Listeria and elicitation of macrophages with killed bacteria) showed enhanced chemiluminescence as compared to control cells upon stimulation with opsonized Listeria or opsonized zymosan (Godfrey and Wilder, 1984). Opsonized zymosan also stimulated the release of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) by LIAE-macrophages. However, only minimal amounts of O_2^- and H_2O_2 were generated upon stimulation of macrophages with either viable or killed, virulent or avirulent, opsonized Listeria. Furthermore, scavengers of oxidative metabolites only had a minimal effect on the bactericidal activity of activated macrophages (Godfrey and Wilder, 1984). Godfrey and Wilder suggested that chemiluminescence in the absence of demonstrable H_2O_2 release might be due to the peroxidase catalysed production of hypochlorous acid (from H_2O_2 and Cl^-) which in turn acts upon the chemiluminescence substrate (luminol). Subsequently, it was found that virulent strains of Listeria produced substantial amounts of O_2^- and H_2O_2 in vitro, whereas only minimal amounts were produced by an avirulent strain. Virulent strains survived intracellularly in resident macrophages, whereas most bacteria of an avirulent strain did not. As addition of exogenous H_2O_2 resulted in a significant loss of macrophage viability, it was suggested that reactive oxygen metabolites might contribute to the pathogenesis and pathology of listerial infection. On the other hand, the deleterious effects of Listeria-derived oxygen metabolites might be counteracted by serum proteins, as opsonized Listeria produced less O_2^- and H_2O_2 than unopsonized bacteria (Godfrey and Wilder, 1985).

In summary, while several reports indicate that reactive oxygen metabolites might contribute to the elimination of other intracellular bacteria such as Mycobacterium lepraemurium, Mycobacterium leprae, Mycobacterium tuberculosis, and Legionella pneumophila (Stach et al., 1984; Sharp, Colston and Banerjee, 1985; Hoffman, Pine and Bell, 1983), they are probably not essential for the elimination of Listeria.

Other bactericidal and bacteriostatic factors

Upon treatment with lymphokines (obtained from the serum of Listeria-immune animals after injection of Listeria-antigen), hamster peritoneal macrophages secreted a factor which inhibited the growth of Listeria (Dumont, 1978).

Caseinate-elicited peritoneal exudate cells from BCG-infected guinea pigs produced a listericidal factor upon stimulation with PPD (purified protein derivative). The listericidal product had the characteristics of a cationic protein. It was relatively heat-stable, non-dialysable, and antagonized by polyanions and iron ions. It adsorbed reversibly to cellulose nitrate but was irreversibly bound by glass and cation-exchangers. The listericidal protein was also active against Corynebacterium pseudodiphtheriticum and Bacillus subtilis, but not against a number of other organisms (Sharma and Middlebrook, 1977). Previously it had been shown that the listericidal protein was produced as a consequence of interactions between BCG-immune lymphocytes and macrophages (Middlebrook, Salmon, and Kreisberg, 1974). Activated guinea pig macrophage monolayers which secreted listericidal products showed only minimal uptake of radiolabeled Listeria (Bast et al., 1974; Middlebrook et al., 1974). Also Listeria-immune murine peritoneal macrophages released soluble listericidal products upon in vitro stimulation with killed Listeria (Sethi, Teschner, and Brandis, 1974).

Davies (1983) reported that activated rat macrophages killed Listeria in vitro without concomittant release of extracellular listericidal products. However, listericidal proteins might also be delivered to phagosomes within which Listeria resides after phagocytosis by macrophages. In fact, intracellular secretion of listericidal protein would be expected to result in higher concentrations of listericidal products and hence more efficient killing of Listeria than extracellular secretion of listericidins.

The listericidin from guinea pig peritoneal exudate cells (Sharma and Middlebrook, 1977) was suggested to be related to the platelet-derived listericidin which is present in rat serum (Czuprynski and Balish, 1981a). In the latter study, also rabbit

serum was found to have listericidal activity. Hamster, guinea pig, beagle, human and mouse sera, however, were not listericidal. Listeria reduced the recalcified clotting time of citrated platelet-rich rat plasma, indicating that the organism can exhibit procoagulant activity (Davies, Ackerman and Nelson, 1981). Live Listeria induced platelet-aggregation in platelet-rich rat plasma, but formalinized bacteria were much less effective. Treatment of platelet-rich plasma with ADP or of washed platelets with thrombin resulted in the release of listericidal activity from the platelets. However, incubation of Listeria with platelet-rich plasma did not kill the bacteria (Czuprynski and Balish, 1981b), indicating that completion of the clotting process may be required for release of listericidal activity. On the other hand, incubation of platelet-rich plasma with Listeria did promote the release of dense granules, which was measured as serotonin-release. Therefore, it was suggested that platelet alpha or lysosomal granules might be the storage site of the platelet-listericidin (Czuprynski and Balish, 1981b). Optimal expression of platelet-derived listericidal activity was found to require a plasma cofactor (Czuprynski and Balish, 1981b; Davies et al., 1981). Rabbit platelets enhanced the postphagocytic destruction of Listeria in cultured mouse peritoneal macrophages. Mouse platelets, however, were reported to be considerably less effective (Wilder and Lubin, 1973). Also the phagocytosis of Listeria by platelets has been described (Absolom et al., 1982).

Carroll and Martinez (1981, a, b, c) described the purification of a platelet-derived low MW cationic protein from rabbit serum. The bactericidal protein was active against Bacillus subtilis, Listeria monocytogenes, Escherichia coli and Salmonella typhimurium, and found to inhibit microbial electron transport.

The clotting system has also been implicated in the expression of delayed-type hypersensitivity, as lymphokines were found to enhance macrophage procoagulant activity, which in turn promotes the deposition of fibrin at the site of a DTH-reaction (Geczy, 1983).

Lehrer et al (1983) and Selsted, Szklarek and Lehrer (1984)

studied the antimicrobial activity of low MW cationic peptides from rabbit macrophages and granulocytes, respectively. Peptide antibiotics from both sources were active against a broad spectrum of bacteria, including L. monocytogenes. Cationic proteins were also shown to function as opsonins for group A streptococci and Candida albicans (Ginsburg et al, 1981) and to induce autolytic enzymes in Staphylococcus aureus (Ginsburg, Lahav, and Giesbrecht, 1982), but whether these properties of cationic proteins are relevant for the elimination of Listeria has not been established.

Theoretically, also acylating enzymes might contribute to the elimination of Listeria as Listeria-peptidoglycan, which is resistant to lysozyme due to the presence of free amino groups, became sensitive to lysozyme-treatment after acetylation (Kamisango et al., 1982).

In conclusion, cationic proteins might be of prime importance for the expression of natural and acquired resistance against Listeria.

Reactive oxygen products might contribute to the elimination of Listeria in an indirect way, because the listericidal product described by Middlebrook et al. (1974) was inactivated by the reducing agent dithiothreitol and partially reactivated by H_2O_2 . Furthermore, optimal production of listericidal activity required the use of H_2O_2 -treated medium. In other studies the bacterium itself may have generated H_2O_2 (Godfrey and Wilder, 1985), thus contributing to its own elimination.

Listeria-vaccines

While enhanced resistance against Listeria is easily induced with a sublethal dose of viable bacteria, results of attempts to immunize animals with killed bacteria have been rather disappointing (Seeliger and Finger, 1976). Medina et al. (1975) reported that C57BL/6J mice could be protected against Listeria infection with a phenol-killed Listeria vaccine. However, in this strain of mice enhanced resistance against Listeria was also observed after treatment with substances bearing no known antigenic relationship to Listeria, indicating that also the protection induced with

killed Listeria may have been due to a nonspecific enhancement of host resistance, rather than to induction of T-cell-dependent immunity.

According to Van Dijk et al. (1980), C3H/HeJ, but not C3HeB/FeJ or BALB/c mice, could be protected against Listeria infection by immunization with heat-killed bacteria. In comparison with BALB/c and C3HeB/FeJ mice, C3H/HeJ mice were found to have relatively high serum concentrations of a polyanionic substance, which was precipitated by protamin sulphate. As polyanions (suramin and dextran sulphate) had been found to be effective adjuvants for killed Listeria vaccines (Van der Meer et al., 1977), Van Dijk et al. (1980) suggested that the polyanionic serum factor of C3H/HeJ mice might function as a kind of intrinsic adjuvant. Alternatively, the immunogenicity of killed bacteria in C3H/HeJ mice might be related to the macrophage defect of these mice, resulting in incomplete degradation of killed bacteria and preservation of immunogenic components (Van Dijk et al., 1980). However, Wirsing von Koenig, Finger and Hof (1982) suggested that the protection observed by Van der Meer et al. (1977) and Van Dijk et al. (1980) might have been due to the small number of viable bacteria left after heat treatment, which is insufficient to induce protection in normal mice, but proliferates to an immunizing dose in mice having a natural (C3H/HeJ) or artificially induced (dextran sulphate treatment) macrophage defect.

Multiple injections of a Listeria cell wall fraction (Rodriguez, McClatchy and Campbell, 1974) or of Listeria-hemolysin (Kingdon and Sword 1970b) induced some protection, but in general use of adjuvants such as lipopolysaccharide (LPS) (Rodriguez et al., 1974), trehalose dimycolate (Le Garrec, Galelli and Audibert, 1981) or dimethyldioctadecylammonium bromide (Van der Meer, Hofhuis and Willers, 1979; Willers et al., 1979) appears to be required for the induction of a substantial level of resistance with killed or radiation-inactivated bacteria, or subcellular fractions of Listeria.

Adjuvants might mimic the deleterious effect of viable Listeria on macrophages resulting in preservation of immunogenic components

(Willers et al. 1979). Conversely, this concept implies that viable bacteria produce their own adjuvant, which might be the hemolysin (Kingdon and Sword, 1970a) or ammonia (Seeliger and Finger, 1976). Ammonia has adjuvant properties which were suggested to be related to its inhibitory effect on the complement system (Klerx, 1985). In addition, ammonia was found to inhibit phagosome-lysosome fusion in macrophages (Gordon, D'Arcy Hart and Young, 1980). Also with other adjuvants a correlation was found between adjuvant activity and anti-complementary effects (Klerx, 1985) or inhibition of phagosome-lysosome fusion (Willers et al., 1979). The immunoenhancing effect of at least some substances appears to be mediated by IL1 (Staruch and Wood, 1983).

In a search for immunogenic subcellular components of Listeria, Willers et al. (1979) found that the highest protective activity was localized in a phenol extract of the cell content of Listeria. In combination with DDA, 1 μ g of this fraction was sufficient to protect mice against 50 LD50. As RNase-treatment completely destroyed its protective properties, the subcellular vaccine of Willers et al. (1979) apparently belongs to a class of RNase-sensitive vaccines the prototype of which is the RNase-sensitive ribosomal vaccine from Mycobacterium tuberculosis described by Youmans and Youmans (1966). Extrapolating from results of experiments with metabolic inhibitors Youmans and Youmans (1974) hypothesized that mycobacterial RNA replicates in host cells in a manner similar to the replication of RNA tumor viruses which involves RNA-directed DNA synthesis. Youmans and Youmans (1974) speculated that replication of mycobacterial RNA might transform a host cell into a specific immune cell. However, direct evidence for the replication of mycobacterial RNA in host cells is lacking.

In studies with ribosomal vaccines from other organisms (reviewed by Eisenstein (1978) and Gonggrijp et al. (1985)) indications were found that RNA does not contribute to the induction of protection (Cooper, Tewari and Bowser, 1980), may have an adjuvant function (Thompson and Snyder, 1971), or nonspecifically enhances host resistance. (Araujo and Remington, 1974). From studies of Medina et al. (1975) and Misfeldt and Johnson (1978) it

has become clear that differences in the strains of mice used to evaluate the protective activity of subcellular vaccines can be a source of conflicting results with respect to the contribution of RNA to the induction of enhanced resistance. On the other hand, Gonggrijp et al. (1980; 1981 a, b; 1983) showed that all three types of vaccine (RNase-sensitive/nonspecific, RNase-insensitive and RNA-adjuvated) could be prepared from Pseudomonas aeruginosa. In these studies RNA-mediated effects were evaluated using DDA as an adjuvant, which might be another source of variation.

Freund's incomplete adjuvant (FIA) appeared to be a particularly effective adjuvant for the mycobacterial RNA vaccine of Youmans and Youmans, whereas only a slight adjuvant effect was observed for several polybasic amines (Youmans and Youmans, 1972). On the other hand, neither Listeria ribosomes in combination with FIA (Coppel and Youmans, 1969b), nor killed Listeria in combination with Freund's complete adjuvant (FCA) (Van der Meer et al., 1979) protected mice against listeriosis. These observations suggest that the specificity attributed to the protection induced with mycobacterial ribosomes plus FIA (which was partially based on the observation that mycobacterial ribosomes plus FIA did not protect mice against listeriosis) (Coppel and Youmans, 1969a) might be related to the use of FIA.

Concluding remarks

Whereas the mechanism of protection against listeriosis induced by immunization with viable Listeria appears to have been largely elucidated, relatively little is known with respect to the mechanism of protection induced by non-viable Listeria-vaccines in combination with adjuvants. Subject of this thesis is the mechanism of protection induced by subcellular preparations from Listeria monocytogenes combined with the adjuvant DDA.

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the $\mathcal{H}^1$ -norm, $\mathcal{H}^1$ -inner product, and $\mathcal{H}^1$ -norm of $\mathbf{u}$ are denoted by $\|\mathbf{u}\|_1$, $(\mathbf{u}, \mathbf{v})_1$, and $\|\mathbf{u}\|_1$, respectively.

Let $\mathbf{u} = (u_1, u_2, u_3, u_4, u_5, u_6, u_7, u_8, u_9, u_{10})^T$ be a vector in $\mathbb{R}^{10}$. Then, the $\mathcal{H}^1$ -inner product and $\mathcal{H}^1$ -norm of $\mathbf{u}$ are defined as follows:

$$(\mathbf{u}, \mathbf{v})_1 = \int_{\Omega} \left(u_1 v_1 + u_2 v_2 + u_3 v_3 + u_4 v_4 + u_5 v_5 + u_6 v_6 + u_7 v_7 + u_8 v_8 + u_9 v_9 + u_{10} v_{10} \right) dx, \\ \|\mathbf{u}\|_1 = \sqrt{(\mathbf{u}, \mathbf{u})_1}.$$

Let $\mathbf{u} = (u_1, u_2, u_3, u_4, u_5, u_6, u_7, u_8, u_9, u_{10})^T$ be a vector in $\mathbb{R}^{10}$. Then, the $\mathcal{H}^1$ -inner product and $\mathcal{H}^1$ -norm of $\mathbf{u}$ are defined as follows:

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Let $\mathbf{u} = (u_1, u_2, u_3, u_4, u_5, u_6, u_7, u_8, u_9, u_{10})^T$ be a vector in $\mathbb{R}^{10}$. Then, the $\mathcal{H}^1$ -inner product and $\mathcal{H}^1$ -norm of $\mathbf{u}$ are defined as follows:

$$(\mathbf{u}, \mathbf{v})_1 = \int_{\Omega} \left(u_1 v_1 + u_2 v_2 + u_3 v_3 + u_4 v_4 + u_5 v_5 + u_6 v_6 + u_7 v_7 + u_8 v_8 + u_9 v_9 + u_{10} v_{10} \right) dx, \\ \|\mathbf{u}\|_1 = \sqrt{(\mathbf{u}, \mathbf{u})_1}.$$

CHAPTER 3

RNase-SENSITIVE AND RNase-INSENSITIVE PROTECTIVE COMPONENTS ISOLATED FROM LISTERIA MONOCYTOGENES

SUMMARY

Crude ribosomes were isolated from Listeria monocytogenes serotype 4b and separated into two fractions by molecular sieve chromatography. Chemical analysis indicated that fraction I contained cell envelope components while fraction II contained the ribosomes. Both fractions protected mice against Listeria, but only in combination with the adjuvant dimethyldioctadecylammonium bromide (DDA). RNase-treatment, but not proteinase K-treatment destroyed the protective properties of fraction II, and RNA purified from fraction II also induced protection. Protection induced by fraction I was not affected by either RNase- or proteinase K-treatment. Both subcutaneous and intraperitoneal, but not intravenous administration of fraction I, fraction II, or purified RNA induced significant protection against intraperitoneal infection, the intraperitoneal route of administration being the most effective. All preparations induced high levels of protection 3 to 7 days after administration, but protection was already decreased after 14 days. Protection induced with RNA appeared to be biphasic, because it also protected mice 1 day, but not 2 days after administration. Protection induced with both fraction I and RNA was at least in part non-specific, because both preparations also protected mice against L. monocytogenes serotype 3, Streptococcus pneumoniae and Pseudomonas aeruginosa.

Results are discussed in relation to previous work with analogous preparations from P. aeruginosa.

INTRODUCTION

Listeria monocytogenes is a Gram-positive bacterium which behaves, when it has entered the body, as a facultative intracellular parasite. Its natural host cell is the macrophage in which the organism is able to survive and even to multiply (World Health Organization Scientific Group, 1973). Protection against experimental listeriosis can be induced with a sublethal dose of living bacteria (Mackaness, 1962), and is assumed to be due to T-cell mediated macrophage-activation (Cole, 1975). Induction of high levels of protection with killed bacteria or bacterial components requires the use of adjuvants such as lipopolysaccharide (LPS) (Rodríguez et al., 1974), trehalose dimycolate (Le Garrec et al., 1981) or the quaternary ammonium compound dimethyldioctadecylammonium bromide (DDA) (Van der Meer et al., 1979; Willers et al., 1979).

In an attempt to identify the protective components of L. monocytogenes, Willers et al. (1979) immunized mice with fractions isolated from disrupted bacteria. Using DDA as an adjuvant, highest protection was obtained with a phenol extract from the cell contents of the bacteria. Enzymatic treatment of this fraction, which contained 60% nucleic acids, indicated that RNA, but not DNA or protein was essential for the induction of protection.

Previously we described the separation of a crude ribosomal preparation from Pseudomonas aeruginosa into RNase-sensitive and RNase-insensitive protective components (Gonggrijp et al., 1980). In the present study we have investigated whether the same purification procedure would yield a similar type of separation when applied to a crude ribosomal preparation from L. monocytogenes. L. monocytogenes was chosen, because, in a sense, it is the opposite of P. aeruginosa, a Gram-negative bacterium which behaves as an extracellular parasite (Peterson, 1980).

It was felt that the purification of ribosomal preparations from such dissimilar organisms as L. monocytogenes and P. aeruginosa according to a standardized procedure and comparison of their protective properties might lead to detection of a general

principle with respect to the mode of action of ribosomal vaccines. Such a general principle has not yet been shown, possibly due to variations with respect to the purification procedures used by different investigators (Eisenstein, 1978).

MATERIALS AND METHODS

Experimental animals

Male BALB/c mice were purchased from TNO, Central Institute for the Breeding of Laboratory Animals, Zeist, The Netherlands, and used at an age of 8-9 weeks.

Bacteria

L. monocytogenes L242, serotype 4b (kindly provided by Dr. H. van Dijk, Department of Immunology, University of Utrecht, The Netherlands) was used throughout this study. Stock suspensions of bacteria were prepared as follows: Listeria was grown for 16 h at 37°C as described previously (Antonissen et al., 1981).

Culture bottles were fitted with 0.22 μ m filters (Millipore) to release pressure. Bacteria were recovered by centrifugation, washed three times with phosphate buffered saline pH 7.0 (PBS) and stored in small portions at -70°C in PBS. To determine the 50% lethal dose (LD_{50}), 0.5 ml of serial dilutions of a stock suspension of bacteria were injected intraperitoneally (i.p.) into mice. Deaths, which occurred almost exclusively within 7 days after infection, were recorded for 14 days. The LD_{50} was calculated according to Reed and Muench (1938). For batches used in this study LD_{50} values varied between 4.8×10^5 and 2.6×10^6 CFU.

L. monocytogenes serotype 3, Streptococcus pneumoniae serotype 1 (NCTC 8314), and Pseudomonas aeruginosa serotype 3 (strain RIV 76-5321) were kindly provided by Drs. J. Oosterom, D.G. Groothuis and J. Borst, respectively (National Institute of Public Health, Bilthoven, The Netherlands). L. monocytogenes serotype 3 was cultured as described for L. monocytogenes serotype 4b. S. pneumoniae was cultured at 37°C in brain heart infusion medium

(Difco Laboratories, Detroit, Mich., U.S.A.) supplemented with 0.2% sucrose and 1% 2.0 M Na_2HPO_4 and enumerated on 5% sheep blood agar plates (Szu et al., 1983). *P. aeruginosa* was cultured as described previously (Gonggrijp et al., 1980). For all bacteria mouse passage (Mackanness, 1962) was used to maintain or increase virulence.

Preparation of crude ribosomes

L. monocytogenes L 242 was grown at 37°C without aeration in 8 liter batches using a Biostat fermentor (B. Braun, Melsungen, W. Germany) fitted with a LF32 miniature line filter (Microflow Ltd., Fleet Hants, England) to release pressure. Bacteria were harvested by centrifugation when the culture had reached a density of approximately 2.4×10^9 CFU/ml. The number of bacteria was estimated by reading the turbidity at 540 nm. Bacteria were washed three times with buffer containing 0.01 M Tris, 0.1 M KCl, 0.015 M MgCl_2 , pH 7.2 (TKM buffer) and used for the preparation of crude ribosomes (Gonggrijp et al., 1980). Briefly, bacteria were frozen at -20°C and mechanically disrupted using a cold Hughes pressure cell. The broken cell paste was suspended in 100 ml of TKM buffer. To reduce viscosity the broken cell suspension was treated with 1 mg of DNase (Merck AG, Darmstadt, W. Germany) for 15 min at 37°C. After DNase-treatment unbroken cells and large cell-envelope fragments were removed by two cycles of centrifugation (20 min 30,000 x g and 20 min 45,000 x g, both at 4°C). The supernatant from the second centrifugation step was filtered through a 0.22 μm filter (Sartorius-Membranfilter GmbH, Göttingen, W. Germany). The filtrate was centrifuged for 2.5 h at 180,000 x g. The resulting pellet containing the ribosomes was resuspended in TKM buffer to a concentration of 100 mg (wet weight) / ml (crude ribosomal preparation).

Preparation of fractions I and II

The crude ribosomal preparation was fractionated by molecular sieve chromatography as described previously (Gonggrijp et al.,

1980) using a Sepharose CL-2B (Pharmacia, Uppsala, Sweden) column (5x40 cm) equilibrated with TKM buffer containing 1 M NH_4Cl . Two peaks were obtained. Fractions of both peaks were pooled and centrifuged at 180,000 x g for 2.5 h. Pellets were resuspended in TKM buffer to a concentration of 50 mg (wet weight)/ml and lyophilized to determine the dry weight. The dry weight was corrected for the amount of salts added. The lyophilized material was reconstituted with distilled water and diluted with TKM buffer to a concentration of 10 mg (dry weight)/ml.

Fraction II was filtered through a 0.22 μm Sartorius filter. In preliminary experiments, filter-sterilization of fraction I was found to cause a loss of protective activity, probably because it contained large amounts of aggregated material. Therefore, fraction I was used without filtration. Unfiltered fraction I was checked for the presence of living Listeria, but none were found. Finally, both fractions were stored at -70°C .

Preparation of RNA from fraction II

RNA was extracted from fraction II with phenol and sodium dodecyl sulphate (SDS) and purified by the method of Poulson (1977).

Chemical analyses

RNA and protein were determined by the orcinol method of Herbert et al. (1971) and the Lowry method as modified by Hartree (1972), using yeast RNA and bovine serum albumin as the standards, respectively. Phospholipids were extracted by the method of Folch et al. (1957) and estimated according to Rouser et al. (1966) using inorganic phosphate as the standard. It was assumed that 1 mg of phospholipid contained 40 μg of phosphorus.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis of fraction II and purified RNA was performed essentially as described by Kastelein et al. (1983). After electrophoresis the gels were scanned at 260 nm. Ribosomal RNA of Pseudomonas faeruginosa 5321 (serotype 3)

(Gonggrijp et al., 1981b) or ribosomal RNA of Escherichia coli MRE-600 (P.L. Biochemicals GmbH, St. Goar, W. Germany) was used as a reference.

Treatment with RNase and with proteinase K

Before the addition of enzymes, fractions I and II were dialyzed overnight at 4°C against 0.005 M EDTA (pH 7.2) followed by dialysis against distilled water for 1 h. For RNase treatment, 125 µg of pancreas-RNase plus 2500 units of RNase T1 (Boehringer Mannheim, Mannheim, W. Germany) was added to 1 ml of fraction I or II (containing 10 mg dry material) and the mixture was incubated for 1 h at 37°C. Treatment with proteinase K was done similarly, using 250 µg of proteinase K (Sigma Chemical Co., St. Louis, M.O.) per ml of fraction I or II. Controls were incubated without the addition of enzymes.

Protection experiments

Unless stated otherwise, preparations to be tested were injected i.p. with or without 300 µg of the adjuvant dimethyldioctadecylammonium bromide (DDA) (Eastman Kodak Co., Rochester, N.Y.) (Van der Meer et al., 1979; Willers et al., 1979). The total volume injected was 0.2 ml and PBS was used as a diluent throughout. Seven days later mice were challenged i.p. with approximately $10 \times LD_{50}$ L. monocytogenes L242, serotype 4b, suspended in 0.5 ml PBS. The challenge inoculum was prepared by direct dilution of a stock suspension of bacteria. The actual number of CFU injected was checked by plating on Trypticase Soy Agar (BBL Microbiology Systems, Cockeysville, MD, U.S.A.). Deaths were recorded for 14 days after challenge.

Statistical evaluation

Significance levels for protection (P values) were determined by the Fisher exact test as described by Bradley (1968). At P values ≥ 0.05 differences were considered to be not significant.

RESULTS

Preparation of fractions I and II

The crude ribosomal preparation was separated into two distinct fractions by molecular sieve chromatography on Sepharose CL-2B. Fraction I was turbid and eluted in the void volume, whereas fraction II eluted later. Fraction II showed high extinction values at 260 nm, indicating the presence of nucleic acids.

Chemical composition of fraction I, fraction II and RNA extracted from fraction II

RNA was the major component of fraction II, but almost absent from fraction I (Table 1). The RNA:protein ratio of fraction II was approximately 2:1 which is characteristic for purified ribosomes. A relatively large amount of phospholipid was found in fraction I, indicating the presence of cell envelope components (in particular membrane fragments) in this fraction. In four batches tested, the RNA:protein ratio of fractions I and II was found to be reproducible. RNA was extracted from a single batch of fraction II. The purified preparation contained RNA and protein in a ratio of 100:1. Upon polyacrylamide gel electrophoresis of fraction II two major bands were found. By comparison with reference rRNA from E. coli or P. aeruginosa, they were tentatively identified as 23S and 16S rRNA (Figure 1). Similar results were obtained with purified RNA.

Protection induced by fraction I, fraction II, and purified RNA

Both fractions protected mice against a lethal Listeria challenge when combined with DDA (Table 2). Preparations administered without DDA, or DDA alone, did not induce protection. Fractions I and II were treated with RNase or proteinase K to test the possible involvement of RNA and protein in the induction of protection. As shown in Table 3, protection induced by fraction II was destroyed by RNase-, but not by proteinase K-treatment. Protection

TABLE 1. Chemical composition of fraction I and fraction II

Preparation	Composition (% by wt)			
	RNA ^a	Protein ^a	Phospholipid ^b	RNA/protein ratio ^a
Fraction I	1 $\pm$ 0.5	37 $\pm$ 11	16	0.03 $\pm$ 0.03
Fraction II	53 $\pm$ 8	29 $\pm$ 5	1	1.85 $\pm$ 0.12

^a Mean of four preparations $\pm$ SD.

^b Pooled material from three preparations.

Fig. 1 Polyacrylamide-SDS gel electrophoresis of fraction II from *Listeria monocytogenes*.

induced by fraction I was not affected by either treatment. Protection induced by RNase-treated fraction I might have been due to protein components, and protection induced by proteinase K-treated fraction I to the small amount of RNA which was present in this fraction. However, also sequential treatment of fraction I with RNase and proteinase K did not reduce its protective properties. Since the results obtained with four batches of fractions I and II were reproducible, these batches were pooled in all further experiments.

TABLE 2. Protection against *L. monocytogenes* induced with fraction I and fraction II

Preparation ^a	Survival (%) ^b	P value ^c
Fraction I	0 + 0	(NS)
Fraction I + DDA	88 + 16	(< 0.001)
Fraction II	0 + 0	(NS)
Fraction II + DDA	96 + 8	(< 0.001)
PBS	0	-
DDA	0	(NS)

^a Fraction I and fraction II were used in a dose of 10 µg.

^b Mean of results obtained with four separate preparations $\pm$ SD, using 6 mice per group; single groups were used for PBS and DDA controls.

^c P Values were calculated with respect to PBS controls; NS, not significant.

Because RNase treatment of fraction II destroyed its protective capacity, RNA was extracted from fraction II and tested in protection experiments. As shown in Table 4, purified RNA protected mice against lethal Listeria challenge in a dose-response relationship. The ability to induce protection against Listeria was not restricted to RNA from the homologous organism, because in a similar experiment 2 μ g RNA from P. aeruginosa fully protected mice against lethal challenge. RNA from either L. monocytogenes or P. aeruginosa was only protective in combination with DDA.

Effect of the route of administration on the level of protection induced with fraction I, fraction II, and purified RNA

Although less effective than i.p. administration, enhanced resistance was also induced by subcutaneous (s.c.) administration of fraction I, fraction II, or RNA in combination with DDA. Intravenous administration did not induce protection (Table 5). Irrespective of the route of administration, all animals treated with PBS or preparations without DDA died from Listeria-infection.

TABLE 3. Effect of treatment with RNase and proteinase K on the induction of protection with fractions I and II

Preparation ^a		Survival (%) ^b	P value ^c
Fraction I (control-treated)	+ DDA	100 $\pm$ 0	-
Fraction I (RNase-treated)	+ DDA	88 $\pm$ 16	(NS)
Fraction I (proteinase K-treated)	+ DDA	88 $\pm$ 8	(NS)
Fraction II (control-treated)	+ DDA	100 $\pm$ 0	-
Fraction II (RNase-treated)	+ DDA	13 $\pm$ 16	(<0.001)
Fraction II (proteinase K-treated)	+ DDA	96 $\pm$ 8	(NS)
DDA		0	-
PBS		0	-

^a All preparations were used in a dose of 10 μ g.

^b Mean of results obtained with four separate preparations $\pm$ SD, using 6 mice per group; single groups were used for PBS and DDA controls.

^c P values were calculated with respect to results obtained with control-treated materials; NS, not significant.

TABLE 4. Protection against *L. monocytogenes* induced with purified RNA isolated from fraction II; dose-response relationship

Preparation ^a	Survival (%) ^b	P value ^c
10.00 µg RNA + DDA	100	(<0.001)
3.33 µg RNA + DDA	63	(0.01)
1.11 µg RNA + DDA	63	(0.01)
0.37 µg RNA + DDA	0	(NS)
0.12 µg RNA + DDA	0	(NS)
DDA	0	-

^a Groups of 8 mice were injected with varying doses of purified RNA with or without DDA and challenged 7 days later. Controls received DDA or PBS.

^b All mice treated with RNA alone or with PBS died from *Listeria* infection.

^c P Values were calculated with respect to DDA controls; NS, not significant.

Time course of protection

Fraction I, fraction II or RNA were administered i.p. in combination with DDA at varying intervals before i.p. challenge with *Listeria*. Mice injected with DDA alone served as negative controls. As shown in Figure 2, all preparations induced high levels of protection when the challenge was given 3-7 days after administration, and resistance decreased after 14 days. Furthermore, a relatively high level of resistance was found one, but not two days after administration of RNA in combination with DDA.

Specificity of the protection induced with fraction I and RNA

Mice were injected i.p. with 10 µg of fraction I or RNA in combination with DDA. Controls received DDA alone. Seven days later mice were challenged i.p. with *L. monocytogenes* serotype 3,

P. aeruginosa serotype 3, or S. pneumoniae serotype 1. As shown in Table 6, in combination with DDA fraction I as well as RNA protected mice against all three organisms.

TABLE 5. Effect of the route of administration on the level of protection; survival (%) after intraperitoneal challenge

Preparation ^a	Route of administration ^b		
	i.p.	s.c.	i.v.
Fraction I + DDA	100	67	0
Fraction II + DDA	100	57	7
RNA + DDA	92	58	25
DDA	4	4	15

^a All preparations were used in a dose of 10 μ g.

^b Preparations were injected intraperitoneally (i.p.), subcutaneously (s.c.) or intravenously (i.v.) and 7 days later all groups were challenged i.p. Each group consisted of at least 12 mice. For all preparations P values with respect to DDA controls were < 0.001 for the i.p. and s.c. route, and > 0.2 (not significant) for the i.v. route.

Fig. 2. Time course of protection against *L. monocytogenes* induced with fraction I, fraction II and RNA. Fraction I, fraction II or RNA were injected i.p. in combination with DDA at varying intervals before i.p. challenge with *Listeria*. At least six mice were used for the day 7 points; for all other points at least twelve mice were used. (○: DDA; ●: 10 µg fraction I + DDA; □: 10 µg fraction II + DDA; ■: 10 µg RNA + DDA).

DISCUSSION

In this study we have investigated the protective properties of ribosomal preparations from *L. monocytogenes*. Crude ribosomes were isolated and separated into two fractions according to a procedure previously described for *P. aeruginosa* (Gonggrijp et al., 1980). Chemical analysis indicated that fraction I contained cell envelope components, while fraction II contained the ribosomes (Table 1, Figure 1).

Although chemically rather dissimilar, both fractions protected mice against *Listeria*. As previously shown for other killed-*Listeria* vaccines (Van der Meer et al., 1979; Willers et al., 1979), fraction I as well as fraction II had to be given in combination with DDA in order to be protective (Table 2).

TABLE 6. Specificity of the protection induced with fraction I and RNA from *L. monocytogenes* serotype 4b

Preparation	Survival (%) ^a after challenge with:		
	<i>Listeria</i> <i>monocytogenes</i> ^b Serotype 3	<i>Streptococcus</i> <i>pneumoniae</i> ^c Serotype 1	<i>Pseudomonas</i> <i>aeruginosa</i> ^d Serotype 3
10 µg fraction I + DDA	95	94	100
DDA	0	22	8
10 µg RNA + DDA	100	100	83
DDA	0	25	8

^a Each group consisted of at least twelve mice. In all cases *P*-values with respect to DDA controls were <0.001.

^b Mean challenge dose 2.8×10^7 CFU ($19 \times LD_{50}$).

^c Mean challenge dose 8.1×10^2 CFU ($26 \times LD_{50}$).

^d Mean challenge dose 1.5×10^8 CFU ($3 \times LD_{50}$).

Experiments with enzymatically treated fractions suggested that neither RNA nor protein was essential for the induction of protection with fraction I (Table 3). In addition, the dose of fraction I tested (10 µg) contained approximately 0.1 µg RNA (Table 1), which was found to be insufficient for the induction of protection with purified RNA (Table 4). Therefore, the major protective components of fraction I remain to be identified. RNA appeared to be the major protective component of fraction II, because protection induced by this fraction was destroyed by RNase-treatment, and could be reproduced with purified RNA (Table 3, 4). Furthermore, even heterologous RNA protected mice against *Listeria*. The presence of RNase sensitive protective components in subcellular fractions from *Listeria* was previously shown by Willers et al. (1979). Medina et al. (1975) found that genetically resistant (C57 BL/6J), but not genetically sensitive (A/J) mice could be protected against *Listeria* with *Listeria* RNA, heterologous RNA, poly

(I:C) and a number of other substances, without the use of adjuvants. Similar results were reported with respect to the effect of RNA on the resistance of mice towards Salmonella typhimurium (Medina et al., 1975; Misfeldt and Johnson, 1978).

In our study BALB/c mice, which are genetically sensitive to Listeria (Cheers et al., 1978), could be protected against a lethal Listeria challenge with a combination of RNA and DDA, but not with RNA or DDA alone. This may indicate that even genetically sensitive mice can be protected against Listeria with a combination of immunostimulants.

Both DDA (Bloksma et al., 1983; Gordon et al., 1980; Willers et al., 1979) and polyanions such as RNA (Cohn and Parks, 1967; Fauve, 1971; Madraso and Cheers, 1978; Schultz et al., 1977; Schultz and Chirigos, 1979) have macrophage-modulating properties. Therefore, enhanced resistance against Listeria induced with a combination of RNA and DDA might be due to a synergistic effect of these materials on macrophage functions.

Enhanced resistance against Listeria induced with fraction I, fraction II or RNA probably contained both systemic and local components, because also s.c. administration protected mice against i.p. challenge, although protection was highest after i.p. administration (Table 5).

The higher protection after i.p. administration may have been due to the use of DDA, which attracts phagocytic cells to the site of injection (Gordon et al., 1980).

For all preparations, protection appeared to be relatively shortlived because resistance was already decreased 14 days after administration, whereas full or nearly full protection was observed 3-7 days after injection of fraction I, fraction II or RNA. In contrast, Listeria-immunized mice continued to show resistance to Listeria at 4 weeks post-immunization (Mackaness, 1964). Protection induced by RNA was biphasic, because enhanced resistance was also observed 1 day, but not 2 days after administration.

Protection induced with RNA as well as fraction I was both serotype- and species-nonspecific (Table 6).

In this respect our preparations resembled nonspecific inflamma-

tory stimuli, which were reported to enhance the resistance of mice towards a variety of pathogens, including L. monocytogenes (Fauve, 1981; Fontan and Fauve, 1983; Fontan et al., 1983). Therefore the contribution of inflammation associated phenomena to the protective properties of our preparations will be subject to further investigation. With respect to the serotype-nonspecificity of the protection induced with fraction I and RNA, it should be noted that also the enhanced resistance against Listeria induced with living bacteria is serotype nonspecific (Wirsing von Koenig et al., 1983). Furthermore, increased resistance against the heterologous organism Brucella abortus was observed until approximately 2 weeks after primary infection with Listeria monocytogenes (Mackaness, 1964). Apparently, the transient nonspecific protection, but not the longer lasting, specific protection induced with living Listeria, could be reproduced with subcellular fractions from Listeria in combination with DDA.

In several respects, the characteristics of the preparations from L. monocytogenes described in this paper were similar to those of the analogous preparations from P. aeruginosa described previously (Gonggrijp et al., 1980, 1981a, 1981b, 1983). Using the same purification procedure, crude ribosomal preparations from both organisms could be separated into cell envelope-components (fraction I) and purified ribosomes (fraction II). Enhanced resistance against homologous challenge induced with fraction I from either organism was insensitive to treatment with RNase or proteolytic enzymes. However, in contrast to the preparation from L. monocytogenes, fraction I from P. aeruginosa induced serotype specific protection without DDA, which was probably due to the presence of LPS in this fraction (Gonggrijp et al., 1980). Also fraction II from P. aeruginosa was most effective when given in combination with DDA, and RNA was identified as its major protective component (Gonggrijp et al., 1980, 1981b). However, in addition to RNA, LPS appeared to contribute to the protection induced with fraction II from P. aeruginosa, and this contribution was serotype-specific (Gonggrijp et al., 1981a, 1981b). Whether components other than RNA contributed to the protection

induced with fraction II from L. monocytogenes remains to be established.

In combination with DDA, also RNA from P. aeruginosa induced serotype- and species-nonspecific protection (Gonggrijp et al., 1981b, 1983). Therefore it is suggested that the mechanisms of enhanced resistance induced with Listeria-RNA and Pseudomonas-RNA are closely related, if not identical.

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CHAPTER 4

*DISSOCIATION BETWEEN ENHANCED RESISTANCE AND DELAYED
HYPERSENSITIVITY INDUCED WITH SUBCELLULAR PREPARATIONS FROM
LISTERIA MONOCYTOGENES AND THE ADJUVANT
DIMETHYLDIOCTADECYLAMMONIUM BROMIDE*

SUMMARY

In this study we investigated the relation between enhanced resistance and delayed hypersensitivity (DH) induced with subcellular preparations from Listeria monocytogenes and the adjuvant dimethyldioctadecylammonium bromide (DDA). Ribosomal RNA as well as cell envelope fragments (fraction I) protected mice against lethal Listeria infection. However, only fraction I induced DH against killed Listeria. For the induction of protection with fraction I or RNA as well as for the induction of DH with fraction I, preparations had to be administered in combination with DDA. Fraction I elicited a DH response in mice immunized with viable Listeria, but RNA did not. These observations pointed to a dissociation between DH and enhanced resistance induced with RNA, and to a dissociation between fraction I and RNA with respect to their ability to induce or elicit DH. Also DH and enhanced resistance induced with fraction I could be dissociated. Intracutaneous administration of fraction I induced high levels of DH without concomittant induction of protection against lethal challenge with Listeria. On the other hand, intraperitoneal administration of fraction I fully protected mice against lethal infection, but only induced a moderate DH response. DH induced with fraction I was largely specific, whereas enhanced resistance induced with this preparation was nonspecific. Finally, proteinase K-sensitive proteins were found to be essential for the induction of DH but not for the induction of protection with fraction I.

INTRODUCTION

Previously we described the separation of a crude ribosomal preparation from Listeria monocytogenes serotype 4b into cell envelope fragments (fraction I) and purified ribosomes (fraction II) (Antonissen et al., 1985). In combination with the adjuvant DDA, both fractions protected mice against lethal Listeria challenge. Ribosomal RNA was identified as the major protective component of fraction II, because its protective properties were destroyed by RNase-treatment, and could be reproduced with purified RNA extracted from this fraction.

Enhanced resistance induced with fraction I was not affected by RNase-treatment, proteinase K-treatment, or sequential treatment with both enzymes. Otherwise, the protective properties of fraction I were rather similar to those of fraction II and/or purified RNA. Subcutaneous as well as intraperitoneal administration of fraction I, fraction II, or purified RNA induced significant protection against intraperitoneal infection, the intraperitoneal route of administration being the most effective. Furthermore, fraction I as well as RNA also protected mice against Streptococcus pneumoniae and Pseudomonas aeruginosa.

In this study we investigated whether fraction I and RNA could be differentiated with respect to their ability to induce or elicit delayed hypersensitivity (DH) against Listeria. As this approach turned out to be successful, a more extensive characterization of DH induced with fraction I and RNA was undertaken to allow comparison of enhanced resistance and DH induced with sub-cellular preparations from Listeria monocytogenes.

MATERIALS AND METHODS

Experimental animals

Male BALB/c mice were purchased from TNO, Central Institute for the Breeding of Laboratory Animals, Zeist, The Netherlands, or from Gl. Bomholtgard Ltd., Ry, Denmark, and used at an age of 8-11 weeks. Animals from both suppliers were employed in DH experiments

but only mice from TNO were used to determine enhanced resistance against Listeria infection.

Bacteria

Listeria monocytogenes L242, serotype 4b, was kindly provided by H. van Dijk, Department of Immunology, University of Utrecht, The Netherlands and used throughout this study. Listeria was grown without aeration for 16 h at 37°C, using the medium described by Antonissen et al. (1981). Culture bottles were fitted with 0.22 µm filters (Millipore) to release pressure. Bacteria were recovered by centrifugation, washed three times with phosphate buffered saline pH 7.0 (PBS) and stored in small portions at -70°C in PBS. Listeria was enumerated by plating on Trypticase Soy Agar (BBL Microbiology Systems, Cockeysville, M.D., U.S.A.).

Streptococcus pneumoniae serotype 1 (NCTC 8314) and Pseudomonas aeruginosa serotype 3 (strain RIV 76-5321) were kindly provided by D.G. Groothuis and J. Borst, respectively (National Institute of Public Health, Bilthoven, The Netherlands).

S. pneumoniae was cultured at 37°C in brain heart infusion medium (Difco Laboratories, Detroit, Mich., U.S.A.) supplemented with 0.2% sucrose and 0.02 M Na₂HPO₄ and enumerated on 5% sheep blood agar plates (Szu, Clarke and Robbins, 1983).

P. aeruginosa was cultured and enumerated as described previously (Gonggrijp et al., 1980). For all bacteria mouse passage (Mackanness, 1962) was used to maintain or increase virulence.

Microbial preparations

Formalin-killed bacteria. Bacteria were killed by overnight exposure to 1.25% formalin in PBS. Killed bacteria were washed three times and stored at -70°C in PBS. Sterility was checked by plating on suitable media.

Subcellular fractions from Listeria monocytogenes. Subcellular fractions from Listeria monocytogenes were prepared as described previously (Antonissen et al., 1985). Briefly, Listeria was grown in 8 liter batches at 37°C without aeration using a Biostat fermentor (B. Braun, Melsungen, W. Germany) fitted with a miniature

line filter (Microflow Ltd., Fleet Hants, England) to release pressure. Cell envelope fragments (fraction I) and purified ribosomes (fraction II) were obtained using procedures originally developed for Pseudomonas aeruginosa (Gonggrijp et al. 1980). However, solubilization with sodium dodecyl sulphate and filtration of fraction I was omitted. Ribosomal RNA was extracted from fraction II with phenol and sodium dodecyl sulphate as described by Poulson (1977). Fraction I contained 37% protein, 16% phospholipids, and 1% RNA. The purified RNA contained less than 1% protein.

Proteinase K treatment of fraction I.

Fraction I was treated with proteinase K as described previously (Antonissen et al., 1985). Briefly, Fraction I (10 mg/ml in PBS) was dialysed overnight at 4°C against 0.005 M EDTA (pH 7.2) followed by dialysis against distilled water for 1 h. Proteinase K (Sigma Chemical Co., St. Louis, M.O., U.S.A.) was added to a concentration of 250 µg/ml and the mixture was incubated for 1 or 2 h at 37°C. Controls were incubated without the addition of enzyme.

Determination of delayed hypersensitivity and enhanced resistance.

DH responses and enhanced resistance against Listeria were determined using modifications of techniques described by Van der Meer et al. (1979). For the induction of DH, preparations were injected intracutaneously (i.c.) with or without 300 µg of the adjuvant DDA (Eastman Kodak Co., Rochester, N.Y., U.S.A.). The total volume injected was 0.1 ml per mouse divided over two sites. PBS was used as a diluent throughout.

Seven days after induction, 20 µl of a suspension of the eliciting antigen was injected into the left hind footpad. DH was measured as the increase in footpad thickness 24 h later. Measurements were made using a semi-electronic footpad-meter (Van Dijk, Versteeg and Hennink, 1976). DH against Listeria was routinely assessed using 10⁸ formalin-killed Listeria as the eliciting antigen.

Increased survival after intraperitoneal (i.p.) injection with

viable Listeria was taken as a measure of enhanced resistance. The mean lethal dose of the batch of Listeria used was 2.6×10^6 colony forming units for mice obtained from TNO. LD₅₀ values were determined according to Reed and Muench (1938). Mice were injected i.p. with approximately 10 LD₅₀ of L. monocytogenes suspended in 0.5 ml PBS and the number of survivors was determined 14 days later.

Statistical evaluation.

Significance levels for protection were determined by the Fisher exact test as described by Bradley (1968). Data from DH experiments were analysed using Student's t-test. For both tests differences were considered to be significant at P-values < 0.05.

RESULTS

Delayed hypersensitivity against fraction I and RNA induced with living Listeria.

Firstly, we investigated whether fraction I or RNA could elicit a DH response in mice immunized i.c. with viable Listeria. As shown in Table 1, living Listeria induced DH against fraction I, but not against the same dose of RNA. Also a tenfold increase in the dose of RNA used for elicitation did not produce a DH response.

Induction of DH against Listeria with fraction I and RNA

Next, we addressed the question whether DH against Listeria could be induced by immunization with fraction I or RNA. Preparations were also tested in combination with the adjuvant DDA, which was previously shown to be required for the induction of protection against Listeria with fraction I or RNA (Antonissen et al. 1985).

TABLE 1. Delayed hypersensitivity against fraction I and RNA induced by living *L. monocytogenes*

Immunization of mice	Footpad challenge	Footpad swelling (0.1 mm) $\pm$ SD	P value ^a
10^6 <i>L. monocytogenes</i>	10 μ g fraction I	5.5 $\pm$ 1.8	0.01
PBS	10 μ g fraction I	2.7 $\pm$ 0.9	
10^6 <i>L. monocytogenes</i>	10 μ g RNA	0.1 $\pm$ 0.9	NS
PBS	10 μ g RNA	1.2 $\pm$ 1.3	
10^6 <i>L. monocytogenes</i>	100 μ g RNA	0.9 $\pm$ 1.1	NS
PBS	100 μ g RNA	0.6 $\pm$ 0.7	

Groups of 5-6 mice were injected intracutaneously with 10^6 living *L. monocytogenes* serotype 4b. Seven days later they were challenged in the left footpad with fraction I or RNA. The increase in footpad thickness was determined after 24 h.

^a P values were calculated with respect to PBS controls, using Student's *t*-test. NS: not significant ($P \geq 0.05$).

TABLE 2. Delayed hypersensitivity against *L. monocytogenes* induced by immunization with fraction I and RNA

Immunizing agent ^a	Footpad swelling ^b (0.1 mm) $\pm$ SD	P value ^d
10 μ g fraction I + DDA	7.9 $\pm$ 2.0	<0.001
10 μ g RNA + DDA	1.1 $\pm$ 1.0	NS
100 μ g RNA + DDA	0.8 $\pm$ 0.5	NS
10 μ g fraction I	0.9 $\pm$ 0.8	NS
10 μ g RNA	0.3 $\pm$ 0.9	NS
100 μ g RNA	0.2 $\pm$ 0.5	NS
10 ⁶ viable <i>Listeria</i>	13.0 $\pm$ 0.8	<0.001
PBS	1.0 $\pm$ 1.0 ^c	-
DDA	1.2 $\pm$ 1.7 ^c	-

^a Groups of 6 mice were injected intracutaneously with preparations to be tested. Seven days later they were challenged in the left footpad with 10⁸ formalin-killed *L. monocytogenes* serotype 4b. The increase in footpad thickness was determined 24 h later. Mice immunized with viable *Listeria* were used as positive controls. Mice treated with DDA alone and PBS alone were used as negative controls for groups treated with DDA-adjuvated and non-adjuvated preparations, respectively.

^b Results represent combined data from 3 experiments and are given as the difference between the means of experimental groups and negative controls. Standard deviations were calculated by square addition of SD values of experimental groups and controls (Moroney, 1968).

^c Absolute values calculated using combined data from 3 experiments.

^d P values were calculated using Student's *t*-test. NS: not significant (*P* > 0.05).

Fraction I induced DH against killed Listeria, but only in combination with DDA (Table 2). RNA, either with or without DDA, did not induce DH. Also a tenfold higher dose of RNA (100 µg) was found to be ineffective.

Effect of the route of administration on the induction of DH and enhanced resistance with fraction I and RNA.

In contrast to i.c. administration, i.p. injection of fraction I only induced a moderate DH response against killed Listeria (Fig. 1). RNA did not induce DH via either route. As i.p. administration of fraction I or RNA was previously found to be quite effective for the induction of protection against lethal infection (Antonissen et al., 1985) it was decided to challenge all groups with viable Listeria after determination of the DH response. Only mice which had received fraction I or RNA via the i.p. route were found to be protected against i.p. challenge with viable Listeria (Fig. 1).

Specificity of DH induced with fraction I.

Formalin-killed Streptococcus pneumoniae and Pseudomonas aeruginosa were used to determine the specificity of DH induced with fraction I.

As shown in table 3, fraction I also induced a low but significant DH-response towards S. pneumoniae and P. aeruginosa. Using two way variance analysis (kindly performed by A. Volovics, Department of Medical Informatics and Statistics) the DH response towards Listeria was found to be significantly higher ($P=0.017$) than the response towards S. pneumoniae and P. aeruginosa.

Fig. 1. Effect of the route of administration on the induction of DH and protection against *Listeria* with fraction I and RNA. Groups of 6 mice were injected intraperitoneally (open symbols) or intracutaneously (closed symbols) with 10 μ g of fraction I (●, ○) or 10 μ g of RNA (■, □). Before injection both preparations were combined with 300 μ g DDA. Controls were injected with DDA (▼, ▼). After seven days DH was elicited by footpad challenge with 10^8 formalin-killed *Listeria* and read as the increase in footpad thickness 24 h later. After determination of the DH response all groups were challenged intraperitoneally with 10 LD₅₀ *Listeria monocytogenes*. The percentage of survivors was determined 14 days later. Significance (S) or insignificance (NS) of protection and DH is indicated by horizontal (→) and vertical (↑) arrows, respectively. *P* values were calculated with respect to DDA-controls.

Effect of enzymatic treatment on the induction of DH with fraction I.

As shown in Table 4, treatment with proteinase K destroyed the ability of fraction I to induce DH against Listeria, suggesting that DH was primarily directed against protein components of this fraction.

DISCUSSION

Results described in this paper clearly point to a dissociation between enhanced resistance and DH induced with subcellular fractions from Listeria and the adjuvant DDA. RNA induced enhanced

TABLE 3. Specificity of delayed hypersensitivity induced with fraction I

Immunization ^a of mice	Footpad challenge	Footpad swelling (0.1 mm $\pm$ SD)	P value ^b
10 μ g FI + DDA	<u>L. monocytogenes</u>	7.8 $\pm$ 2.5	< 0.001
DDA	<u>L. monocytogenes</u>	1.9 $\pm$ 0.5	-
10 μ g FI + DDA	<u>S. pneumoniae</u>	4.7 $\pm$ 1.0	0.01
DDA	<u>S. pneumoniae</u>	2.5 $\pm$ 1.6	-
10 μ g FI + DDA	<u>P. aeruginosa</u>	5.9 $\pm$ 2.4	0.02
DDA	<u>P. aeruginosa</u>	3.6 $\pm$ 1.2	-

^a Groups of 6-7 mice were injected intracutaneously with 10 μ g fraction I (FI) + DDA or with DDA alone. Seven days later they were challenged in the left footpad with 10⁸ formalin-killed L. monocytogenes serotype 4b, S. pneumoniae or P. aeruginosa. The increase in footpad thickness was determined after 24 h.

^b P values were calculated with respect to DDA controls.

TABLE 4. Effect of proteinase K-treatment on the induction of delayed hypersensitivity with fraction I

Treatment of fraction I ^a	Footpad swelling (0.1 mm $\pm$ SD)	P value ^b
none	7.8 $\pm$ 2.5	< 0.001
37°C, 2h	8.8 $\pm$ 2.4	< 0.001
proteinase K, 37°C, 1h	2.5 $\pm$ 0.7	NS
proteinase K, 37°C, 2h	2.4 $\pm$ 1.2	NS
no fraction I (DDA control)	1.9 $\pm$ 0.5	-

^a Fraction I was treated with proteinase K for 1 or 2 h at 37°C. Untreated fraction I and fraction I incubated for 2 h without the addition of enzyme were used as controls. After appropriate dilution and combination with DDA, preparations were injected i.c. in a dose equivalent to 10 μ g untreated fraction I, using 6-7 mice per group. Controls received DDA alone. Seven days later DH was elicited by footpad challenge with 10⁸ formalin-killed *Listeria*. The increase in footpad thickness 24 h after elicitation was used as a measure of DH.

^b P values were calculated with respect to DDA controls.
NS: Not significant ($P \geq 0.05$).

resistance against lethal *Listeria*-challenge (Fig. 1) but it did not induce or elicit DH (Table 1,2; Fig. 1). In contrast, a highly significant DH response was induced by immunization of mice with viable *Listeria* (Table 2). DH as well as protection against *Listeria* induced by immunization with viable bacteria are mediated by T-cells (Kaufmann, Simon and Hahn, 1979), but the relation between both phenomena of cell-mediated immunity is controversial. According to Kaufmann and Hahn (1982) both DH and enhanced resistance against *Listeria* could be transferred to normal recipient

mice with the same clone of Listeria-specific T-cells. On the other hand, Chen-Woan, Sajewski and McGregor (1985) showed that T-cells from rats immunized with viable Listeria could be separated into mutually exclusive subsets of T-cells mediating DH and T-cells mediating protection against Listeria. However, T-cells mediating DH were not completely irrelevant for the expression of protection, as a combination of T-cells mediating DH and T-cells mediating protection conferred a higher level of resistance than the protective subset alone (Chen-Woan et al, 1985). As RNA did not induce DH, T-cells mediating DH probably did not contribute to the expression of protection induced with RNA. Protection against Listeria induced with Listeria RNA might in fact be entirely due to non-immunological mechanisms because also heterologous ribosomal RNA (Antonissen et al., 1985) and poly(A). Poly(U) (Antonissen et al., manuscript in preparation) protected mice against Listeria infection. Furthermore, cyclosporin A, which inhibits the induction of T-cell dependent protection with viable Listeria (Schaffner, Douglas and Davis, 1983) did not affect the induction of enhanced in vivo killing of Listeria with RNA (van den Bosch et al., manuscript in preparation). Conceivably, RNA directly activates macrophages as suggested by Madraso and Cheers (1978) with respect to the mechanism of protection against Brucella abortus induced with poly (A). poly (U).

Also ribosomal RNA from Mycobacterium tuberculosis induced protection against homologous challenge without induction of DH (Youmans and Youmans, 1969, a, b). However, protection against M. tuberculosis induced with mycobacterial RNA was attributed to lymphocyte-mediated macrophage activation (Patterson and Youmans, 1970), and therefore might be closely related to the T-cell-dependent immunity induced with viable mycobacteria (North, 1974). Both DH and enhanced resistance against Listeria could be induced with fraction I, and this fraction also elicited DH in mice immunized with viable Listeria (Fig. 1; Table 1,2). However, DH was highest when fraction I was administered i.c., whereas i.p. injection was required for the induction of enhanced resistance against i.p. challenge (Fig. 1). Similar results with respect to the effect of

the route of administration were reported by Van der Meer et al. (1979), who studied the induction of DH and enhanced resistance with unfractionated killed Listeria and DDA.

The relative ineffectiveness of the i.p. route of administration for the induction of DH may have been due to restricted recirculation from the peritoneal cavity of Listeria specific T-cells which tend to accumulate in inflammatory foci (North and Spitalny, 1974). In this respect it should be noted that DDA causes a relatively mild inflammatory reaction leading to accumulation of predominantly macrophages/monocytes at the site of injection (Gordon, Prager and Carroll, 1980). Thus, i.p. administration of fraction I plus DDA might be more effective for the induction of protection against i.p. challenge than i.c. administration because in the former case Listeria specific T-cells and increased numbers of effector macrophages/monocytes are already present at the site of infection at the time of challenge.

On the other hand, limited space might restrict the intracutaneous accumulation of Listeria-specific T-cells and thus explain the relative effectiveness of i.c. administration for the induction of DH.

Previously we showed that also subcutaneous immunization with fraction I plus DDA protected mice against i.p. challenge with Listeria (Antonissen et al., 1985). Therefore the absence of protection against i.p. challenge after i.c. immunization was probably not exclusively due to the distance between the site of immunization and the site of challenge. Conceivably, i.c. immunization induces the proliferation of T-cells mediating DH, but not of T-cells mediating cellular resistance.

Attempts to induce DH against Listeria by subcutaneous immunization with fraction I plus DDA produced erratic results (data not shown).

Enhanced resistance induced with fraction I was nonspecific, as fraction I also protected mice against S. pneumoniae and P. aeruginosa (Antonissen et al., 1985). DH responses against S. pneumoniae and P. aeruginosa induced with fraction I were by themselves significant (Table 3), but at the same time signifi-

cantly lower than the DH response against Listeria. Therefore DH induced with fraction I appeared to be largely but not completely specific.

Proteinase-K sensitive proteins were identified as the components of fraction I which induced DH against Listeria (Table 4). However, the protective components of fraction I were proteinase K-resistant (Antonissen et al, 1985), indicating that protection induced with fraction I was due to non-protein components or to proteinase K-resistant peptides. Therefore DH and protection against Listeria induced with fraction I not only had different characteristics, but were also due to chemically different components of this fraction.

Our observations corroborate several other studies in which a dissociation between enhanced resistance and DH against Listeria was observed (Barry and Hinrichs, 1983; Kerckhaert, Hofhuis and Willers, 1977; Osebold, Pearson and Medin, 1974). In addition we identified the chemical nature of the DH-inducing components of fraction I. Consequently this preparation might be a suitable source for the purification of DH-inducing components from Listeria monocytogenes.

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CHAPTER 5

*PURIFICATION OF A DELAYED HYPERSENSITIVITY-INDUCING
PROTEIN FROM LISTERIA MONOCYTOGENES*

SUMMARY

Cell-envelope fragments were prepared from Listeria monocytogenes L242, serotype 4b. Delayed hypersensitivity (DH) inducing proteins were extracted with deoxycholate and separated into two fractions by filtration through a Sephacryl S-200 column equilibrated with deoxycholate buffer. The second peak eluting from the Sephacryl column was fractionated using ion exchange chromatography on a DEAE Sepharose CL-6B column in the presence of 6 M urea. A purified 20,400 MW protein which induced DH against Listeria was obtained by isocratic elution. Three other DH inducing fractions containing several protein bands were eluted by a gradient of potassium thiocyanate (KSCN) in urea buffer. Our results indicate that denaturing conditions can be employed for the fractionation and purification of DH inducing proteins from Listeria monocytogenes. In addition it is suggested that the procedure described might also be useful for the purification of other antigens involved in cellular immune reactions.

INTRODUCTION

Previously we described the separation of a crude ribosomal preparation from Listeria monocytogenes into cell-envelope fragments (fraction I) and purified ribosomes (fraction II). In combination with the adjuvant dimethyldioctadecylammonium bromide (DDA) both fractions protected mice against lethal Listeria infection. RNA was identified as the major protective component of fraction II, because its protective properties were destroyed by

RNase-treatment and could be reproduced with purified RNA. Enhanced resistance induced by fraction I was not affected by either RNase- or proteinase K-treatment. In combination with DDA, fraction I as well as RNA also protected mice against heterologous challenge, suggesting that protection induced with these preparations was primarily due to a nonspecific stimulation of host resistance (1).

Fraction I plus DDA induced delayed hypersensitivity (DH) against killed Listeria, and DH could also be elicited with fraction I in mice immunized with viable Listeria. DH could not be induced or elicited with RNA. In contrast to enhanced resistance, DH induced with fraction I appeared to be largely specific. Furthermore, proteinase K-treatment destroyed the ability of fraction I to induce DH against Listeria (Antonissen et al., submitted for publication). Therefore, DH induced with fraction I was apparently due to protein components present in this fraction. With this observation the purification of DH-inducing components from Listeria monocytogenes has become feasible. Experiments aimed at such a purification are the subject of this paper.

MATERIALS AND METHODS

Experimental animals

Male BALB/c mice were obtained from TNO, Central Institute for the Breeding of Laboratory Animals, Zeist, The Netherlands, and used at an age of 8-11 weeks.

Determination of delayed hypersensitivity

DH against Listeria was determined as described previously (Antonissen et al., submitted for publication) using modifications of published procedures (2).

In brief, mice were injected intracutaneously (i.c.) with preparations to be tested, suspended in phosphate buffered saline, pH 7.0 (PBS). Before injection materials were combined with 300 µg of the adjuvant DDA (Eastman Kodak Co., Rochester, N.Y., U.S.A.). Controls received DDA alone. After seven days DH was elicited by

injection of 10^8 formalin-killed Listeria into the left footpad and measured as the increase in footpad thickness 24 h later. Measurements were made using a semi-electronic footpad-meter (3). Results were analysed statistically using Student's t-test. Footpad swelling was considered to be significant at P-values <0.05 relative to DDA controls.

Preparation of fraction I

Cell-envelope fragments (fraction I) were prepared from Listeria monocytogenes L242, serotype 4b, as previously described (1). Fraction I contained 37% protein, 16% phospholipids, and 1% RNA (1). Preliminary experiments indicated that DH inducing proteins could be extracted from fraction I with deoxycholate (4) or with 6 M urea. Therefore, it was decided to attempt purification of DH inducing proteins by gel filtration in the presence of deoxycholate and ion exchange chromatography in 6 M urea.

Extraction with deoxycholate

Fraction I (100 mg; 5 mg/ml in PBS) was dialysed overnight against 10 mM Tris-HCl, pH 7.5, containing 0.5% sodium deoxycholate and 1 mM 2-mercaptoethanol (DOC-buffer). Insoluble material was removed by centrifugation (30 min 31,000xg), washed once with DOC-buffer, dialysed against PBS for 2 days and stored at -70°C (DOC-sediment). The supernatant from the first centrifugation step (DOC-supernatant) was used for further fractionation.

Gel filtration

The DOC-supernatant fraction was supplemented with 100 mM 2-mercaptoethanol, heated for 30 min at 37°C , cooled to 4°C and applied to a 90x2.6 cm column of Sephacryl S-200 (Pharmacia P-L Biochemicals, Uppsala, Sweden), equilibrated with DOC-buffer. The eluate was scanned at 280 nm and collected in fractions of 2.3 ml. Peaks were pooled, concentrated and transferred to PBS.

Concentration and buffer exchange

In general, a 50 ml Amicon concentration cell fitted with a Millipore PTGC membrane was used for concentration and buffer exchange. Buffer exchange was achieved by several steps of concentration and dilution with the buffer to which the sample had to be transferred. Fractions containing deoxycholate were dialysed for 24-48 h against 10 mM Tris-HCl, pH 7.5 (Tris-buffer) to remove most of the deoxycholate. Residual deoxycholate was removed by several steps of concentration and dilution with Tris-buffer, using the Amicon concentration cell. The removal of deoxycholate was monitored by mixing a drop of concentrate with 1 M $MgCl_2$, which precipitates deoxycholate. Subsequently fractions were transferred to PBS, using the concentration/dilution procedure already described.

Ion exchange chromatography

One of the fractions obtained by gel filtration was selected for further purification using ion exchange chromatography in the presence of 6 M urea (Bio-Rad electrophoresis quality). DEAE-Sephacrose CL-6B (Pharmacia P-L Biochemicals, Uppsala, Sweden) was equilibrated three times with 0.5 M Tris-HCl, pH 7.5). Subsequently it was treated with three cycles of end buffer (6 M urea, 1 M KSCN, 1 mM 2-mercaptoethanol, 50 mM Tris-HCl, pH 7.5) and starting buffer (6 M urea, 1 mM 2-mercaptoethanol, 50 mM Tris-HCl, pH 7.5). A 15x1.6 cm column was prepared from precycled ion exchanger and equilibrated with starting buffer. The material to be separated was treated with 100 mM 2-mercaptoethanol (30 min, 37°C) and transferred to starting buffer.

After removal of insolubles by centrifugation (30 min. 31,000 x g), the material was applied to the column. After two column volumes of starting buffer, the column was developed with a linear gradient (2 x 150 ml) of 0-1 M KSCN in starting buffer. The eluate was scanned at 280 nm and fractions of 0.9 ml were collected. Pooled fractions were concentrated and transferred to PBS.

Chemical analyses

Protein was determined by the Lowry method as modified by Hartree (5) using bovine serum albumin as the standard. Fractions were analysed qualitatively on 11% polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS-PAGE) (6). The Pharmacia low molecular weight electrophoresis calibration kit (Pharmacia Fine Chemicals AB, Uppsala, Sweden) was used for molecular weight estimation.

RESULTS

Extraction with deoxycholate

Approximately 50% of the protein present in fraction I was solubilised by extraction with deoxycholate. The extraction procedure appeared to be partially selective, although both the extract and the sediment left after extraction contained a large number of different proteins (Fig. 1).

Gel filtration

Two peaks (S1 and S2) were obtained upon gel filtration of a deoxycholate extract of fraction I (Fig. 2). In combination with DDA, both fractions induced DH against Listeria (Table 1). As fraction S2 appeared to be less complex than fraction S1, the former was selected for further purification. This was done using pooled material from two batches, which were essentially identical as assessed by SDS-PAGE (not shown).

Fig. 1. SDS-PAGE analysis of cell-envelope fragments (fraction I) isolated from Listeria monocytogenes and fractions obtained by deoxycholate extraction, gel filtration and ion exchange chromatography. (a: fraction I; b: DOC-sediment; c: DOC-supernatant; d, e: Sephacryl fractions S1, S2; f, g, h, i: DEAE fractions D1, D2, D3, D4; j: Pharmacia low molecular weight electrophoresis calibration kit).

Fig. 2. Gel filtration of the DOC-supernatant fraction of fraction I on a Sephacryl S-200 column in DOC-buffer.

TABLE 1. Induction of delayed hypersensitivity against *Listeria monocytogenes* with fractions obtained by gel filtration of a deoxycholate extract of fraction I.

Preparation ^a	Footpad swelling (0.1 mm) $\pm$ SD	P value ^b
S1 + DDA	13.3 $\pm$ 1.4	< 0.001
S2 + DDA	8.3 $\pm$ 1.8	< 0.001
fraction I + DDA	8.7 $\pm$ 1.7	< 0.001
DDA	1.2 $\pm$ 1.1	-

^a Groups of six mice were injected i.c. with fractions to be tested in a dose containing 2 μ g protein. Positive controls received 10 μ g (dry weight) fraction I. Before injection all preparations were combined with 300 μ g DDA. Negative controls received DDA alone. After seven days DH was elicited by footpad challenge with 10^8 formalin-killed *Listeria* and read as the increase in footpad thickness 24 h later.

^b P values were calculated relative to DDA controls.

Ion exchange chromatography

Ion exchange chromatography of fraction S2 resulted in the elution profile presented in figure 3. The eluate was fractionated as indicated in the figure. Fraction D1 was eluted with starting buffer and found to be homogeneous by SDS-PAGE (MW 20,400). Upon gradient elution fractions were obtained with a gradually decreasing content of low MW proteins. Fractions D3 and D4 contained a 67,000 MW protein which was not found in fractions D1 and D2 (fig. 1). All fractions obtained by ion exchange chromatography induced DH against Listeria (table 2).

Fig. 3. Ion exchange chromatography of Sephacryl fraction S2 on a DEAE Sepharose CL-6B column in 6M urea buffer. (---KSCN gradient).

TABLE 2. Induction of delayed hypersensitivity against *Listeria* monocytogenes with fractions obtained by ion exchange chromatography of fraction S2.

Preparation ^a	Foodpad swelling (0.1 mm) $\pm$ SD	<u>P</u> value ^b
D1 + DDA	6.4 $\pm$ 3.6	0.01
D2 + DDA	10.9 $\pm$ 1.4	< 0.001
D3 + DDA	8.6 $\pm$ 2.8	< 0.001
D4 + DDA	8.3 $\pm$ 2.8	< 0.001
fraction I + DDA	7.1 $\pm$ 3.9	0.01
DDA	2.3 $\pm$ 0.1	-

^a Preparations were tested in a dose containing 2 μ g protein as described in the legend to Table 1. Positive controls received 10 μ g (dry weight) fraction I.

^b P values were calculated relative to DDA controls.

DISCUSSION

Experiments described in this paper resulted in the purification of a 20,400 MW protein which induced DH against *Listeria*. Despite the rather drastic conditions used in the ion exchange procedure all fractions obtained by ion exchange chromatography induced DH.

In this respect it should be noted that the DH response is a T-cell mediated reaction. In contrast to the interaction between antigen and antibody which is rather sensitive towards denaturation of the antigen, recognition of protein-antigens by T-cells appears to depend mainly on the amino acid sequence of the antigen (7).

Therefore the purification procedure described might also be useful for the purification of other antigens involved in cellular

immune reactions.

Apparently the 20,400 MW protein was not the only antigen involved in the induction of DH against Listeria, because DH could also be induced with fraction D4 which lacked the 20,400 MW component. Nevertheless the availability of purified antigen should facilitate the study of the DH response against Listeria which thusfar has been done using rather complete antigenic mixtures such as complete killed bacteria (2) or concentrated and dialysed culture filtrate (8).

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CHAPTER 6

TRANSFER OF ENHANCED RESISTANCE AGAINST
LISTERIA MONOCYTOGENES INDUCED WITH RIBOSOMAL
RNA AND THE ADJUVANT DIMETHYLDIOCTADECYLAMMONIUM BROMIDE

SUMMARY

In this study we investigated the mechanism of enhanced resistance against Listeria monocytogenes induced with Listeria ribosomal RNA and the adjuvant dimethyldioctadecylammonium bromide (DDA). Mice immunized with DDA alone (which were not protected against Listeria-infection) were used as negative controls. Mice immunized with RNA plus DDA were found to have an increased capacity to mobilize polymorphonuclear leukocytes (PMNs) and macrophages to the inflamed peritoneal cavity compared to mice immunized with adjuvant alone. Intraperitoneal (i.p.) inflammation was induced by injection of the sterile irritant proteose peptone. The protective capacity of various cell-populations was investigated by i.p. transfer of cells to normal recipient mice and concomitant challenge of recipient animals with a lethal dose of viable Listeria. Inflammatory PMNs as well as inflammatory macrophages from mice immunized with RNA plus DDA protected recipient animals against listeriosis whereas cells from mice immunized with DDA alone failed to do so. Therefore, enhanced mobilization as well as activation of PMNs and macrophages may have contributed to the expression of protection against Listeria monocytogenes induced with RNA plus DDA.

INTRODUCTION

Listeria monocytogenes is a Gram-positive bacterium which behaves, when it has entered the body, as a facultative intracellular parasite. Its natural host cell is the macrophage in

which the organism is able to survive and even to multiply (World Health Organization Scientific Group, 1973). Expression of protection against Listeria induced by immunization with a sublethal dose of viable bacteria is generally considered to require T-cell dependent macrophage activation (Mackanness, 1962, 1969; North and Spitalny, 1974). However, recently it was suggested that increased numbers of bactericidal phagocytes accumulating at sites of Listeria-infection rather than macrophage activation (i.e., increased bactericidal activity per cell) might be of crucial importance for the expression of immunity against Listeria (Czuprynski et al., 1984).

In the latter study the number of polymorphonuclear leukocytes (PMNs) and macrophages accumulating in the peritoneal cavity of Listeria-immune mice after induction of intraperitoneal inflammation by injection of killed Listeria or the sterile irritant proteose peptone was found to be considerably higher than the number of cells which accumulated in the peritoneal cavity of non-immunized mice. However, equal numbers of phagocytes from Listeria-immune and non-immunized mice did not differ in their capacity to kill Listeria in vitro, i.e., inflammatory PMNs and inflammatory macrophages from either source killed Listeria, whereas resident peritoneal macrophages failed to do so (Czuprynski et al., 1984).

Previously we reported that mice could be immunized against lethal Listeria infection with ribosomal RNA isolated from Listeria monocytogenes. RNA had to be combined with the adjuvant dimethyldioctadecylammonium bromide (DDA) to induce protection (Antonissen et al., 1985). Whereas considerable progress has been made with the elucidation of the mechanism of protection induced by immunization with viable Listeria (for review, see Hahn and Kaufmann, 1981; Kaufmann et al., 1985; Chen-Woan et al., 1985), relatively little is known about the mechanism by which Listeria-RNA (Antonissen et al., 1985) or other non-viable Listeria-vaccines (Rodriguez et al., 1974; Van der Meer et al., 1979; Willers et al., 1979) provide protection. In this paper we report that mice immunized with RNA plus DDA have an increased capacity to mobilize

phagocytes to the inflamed peritoneal cavity as compared to mice treated with DDA alone. Results from passive transfer-experiments indicated that inflammatory PMNs as well as inflammatory macrophages from mice treated with RNA plus DDA are activated compared to cells from adjuvant-treated mice.

MATERIALS AND METHODS

Experimental animals

Male BALB/c mice were obtained from TNO, Central Institute for the Breeding of Laboratory Animals, Zeist, The Netherlands, and used at an age of 8-11 weeks.

Bacteria

Listeria monocytogenes L242, serotype 4b, was kindly provided by H. van Dijk, Department of Immunology, University of Utrecht, The Netherlands. Mouse-passaged bacteria were grown for 16 h at 37°C as described previously (Antonissen et al., 1981). Culture bottles were fitted with 0.22 μ m filters to release pressure. Bacteria were recovered by centrifugation, washed three times with phosphate-buffered saline pH 7.0 (PBS) and stored in small portions at -70°C in PBS. The concentration of viable bacteria in thawed stock suspensions was estimated by plating on Trypticase Soy Agar (BBL Microbiology Systems, Cockeysville, MD, U.S.A.) To determine the 50% lethal dose (LD₅₀), 0.5 ml of serial dilutions of a stock suspension of bacteria were injected intraperitoneally (i.p.) into mice. Deaths were recorded for 14 days. The LD₅₀ was calculated according to Reed and Muench (1938). For the batch used in this study the LD₅₀ was 2.6×10^6 colony forming units.

Ribosomal RNA; DDA

Ribosomal RNA from Listeria monocytogenes was prepared as described previously (Antonissen et al., 1985) using procedures originally developed for the purification of rRNA from Pseudomonas aeruginosa (Gonggrijp et al., 1980, 1981). The purified RNA con-

tained less than 1% residual protein. It was stored at -70°C in PBS.

DDA (Eastman Kodak Co., Rochester, N.Y.) was suspended in PBS by brief sonication immediately before use. The suspension contained 3 mg DDA per ml PBS.

Immunization with RNA

Mice were injected i.p. with 10 μg RNA plus 300 μg DDA in 0.2 ml PBS. Controls received DDA alone (Antonissen et al., 1985).

Immunization with viable *Listeria*

Mice were immunized i.p. with 0.1 LD₅₀ *Listeria* (Czuprynski et al., 1984). Each mouse received 0.2 ml *Listeria*-vaccine, which was prepared by appropriate dilution of a stock suspension of bacteria with PBS. Controls received 0.2 ml PBS.

Collection of resident and inflammatory peritoneal cells

Resident and inflammatory peritoneal cells were collected using modifications of procedures described by Czuprynski et al. (1984). To obtain inflammatory cells, mice were injected i.p. with 1 ml 10% proteose peptone (Oxoid Ltd., Basingstoke, Hants, England). At 4, 24 or 48 h after injection of proteose peptone mice were killed by ether overdose. Inflammatory cells were collected by peritoneal lavage with 4 ml cold Hanks balanced salt solution (HBSS). Cells were recovered by centrifugation (300xg for 10 min.), washed 3 times with HBSS containing 0.25% bovine serum albumin (HBSA), and counted with a haemocytometer. Differential counts were performed on smears prepared from cells suspended in newborn calf serum. Smears were stained with May Grunwald-Giemsa, and scored for PMNs, lymphocytes and monocytes/macrophages (macrophages). The same procedure was used for the collection of resident peritoneal cells, except that the injection of proteose peptone was omitted.

Separation of polymorphonuclear leukocytes and macrophages

PMNs and macrophages were separated using a discontinuous gradient of Nycodenz (Nyegaard & Co., Oslo, Norway). A stock

solution of 90% (v/v) Nycodenz containing 10 mM EDTA was prepared by 9 to 1 dilution of isotonic Nycodenz (as supplied by the manufacturer) with 100 mM EDTA, pH 7.0. Solutions of 70% and 53% Nycodenz were prepared by dilution of 90% Nycodenz with HBSS containing 10 mM EDTA (HEDTA). Peritoneal cells were collected with HEDTA, sedimented by centrifugation and suspended in 70% Nycodenz to a maximum of 1.25×10^7 cells/ml. Three-layer gradients were prepared in 50 ml conical polypropylene tubes (type 2070F, Falcon Plastics, Oxnard, California, USA) and consisted of 12 ml HEDTA, 16 ml 53% Nycodenz and 12 ml 70% Nycodenz containing the cells.

Gradients were spun at 4°C for 30 min at 400xg. Macrophages and PMNs were collected from the HEDTA/53% Nycodenz and the 53%/70% Nycodenz interface, respectively, and washed three times with HBSS. Macrophages and PMNs were obtained in 80-92% and 87-93% purity, respectively. The yield of purified cells was approximately 40%. More than 90% of the cells were viable as measured by trypan blue exclusion.

Transfer of protection

The protective capacity of various cell populations was determined by transfer of cells to normal recipient mice and concomitant challenge of recipient animals with a lethal dose of viable Listeria. Each mouse was injected i.p. with the appropriate number of cells and approximately 4.5 LD₅₀ Listeria in 0.5 ml PBS. Cells were combined with the Listeria-challenge by resuspension of sedimented cells in a diluted stock suspension of bacteria in PBS. Controls received the Listeria-challenge without cells. The percentage of survivors was determined after 14 days.

Statistical evaluation

Significance levels for protection were determined by the Fisher exact test (Bradley, 1968). Differences were considered to be significant at P values < 0.05.

RESULTS

Correlation of protection induced with RNA plus DDA with enhanced phagocyte inflammatory responses

Previously we reported that mice were highly protected against Listeria-infection seven days after i.p. injection of 10 μ g RNA plus 300 μ g DDA, whereas mice treated with DDA alone, RNA alone, or PBS died after challenge with Listeria (Antonissen et al., 1985). To investigate whether protection induced with RNA plus DDA was associated with enhanced phagocyte inflammatory responses, groups of 28 mice were immunized i.p. with 10 μ g RNA plus 300 μ g DDA. Controls received DDA alone. To obtain a positive control for increased inflammatory responsiveness, mice immunized with 0.1 LD₅₀ viable Listeria were included in the experiment (Czuprynski et al., 1984). Mice treated with PBS were used as negative controls. Seven days after various treatments seven mice of each group were used to collect resident peritoneal cells. The remaining mice were injected i.p. with 1 ml 10% proteose peptone to obtain inflammatory peritoneal cells. Inflammatory cells were collected at 4, 24, and 48 h after injection of proteose peptone, using seven mice of each group at each time. Total and differential counts were performed on pooled cells from seven mice. The mean result of two experiments is given in Fig. 1A. The most striking effect of immunization of mice with RNA plus DDA was the massive number of PMNs collected 4 h after injection of proteose peptone which was more than fourteen times as high as the number of resident peritoneal PMNs and more than three times as high as the number of inflammatory PMNs collected from DDA-pretreated mice. Four hours exudates from mice immunized with RNA plus DDA also contained elevated numbers of macrophages compared to resident peritoneal cells as well as compared to 4 h exudates from DDA-immunized mice. Therefore, protection induced with RNA plus DDA was positively correlated with enhanced inflammatory responses of PMNs and macrophages. Differences between mice treated with RNA plus DDA and DDA-treated mice with respect to the number of inflammatory peritoneal cells appeared to be of a transient

nature, as mice subjected to either treatment contained approximately equal numbers of peritoneal cells (predominantly macrophages) at 48 h after injection of proteose peptone. As expected, mice immunized with viable Listeria mobilized increased numbers of PMNs and macrophages to the inflamed peritoneal cavity compared to mice treated with PBS.

Transfer of protection with resident and inflammatory peritoneal cells

To investigate the contribution of resident and inflammatory peritoneal cells to the expression of protection against Listeria, the cell populations described in the previous section (Fig. 1A) were transferred to normal recipient mice using groups of six animals for each cell population to be tested. Each recipient mouse received one seventh part of pooled cells from seven mice, resulting in a donor to acceptor ratio of 1:1. The Listeria-challenge (approximately 4.5 LD₅₀ per mouse) was incorporated in the suspension of cells to be transferred. Recipient mice were injected i.p. with 0.5 ml of a mixture of cells and Listeria in 0.5 ml PBS. Controls received the Listeria-challenge without cells. The mean result of two experiments is presented in Fig. 1B. Resident as well as 4 and 48 h inflammatory cells collected from mice pretreated with RNA plus DDA conferred a significantly higher level of protection to recipient mice than the corresponding cell populations collected from mice pretreated with DDA. However, protection conferred by 4 and 48 h inflammatory cells was higher and less variable than the protection conferred by resident peritoneal cells. All cell populations collected from mice immunized with viable Listeria conferred a high level of protection to Listeria-infection compared to cells collected from PBS-pretreated mice. Forty five out of 48 mice (94%) receiving the Listeria-challenge but no cells died from listeriosis (data not shown).

Contribution of inflammatory macrophages and inflammatory PMNs to the expression of protection induced with RNA plus DDA

At 48 h after the injection of proteose peptone peritoneal

FIG. 1. Modulation of phagocyte inflammatory responses with RNA plus DDA and transfer of protection with resident and inflammatory peritoneal cells.

Legends to Fig. 1

- A Groups of seven mice were injected i.p. with 10 μ g RNA plus DDA or with DDA alone. Mice immunized i.p. with 0.1 LD₅₀ viable Listeria and mice treated with PBS were used as positive and negative controls, respectively. Seven days later resident peritoneal cells (-) were collected by peritoneal lavage. Inflammatory cells were obtained by i.p. injection of proteose peptone seven days after various pretreatments and collection of cells 4, 24 or 48 h later (mean result of two experiments).
- B The protective capacity of resident and inflammatory cells as described in Fig. 1A was investigated by i.p. transfer to groups of six normal recipient mice, using a donor to acceptor ratio of 1:1. Before transfer donor cells were combined with 4.5 LD₅₀ Listeria monocytogenes. The percentage of survivors was determined 14 days later. Results represent the mean of two experiments + SD. SD values not indicated were zero. Significance levels of protection conferred by cells derived from RNA plus DDA treated mice and by cells from Listeria immunized mice were calculated relative to DDA and PBS controls, respectively. (S: significant; NS: not significant).

exudates from mice immunized with RNA plus DDA or DDA alone contained approximately equal numbers of cells which were predominantly macrophages (Fig. 1A). However, only cells collected from mice immunized with RNA plus DDA protected recipient animals against listeriosis (Fig. 1B). Therefore, protection conferred by 48 h inflammatory cells from mice immunized with RNA plus DDA was most likely due to activated macrophages.

To investigate whether protection conferred by 4 h inflammatory cells from mice immunized with RNA plus DDA was due to PMNs or macrophages, these cells were separated by gradient-centrifugation. Groups of six normal recipient mice were injected i.p. with 10^7 purified PMNs or 10^7 purified macrophages from mice immunized with RNA plus DDA or with the same number of purified cells from mice immunized with DDA alone. Before transfer cells were combined with a lethal dose of viable Listeria as already described. Controls consisted of groups of six mice treated with 10 μ g RNA plus DDA or DDA alone and a group of six mice not subjected to any treatment at all. Controls received the Listeria-challenge but no cells. The mean result of two experiments is presented in Fig. 2. PMNs from mice pretreated with RNA plus DDA protected recipient mice against listeriosis, but PMNs from DDA-pretreated mice and macrophages from either source did not. Therefore, protection conferred by 4 h inflammatory cells from mice immunized with RNA plus DDA appeared to be largely due to PMNs which were activated compared to PMNs from mice immunized with DDA alone.

DISCUSSION

Data presented in this paper indicate that mice immunized with RNA plus DDA had an increased capacity to mobilize phagocytes (PMNs as well as macrophages) to the inflamed peritoneal cavity compared to mice treated with DDA alone. The most striking effect of treatment of mice with RNA plus DDA was the massive number of PMNs which could be collected 4 h after i.p. injection of proteose peptone. In fact, mice treated with RNA plus DDA mobilized even more PMNs to the inflamed peritoneal cavity than mice immunized

FIG. 2. Transfer of protection with purified inflammatory PMNs and macrophages.

Mice were injected i.p. with 10 μ g RNA plus 300 μ g DDA or with DDA alone. After seven days mice were injected i.p. with 1 ml 10% proteose peptone. Inflammatory cells were collected 4 h later and separated by gradient centrifugation.

Groups of six normal mice were injected i.p. with 10^7 purified PMNs or macrophages (M ϕ) in combination with 4.5 LD₅₀ viable *Listeria*. Groups of six mice immunized seven days previously with RNA plus DDA or DDA alone and a group of mice having received no treatment at all (-) were used as controls. Controls received the *Listeria*-challenge but no cells. The percentage of survivors was determined 14 days later. Results represent the mean of two experiments + SD. P values were calculated relative to untreated controls (-). (S: significant; NS: not significant).

with viable Listeria (Fig. 1A). Furthermore, PMNs from mice treated with RNA plus DDA were found to be activated compared to cells from mice treated with DDA alone (Fig. 2). To our knowledge this is the first report implicating the phenomenon of PMN-activation in the expression of protection against Listeria monocytogenes. With few exceptions (Czuprynski et al., 1984; McGowan et al., 1983) the contribution of PMNs to the expression of protection against Listeria seems to have been largely overlooked, possibly because these cells rapidly disappear after entering a site of inflammation (Fig. 1A; Czuprynski et al., 1984). Transfer experiments with 48 h inflammatory cells indicated that in addition to PMN-activation, activation of macrophages contributed to the expression of protection with RNA plus DDA (Fig. 1A, B). In this study the protective capacity of various cell-populations was investigated by passive transfer rather than by in vitro bactericidal assays because the latter tend to produce highly conflicting results (for review, see Czuprynski et al., 1984) and may not reflect differences in antibacterial resistance observed in vivo (Czuprynski et al., 1984; Gonggrijp et al., 1985).

As enhanced inflammatory responsiveness and activation of phagocytes (at least of macrophages) appear to be common features of protection induced with RNA plus DDA and protection induced by immunization with viable Listeria (Mackanness, 1962, 1969; Czuprynski et al., 1984; North and Spitalny, 1974) one might speculate that the mechanism of protection induced with RNA plus DDA is similar if not identical to the mechanism of protection induced by immunization with viable Listeria. However, several observations point to the contrary. In contrast to viable Listeria, RNA plus DDA did not induce delayed hypersensitivity against Listeria-antigens (Antonissen et al., 1986). Furthermore, cyclosporin A, which inhibits the induction of T-cell dependent immunity against Listeria (Schaffner, Douglas and Davis, 1983), did not inhibit the induction of protection with RNA plus DDA (Van den Bosch et al., 1985). Therefore, protection induced with RNA plus DDA was probably due to a T-cell independent rather than to a T-cell dependent modulation of phagocyte functions which might

also explain why the overall protection conferred by cell populations from mice immunized with viable Listeria was higher than the overall protection conferred by cells from mice immunized with RNA plus DDA (Fig. 1A, 1B).

Finally, our observations with respect to the effect of RNA plus DDA on phagocyte inflammatory responses corroborate several other reports indicating that enhanced inflammatory responsiveness is a highly conserved feature of various types of resistance to listeriosis including nonspecifically enhanced resistance (i.e., protection induced by preparations unrelated to Listeria monocytogenes) (Fauchère et al., 1982), genetically determined natural resistance (Czuprynski et al., 1985; Stevenson et al., 1981), and protection induced by immunization with viable Listeria (Czuprynski et al., 1984).

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CHAPTER 7

*PROTECTION AGAINST LISTERIA MONOCYTOGENES INDUCED WITH
NATURAL AND SYNTHETIC RNA COMBINED WITH THE ADJUVANT
DIMETHYLDIOCTADECYLAMMONIUM BROMIDE:
CORRELATION WITH ENHANCED PHAGOCYTE INFLAMMATORY RESPONSES*

SUMMARY

In this study we investigated the effect of synthetic RNA's and polyanions on the resistance of mice to infection with Listeria monocytogenes to gain further insight with respect to the mechanism of protection against listeriosis induced with ribosomal RNA from Listeria monocytogenes. All substances were tested in combination with the adjuvant dimethyldioctadecylammonium bromide (DDA) which is required for the induction of protection with Listeria-RNA. From a number of substances tested, only poly A:U approached the protective capacity of Listeria-RNA.

Mice treated with protective RNA's (poly A:U and Listeria-RNA) plus DDA had an increased capacity to mobilize polymorphonuclear leukocytes to the inflamed peritoneal cavity as compared to mice treated with a non-protective RNA (poly I:C) plus DDA or mice treated with adjuvant alone. Therefore, the induction of a state of enhanced inflammatory responsiveness appeared to be a crucial factor with respect to the mechanism of protection induced with RNA plus DDA.

INTRODUCTION

Previously we reported that mice could be protected against lethal Listeria infection with ribosomal RNA from Listeria monocytogenes combined with the adjuvant dimethyldioctadecylammonium bromide (DDA) (Antonissen et al., 1985). Mice immunized with RNA plus DDA had an increased capacity to mobilize phagocytes

(in particular polymorphonuclear leukocytes (PMNs)) to the inflamed peritoneal cavity which compared favourably with the enhanced inflammatory responsiveness of mice immunized with viable Listeria (Chapter 6). Therefore, enhanced inflammatory responsiveness appeared to be a common feature of the effector phase of protection against Listeria induced by immunization with viable Listeria and enhanced resistance induced with RNA plus DDA. To gain further insight with respect to the mechanism of protection induced with RNA plus DDA, it was decided to investigate whether Listeria-RNA could be substituted by synthetic RNA's or other polyanions. From a number of substances tested only poly A:U approached the protective capacity of Listeria-RNA. Protection induced with poly A:U was associated with enhanced inflammatory responsiveness as previously described with respect to the enhanced resistance induced with Listeria-RNA (Chapter 6).

MATERIALS AND METHODS

Experimental animals

Male BALB/c mice were obtained from TNO, Central Institute for the Breeding of Laboratory Animals, Zeist, The Netherlands, and used at an age of 8-11 weeks.

Bacteria

Listeria monocytogenes L242, serotype 4b, was obtained and cultured as described previously (Antonissen et al., 1985). Challenge inocula were prepared from stock suspensions of bacteria kept at -70°C in phosphate buffered saline, pH 7.0 (PBS). The mean lethal dose (LD_{50}) of the batch of bacteria used was 2.6×10^6 colony forming units for the intraperitoneal (i.p.) route of infection.

Polyribonucleotides and other polyanions; DDA

Ribosomal RNA from Listeria monocytogenes was prepared as described previously (Antonissen et al., 1985). The synthetic double-stranded RNA poly A:U was purchased from Boehringer

Mannheim (Mannheim, W. Germany). Poly I:C, poly A, poly U, and the random copolymers poly (A, U) and poly (I, C) were purchased from Pharmacia P-L Biochemicals (Uppsala, Sweden). Synthetic polynucleotides were dissolved in PBS by heating to 50°C, allowed to cool to room temperature, and stored at -70°C. The polyanionic dyes poly B-411 and poly R-478 were obtained from Janssen Chimica (Beerse, Belgium).

Lipoteichoic acid (LTA) from L. monocytogenes L242 was prepared as follows: Bacteria were suspended in 0.9% NaCl, frozen at -20°C, and disrupted using a cold Hughes pressure cell. The broken cell suspension was supplemented with 10 mM MgCl₂ and treated with DNase to reduce viscosity (Gonggrijp et al., 1980). After DNase-treatment and addition of EDTA to a concentration of 20 mM, the preparation was centrifuged (16,000 x g, 30 min) to remove cell walls and unbroken cells.

The supernatant solution containing fragmented membranes was lyophilized and delipidated by extraction with chloroform-methanol (Coley et al., 1975). Crude LTA was extracted with hot (60°C) aqueous phenol (Coley et al., 1975) and digested with RNase and proteinase K (Antonissen et al., 1985). Enzymes were removed by extraction with phenol at room temperature (Coley et al., 1975). The aqueous phase of the second phenol extraction was dialysed against water and lyophilized. The lyophilized material was fractionated using a column of Sephacryl S-400 equilibrated with 0.2 M ammonium acetate. Three phosphorus containing fractions were obtained, the first of which was identified as LTA. It contained hexoses, fatty acid residues, glycerol and phosphate in molar ratio's of 0.39, 0.14, 1.04, and 1.00 to phosphate, respectively. Hexoses were determined using the anthrone method as described by Herbert et al. (1971), with glucose as the standard. Fatty acid residues were converted to methyl esters with BF₃-methanol (Morrison and Smith, 1964) and estimated colorimetrically (Snyder and Stephens, 1959) using glycerol tributyrat as the standard. Glycerol was determined enzymatically (Hagen and Hagen, 1962) after acid hydrolysis of samples and conversion of glycerol phosphate to glycerol with alkaline phosphatase (Decker et al., 1972).

Phosphate was determined as described by Rouser et al. (1966). DDA (Eastman Kodak Co., Rochester, N.Y.) was suspended in PBS by brief sonication immediately before use. The suspension contained 3 mg DDA per ml PBS.

Immunization and challenge of mice

Procedure for the immunization and challenge of mice were as described previously (Antonissen et al., 1985). Briefly, preparations to be tested were appropriately diluted with PBS and injected i.p. into mice. Before injection preparations were combined with 300 µg of the adjuvant DDA. Controls received DDA alone. After seven days mice were challenged i.p. with 10 LD₅₀ Listeria monocytogenes suspended in 0.5 ml PBS. The percentage of survivors was determined 14 days after challenge.

Assay for enhanced inflammatory responsiveness

The total and differential score of resident and 4 h inflammatory peritoneal cells was determined seven days after various treatments as described previously (Chapter 6; Czuprynski et al., 1984). Briefly, resident peritoneal cells were obtained by peritoneal lavage of mice with 4 ml cold Hanks' balanced salt solution. Inflammatory peritoneal cells were collected 4 h after i.p. injection of 1 ml 10% proteose peptone. Differential counts were performed on smears prepared from cells suspended in newborn calf serum after staining with May Grunwald-Giemsa.

Statistical evaluation

Significance levels for protection were determined by the Fisher exact test (Bradley, 1968). The effects of various treatments on phagocyte inflammatory responses were analysed statistically using Wilcoxon's rank-sum test (Bradley, 1968). For both tests differences were considered to be significant at P values < 0.05.

RESULTS

Induction of protection with synthetic RNA's and polyanions

To investigate whether protection against Listeria could be induced with synthetic RNA's, mice were injected i.p. with poly A:U or poly I:C combined with the adjuvant DDA. Negative controls received DDA alone. Positive controls received 10 μ g Listeria-RNA plus DDA. Seven days later all groups were challenged i.p. with 10 LD₅₀ Listeria monocytogenes. As shown in Table 1, mice treated with poly A:U plus DDA were protected against Listeria infection, whereas mice treated with poly I:C plus DDA or DDA alone died from listeriosis. Other substances which were non-protective in the dose in which they were tested (which is given parenthetically) were: poly (I, C) (10 μ g, 100 μ g), poly B-411 (10 μ g, 100 μ g), poly R-478 (10 μ g, 100 μ g), and Listeria-LTA (10 μ g). For further characterization of the protection induced with poly A:U, we also tested poly A alone, poly U alone, and the random copolymer poly (A, U). As shown in Table 2, all of these substances failed to induce protection against Listeria.

Correlation of protection induced with synthetic RNA's with phagocyte inflammatory responses

Groups of 12 mice were injected i.p. with 100 μ g poly A:U plus DDA, 100 μ g poly I:C plus DDA, 10 μ g Listeria-RNA plus DDA or DDA alone. Seven days later six mice of each group were used to determine the number of resident peritoneal PMNs and macrophages. To investigate the effect of various treatments on phagocyte inflammatory responses, the remaining mice were injected i.p. with 1 ml 10% proteose peptone.

Inflammatory peritoneal cells were collected 4 h after the injection of proteose peptone. As shown in Fig. 1, the effects of treatment of mice with 100 μ g poly A:U plus DDA were similar to the effects of 10 μ g Listeria-RNA plus DDA. Upon i.p. injection of proteose peptone mice treated with RNA plus DDA or poly A:U plus DDA mobilized increased numbers of PMNs to the inflamed peritoneal cavity as compared to mice treated with DDA alone. Furthermore,

TABLE 1: Induction of protection against *Listeria* with synthetic RNA

Treatment of mice ^a	Survival ^b	P value ^c
10 µg poly A:U + DDA	4/6	0.01
100 µg poly A:U + DDA	16/18	<0.001
10 µg poly I:C + DDA	0/6	NS ^d
100 µg poly I:C + DDA	0/12	NS ^d
10 µg RNA + DDA	12/12	<0.001
DDA	1/18	-

a Mice were i.p. injected with poly A:U or poly I:C. Positive controls received RNA from *Listeria monocytogenes*. Before injection all preparations were combined with 300 µg DDA. Negative controls received DDA alone. Seven days later all groups were challenged i.p. with 10 LD₅₀ *Listeria monocytogenes*. The number of survivors was determined 14 days after challenge.

b Number of survivors/number of mice used.

c P values were calculated relative to DDA controls.

d NS: not significant ($P \geq 0.05$).

TABLE 2: Induction of protection with single-stranded polyribonucleotides

Treatment of mice ^a			Survival ^b	P value ^c
100 µg poly A:U	+ DDA	5/6		0.008
100 µg poly (A,U)	+ DDA	3/6		NS ^d
50 µg poly A	+ DDA	1/6		NS
50 µg poly U	+ DDA	3/6		NS
	DDA	0/6		---

a Mice were i.p. injected with the single stranded polyribonucleotides poly (A,U), poly A, or poly U, combined with 300 µg DDA. Positive controls received poly A:U plus DDA. Negative controls received DDA alone. Seven days later all groups were challenged i.p. with 10 LD₅₀ Listeria monocytogenes. The number of survivors was determined 14 days after challenge.

b number of survivors/number of mice used.

c P values were calculated relative to DDA controls.

d NS: not significant ($P \geq 0.05$).

FIG. 1. Effect of poly (A:U) and poly(I:C) on phagocyte inflammatory responses.

Groups of 12 mice were injected i.p. with 100 μ g poly(A:U) or poly(I:C) and 300 μ g DDA. Controls received 10 μ g RNA plus DDA or DDA alone. Seven days later half of the mice were used to determine the number of resident peritoneal macrophages (Mφ) and PMNs. The other half was injected i.p. with proteose peptone and the number of inflammatory PMNs and macrophages was determined 4 h later. Open (○) and closed (●) symbols represent cell numbers before and after injection of proteose peptone, respectively. (AU: poly (A:U) plus DDA; IC: poly (I:C) plus DDA; RNA: RNA plus DDA; DDA: DDA; *: number of PMNs/Mφ significantly higher than DDA control by Wilcoxon's rank sum test).

both groups contained elevated numbers of resident peritoneal PMNs as compared to DDA controls.

Whereas the injection of proteose peptone induced a massive mobilization of PMNs to the peritoneal cavity, it had only a moderate effect on the number of peritoneal macrophages. Only mice treated with RNA plus DDA contained a significantly higher number of inflammatory macrophages than mice treated with DDA alone. However, mice treated with poly A:U plus DDA as well as mice treated with RNA plus DDA contained a significantly higher number of resident peritoneal macrophages than DDA controls. The effects of treatment of mice with poly I:C plus DDA were essentially identical to the effects of DDA alone.

Thus, the synthetic RNA which mimicked the protective properties of Listeria-RNA (i.e., poly A:U) also had approximately the same effect on phagocyte inflammatory responses as Listeria-RNA. Conversely, the non-protective poly I:C failed to enhance phagocyte inflammatory responses. Therefore, protection against listeriosis induced with synthetic RNA plus DDA appeared to be intimately linked with enhanced phagocyte inflammatory responses, as previously described with respect to the enhanced resistance against Listeria-infection induced with Listeria-RNA plus DDA (Chapter 6).

DISCUSSION

Experiments described in this paper indicate that the enhanced resistance against listeriosis induced with Listeria-RNA could be reproduced with poly A:U. Protection could not be induced with the random copolymer poly (A, U) or with the double stranded poly I:C, indicating that base composition as well as secondary structure are of relevance with respect to the mechanism of protection induced with RNA plus DDA.

As RNA is a polyanionic substance, and polyanions have been reported to protect mice against listeriosis (Remington and Merigan, 1970) we also tested Listeria-LTA and the synthetic polyanions poly B-411 and poly R-478. However, neither Listeria-

LTA, nor poly B-411, nor poly R-478 protected mice against listeriosis. Therefore, the chemical and physicochemical properties which make a particular substance protective remain somewhat enigmatic. However, at the functional level enhanced inflammatory responsiveness (in particular of PMNs) appeared to be a common feature of enhanced resistance induced with Listeria-RNA plus DDA and protection induced with poly A:U plus DDA. Consequently, further study of the effects of poly A:U plus DDA on PMN functions might be useful for the elucidation of the mechanism of protection induced with RNA plus DDA.

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CHAPTER 8

PROTECTION AGAINST LISTERIOSIS INDUCED WITH RIBOSOMAL RNA FROM
LISTERIA MONOCYTOGENES COMBINED WITH THE ADJUVANT
DIMETHYLDIOCTADECYLAMMONIUM BROMIDE (DDA) IS DUE TO A
T-CELL INDEPENDENT MECHANISM

SUMMARY

Purified ribosomal RNA from Listeria monocytogenes injected in combination with dimethyldioctadecylammonium bromide (DDA), protects mice nonspecifically against a lethal challenge of various extra- and intracellular bacteria. In the present study we have shown that vaccination of BALB/c as well as C57BL/Ka mice with listerial RNA+DDA resulted into an enhanced in vivo Listeria killing in spleen and liver. Evidence was found that enhanced resistance induced by vaccination with ribosomal RNA+DDA occurred by a T-cell independent mechanism: (i) Treatment of mice with Cyclosporin A had no effect on the enhanced Listeria killing induced with RNA+DDA; (ii) In vitro exposure to RNA+DDA did not induce lymphocyte proliferation in spleen cell cultures from unvaccinated mice; (iii) Exposure to Listeria-antigens (formalin-killed Listeria) induced a massive proliferative response in spleen cell cultures from mice vaccinated with live Listeria, but did not give rise to any lymphocyte proliferation response in spleen cell cultures from mice vaccinated with RNA+DDA.

INTRODUCTION

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's 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: 7, 10). A few reports showed that ribosomal RNA also protected mice against heterologous challenge (1, 4, 12, 16, 17). The RNase sensitivity of some ribosomal vaccines, as well as the nonspecifically increased resistance induced with ribosomal RNA, point at RNA as an immunomodulating agent.

Recently we reported that highly purified ribosomal RNA from Listeria monocytogenes protected BALB/c mice against homologous and heterologous challenge, provided that the RNA was injected in combination with the adjuvant dimethyldioctadecylammonium bromide (DDA) (1). Vaccination with RNA or DDA alone was ineffective. Intraperitoneal vaccination with RNA+DDA protected 92-100% of mice against intraperitoneal challenge with L. monocytogenes. Subcutaneous vaccination was less effective but still induced 58% protection. Therefore, protection induced with RNA+DDA appeared to be at least partially due to systemic effects. In this study the systemic protection induced with RNA+DDA is analyzed by subcutaneous vaccination of mice and intravenous challenge with L. monocytogenes. It is shown that vaccination with RNA+DDA induced an accelerated elimination of Listeria from spleen and liver. Furthermore, evidence from several in vivo and in vitro experiments lead us to conclude that protection induced with RNA+DDA was due to a T-cell independent mechanism.

MATERIALS AND METHODS

Bacterial strain.

L. monocytogenes strain 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 Listeria were supplemented with 15% glycerol, and aliquots of 1 ml containing 2×10^9 CFU/ml were stored at -70° C. The Listeria 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 Listeria was used for all animal experiments, with a 50% lethal dose after intravenous (i.v.) injection of approx. 1×10^4 CFU for BALB/c mice.

Animals.

Male BALB/c mice were purchased from TNO, Zeist, The Netherlands, and used at an age of 7-8 weeks. Female C57BL/Ka nude (nu/nu) mice, and their immunologically normal controls, heterozygous for the mutation nude (nu/+), were purchased from TNO, Rijswijk, and used at an age of 7-8 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 (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 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, Cockeysville, MD). The mean CFU per organ was calculated for at least 5 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 Listeria, or 100 µg RNA plus 300 µg DDA (Eastman Kodak Co., Rochester, N.Y.), 100 µg RNA, or 300 µg DDA. DDA was sonicated briefly before use. Injection of 0.1 ml PBS served as a control.

Cyclosporin A.

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 one 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 5×10^6 cells/ml in 0.2 ml Dulbecco Minimal Essential Medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were incubated in the presence of various concentrations RNA and DDA for 72h at 37°C in a humidified atmosphere of 7% CO₂ in air. During the last 4h of the incubation 0.5 µCi [³H]thymidine (specific activity 2 Ci/mmol) was added. The cells were harvested with a microharvester and [³H]thymidine uptake was measured in a liquid scintillation counter. As controls were included incubation of spleen cells with either 2.5 µg/ml concanavalin A (ConA) or 10^7 /ml formalin-killed Listeria (FKLM). Spleen cells were used from untreated mice and from mice vaccinated with RNA+DDA or live Listeria. 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 *Listeria* in liver and spleen.

BALB/c mice were injected s.c. with the various preparations, and 7 days later challenged i.v. with *Listeria*. The kinetics of *Listeria* in liver and spleen were followed up to 3 days after challenge. As shown in Fig. 1 (liver) and Fig. 2 (spleen), pre-treatment of the mice with RNA or DDA alone had no effect on the viable counts of *Listeria* as compared with control mice pretreated with PBS. However, vaccination with the combination of RNA+DDA resulted in an enhanced in vivo killing of *Listeria* in both liver and spleen. In the liver the *Listeria* counts in mice vaccinated with RNA+DDA were significantly lower compared with mice vaccinated with DDA alone, at 48 h ($P=0.0001$) as well as 72 h ($P=0.0006$) after challenge. Also the spleen counts in RNA+DDA-mice were significantly lower than in DDA-mice, both at 48 h ($P<0.0001$) and 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 prior to vaccination with RNA+DDA resulted at 72 h after challenge in a log. C.F.U. 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 *Listeria* resulted in a very efficient killing of *Listeria*, especially in the spleen.

Effect of T-cell deficiency on vaccination.

To investigate the contribution of T-lymphocytes to the enhanced in vivo killing of *Listeria* induced with RNA+DDA vaccination

FIG. 1. Fate of L. monocytogenes in livers of mice after i.v. injection (log. dose = 3.74 ± 0.03). Seven days before challenge the mice were vaccinated s.c. with live Listeria (L. mon.), ribosomal RNA+DDA, RNA or DDA alone, or with PBS (controls). Vertical bars indicate the SD values.

FIG. 2. Fate of *L. monocytogenes* in spleens of mice after i.v. injection (log. dose = 3.74 ± 0.03). Seven days before challenge the mice were vaccinated s.c. with live *Listeria* (*L. mon.*), ribosomal RNA+DDA, RNA or DDA alone, or with PBS (controls). Vertical bars indicate the SD values.

experiments were carried out in two T-cell deficient animal models. Firstly, T-cells were suppressed by treating mice with CyA, an inhibitor of T-cell functions. Secondly, 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 Listeria. Three days after challenge the numbers of viable Listeria in spleen and liver were determined. Since CyA was dissolved in olive oil, control mice received also olive oil i.p. As shown in Table 1, CyA treatment of the mice had no effect on the enhanced in vivo killing of Listeria induced with RNA+DDA. In contrast, the enhanced in vivo killing induced by vaccination with live Listeria was abolished completely when the mice were treated with CyA.

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

Lymphocyte proliferation in vitro.

The possible effect of RNA and DDA on T lymphocytes was also tested in two in vitro proliferation assays. Firstly, spleen lymphocytes from normal mice were incubated with RNA+DDA (Table 3). For positive controls, lymphocytes were treated in vitro with ConA. As expected, exposure of lymphocytes to ConA resulted into a marked proliferative response. In contrast, RNA+DDA failed to induce lymphocyte proliferation in the concentrations tested (1, 5 and 10 $\mu\text{g/ml}$). The concentrations RNA and DDA used did not affect cell viability as judged by trypan blue exclusion.

Secondly, to find out whether vaccination with RNA+DDA primed lymphocytes for a proliferative response to Listeria-antigens, spleen cells from vaccinated mice were incubated with FKLM (Table 4). Vaccination with live Listeria was used as a positive control. As expected, vaccination of mice with live Listeria resulted into

TABLE 1: Effect of Cyclosporin A (CyA) on the viable counts^a in spleen and liver of mice 3 days after i.v. injection of *Listeria* (log.dose = 3.27 + 0.13)

Organ and treatment of mice ^b	Vaccination ^c of mice with		
	PBS	RNA+DDA	<i>L. monocytogenes</i>
<u>Liver:</u>			
Olive oil	5.62 ± 0.36	4.15 ± 0.52 (P=0.001)	2.98 ± 0.42 (P<0.0001)
CyA	5.68 ± 0.20	4.52 ± 0.27 (P=0.0001)	6.55 ± 0.33 (P=0.003)
<u>Spleen:</u>			
Olive oil	5.11 ± 0.35	4.14 ± 0.45 (P=0.0025)	0.48 ± 1.07 (P<0.0001)
CyA	5.44 ± 0.26	4.47 ± 0.25 (P=0.0004)	5.03 ± 0.41 (NS)

^a The figures represent the mean log. C.F.U. ± S.D. 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*, RNA+DDA, or PBS seven days before challenge.

TABLE 2: The viable counts^a in spleen and liver of athymic nude (nu/nu) mice and their immunologically normal controls (nu/+), 3 days after i.v. injection of *Listeria* (log. dose = 3.79 ± 0.18)

Organ and type of mice	Vaccination ^b of mice with		
	PBS	RNA+DDA	<i>L. monocytogenes</i>
<u>Liver:</u>			
nu/+	4.99 ± 0.41	3.51 ± 0.34 ($P=0.001$)	1.21 ± 0.48 ($P<0.0001$)
nu/nu	4.64 ± 0.56	4.54 ± 0.39 (NS)	4.85 ± 0.32 (NS)
<u>Spleen:</u>			
nu/+	5.52 ± 0.26	4.10 ± 0.41 ($P=0.0008$)	1.43 ± 0.22 ($P<0.0001$)
nu/nu	4.65 ± 0.50	4.48 ± 0.43 (NS)	4.36 ± 0.52 (NS)

^a The figures represent the mean log. C.F.U. $\pm$ S.D. The P values were calculated compared with PBS controls; NS, not significant.

^b Mice were vaccinated s.c. with live *L. monocytogenes*, RNA+DDA, or PBS seven days before challenge.

an increased proliferative response to FKLM compared with unvaccinated controls. Vaccination with RNA+DDA did not result into an increased response to FKLM.

DISCUSSION

In the present study we have shown that s.c. injection of purified ribosomal RNA in combination with the adjuvant DDA enhanced the in vivo killing of Listeria in liver and spleen after an i.v. challenge with a sublethal dose of Listeria. 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: 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/6J) 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 Listeria (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 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 interferon- γ has been identified as the macrophage activating factor (9, 18, 23). Cyclosporin A (CyA),

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

<u>In vitro</u> exposure with	[³ H]Thymidine uptake (cpm $\pm$ SD)	<u>P</u> value
-	4,106 $\pm$ 944	-
RNA + DDA (1 μ g/ml)	4,813 $\pm$ 3,137	N.S. ^a
RNA + DDA (5 μ g/ml)	2,558 $\pm$ 397	N.S.
RNA + DDA (10 μ g/ml)	3,101 $\pm$ 762	N.S.
ConA (2.5 μ g/ml)	79,086 $\pm$ 7,991	<0.0001

^a N.S., not significant ($P > 0.05$).

TABLE 4: Lymphocyte proliferation after in vitro exposure of spleen cells from vaccinated mice

<u>Vaccination</u> ^a	<u>In vitro</u> exposure with	[³ H]Thymidine uptake (cpm $\pm$ SD)	<u>P</u> value
-	FKLM ^b	6,780 $\pm$ 141	-
<u>Listeria</u>	FKLM	18,563 $\pm$ 1,931	<0.0001
RNA+DDA	FKLM	5,591 $\pm$ 1,558	N.S.

^a Vaccination s.c. with live Listeria or RNA+DDA 7 days before spleen cell harvesting.

^b FKLM, formalin-killed Listeria.

a specific inhibitor of T-cell functions (22, 25), has been found to inhibit lymphokine production including interferon- γ (15, 21, 24). In the present study it was shown that CyA indeed inhibited the in vivo macrophage activation induced by vaccination with live Listeria. In contrast, treatment of mice with CyA did not inhibit the enhanced in vivo killing of Listeria induced with RNA+DDA. Therefore it is concluded 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 normal spleen cells to RNA+DDA, nor after exposure of spleen cells from RNA+DDA vaccinated mice to FKLM.

Seemingly conflicting results were obtained in the experiments with athymic nude mice, since enhanced in vivo killing of Listeria induced by vaccination with RNA+DDA was not observed in nude mice. If macrophage activation by RNA+DDA is supposed to be 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.

Alternatively, the ineffectiveness of RNA+DDA in nude mice might be due to defects other than the well-documented T-cell deficiency. Recently, indications were found that the initial phase of the expression of protection against listeriosis induced with RNA+DDA is due to activated polymorphonuclear leukocytes (PMNs) rather than to activated macrophages (3).

Therefore, the ineffectiveness of RNA+DDA in nude mice might also be related to anomalous PMN functions, either because nude mice

contain PMNs which are already activated, or because RNA+DDA does not induce PMN-activation in nude mice.

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CHAPTER 9*SUMMARY AND GENERAL DISCUSSION*

Subject of this thesis is the modulation of antibacterial resistance by subcellular preparations from Listeria monocytogenes (L. monocytogenes). A crude ribosomal preparation from L. monocytogenes L242, serotype 4b was separated into cell envelope fragments (fraction I) and purified ribosomes (fraction II) by molecular sieve chromatography. In combination with the adjuvant dimethyldioctadecylammonium bromide (DDA) both preparations protected mice against lethal infection with L. monocytogenes. Preparations without DDA or DDA alone did not induce protection. Ribosomal RNA was identified as the major protective component of fraction II, as the protective properties of this fraction were destroyed by RNase-treatment but not by treatment with proteinase K. Furthermore, also purified rRNA extracted from fraction II protected mice against listeriosis. The protective properties of fraction I were not affected by RNase-treatment, proteinase K treatment, or sequential treatment with both enzymes. Fraction I plus DDA as well as RNA plus DDA also protected mice against L. monocytogenes serotype 3, Pseudomonas aeruginosa and Streptococcus pneumoniae.

As immunity to listeriosis is frequently associated with a state of delayed-hypersensitivity (DH) to Listeria-antigens, we also investigated whether DH could be induced or elicited with fraction I or RNA. Fraction I plus DDA induced DH to killed Listeria. Conversely, DH induced by immunization with viable Listeria could be elicited with fraction I. However, DH could not be induced or elicited with RNA. Proteinase K-treatment of fraction I destroyed its ability to induce DH against killed Listeria. Therefore DH induced with fraction I was apparently due to proteinase K-sensitive proteins, whereas enhanced resistance induced with this preparation was due to non-protein components or

to proteinase K-resistant peptides/proteins. Experiments with protein fractions isolated from fraction I suggested that several proteins contributed to the induction of DH with fraction I. However, DH could also be induced with a single purified protein (MW 20,400).

As attempts to identify the chemical nature of the protective components of fraction I were inconclusive, further experiments were restricted to elucidation of the mechanism of protection induced with RNA plus DDA. In addition to Listeria rRNA also heterologous rRNA (from P. aeruginosa) and the synthetic RNA poly A:U protected mice against listeriosis. These observations suggested that the mechanism of protection induced with RNA plus DDA might be different from the mechanism of protection induced by immunization with viable Listeria which depends on recognition of Listeria-antigens by Listeria-specific T-cells.

The immunosuppressive drug cyclosporin A, which inhibits the induction of T-cell-dependent immunity with viable Listeria, did not affect the induction of protection with RNA plus DDA. Therefore, enhanced resistance induced with RNA plus DDA was apparently due to a T-cell-independent modulation of phagocyte functions.

Upon intraperitoneal injection of the sterile irritant proteose peptone, mice treated seven days previously with RNA plus DDA were found to mobilize increased numbers of polymorphonuclear leukocytes (PMNs) and macrophages to the inflamed peritoneal cavity as compared to mice treated with DDA alone. Also protection to listeriosis induced with poly A:U plus DDA was found to be associated with enhanced phagocyte inflammatory responsiveness. Enhanced resistance induced with RNA plus DDA could be transferred to normal recipient mice with inflammatory macrophages (collected 48 h after the injection of proteose peptone) as well as with 4 h inflammatory PMNs. Inflammatory PMNs as well as inflammatory macrophages from mice treated with RNA plus DDA appeared to be activated, as recipient mice could not be protected against listeriosis with inflammatory PMNs or macrophages from mice treated with DDA alone.

Whereas protection to Listeria monocytogenes induced by immu-

nization with viable Listeria depends on T-cell mediated macrophage activation, RNA plus DDA protected mice against listeriosis through a T-cell-independent mechanism which was partially based on activation of PMNs, cells which are generally considered to be only of minor importance for the elimination of intracellular parasites such as Listeria monocytogenes.

SAMENVATTING

Listeria monocytogenes is een bacterie die na het binnendringen van het lichaam snel wordt opgenomen door macrofagen. Terwijl vele micro-organismen na opname door macrofagen door deze cellen worden gedood, is Listeria monocytogenes in staat binnen macrofagen te overleven en zich intracellulair te vermenigvuldigen. Dit proces leidt veelal tot de dood van de macrofaag, waarna de infectie zich verder door het lichaam kan verspreiden. Infecties die niet tijdig tot staan worden gebracht, leiden tot de dood of tot restverschijnselen die voornamelijk van neurologische aard zijn. Bij de bestrijding van Listeria-infecties kunnen zowel het afweersysteem van de gastheer als antibiotica-therapie een rol spelen.

Uit onderzoeken van Mackaness is gebleken dat proefdieren tegen een dodelijke infectie met Listeria monocytogenes kunnen worden beschermd door immunisatie met een niet-dodelijke dosis levende bacteriën. In tegenstelling tot macrofagen geïsoleerd uit niet-geïmmuniseerde dieren bleken macrofagen uit geïmmuniseerde dieren wel in staat te zijn Listeria te doden. Dit verschijnsel wordt aangeduid met de term "macrofaag-activering". De bescherming tegen Listeria monocytogenes geïnduceerd door immunisatie met levende bacteriën kan op niet-geïmmuniseerde dieren worden overgedragen met behulp van miltcellen geïsoleerd uit geïmmuniseerde dieren. Bij nadere analyse bleek de overdracht van bescherming door miltcellen te berusten op in de milt aanwezige T-cellen. Door contact met macrofagen die Listeria-bacteriën hebben opgenomen, worden deze T-cellen aangezet tot het uitscheiden van factoren (lymfokinen) die de antibacteriële eigenschappen van macrofagen dusdanig kunnen versterken dat deze cellen wel in staat zijn om Listeria te doden. De interactie tussen T-cellen uit tegen Listeria geïmmuniseerde proefdieren (Listeria-immuun T-cellen) en met macrofagen geassocieerde Listeria-antigenen is specifiek, dat wil zeggen lymfokine-productie en macrofaag-activering treden niet

op indien aan Listeria-immuun T-cellen andere bacteriële antigenen worden aangeboden. Eenmaal geactiveerde macrofagen zijn echter in staat om naast L. monocytogenes ook andere intracellulaire bacteriën te doden. Een karakteristiek kenmerk van de afweer tegen L. monocytogenes is, dat immuniteit niet kan worden opgewekt met behulp van dode bacteriën tenzij het dode vaccin wordt gecombineerd met adjuvantia zoals het dimethyldioctadecylammonium bromide (DDA). Terwijl het mechanisme van de bescherming geïnduceerd door immunisatie met levende bacteriën grotendeels is opgehelderd, is betrekkelijk weinig bekend met betrekking tot het mechanisme van de bescherming geïnduceerd door dode Listeria-vaccins in combinatie met adjuvantia.

Onderwerp van dit proefschrift is het mechanisme van de bescherming geïnduceerd door subcellulaire vaccins geïsoleerd uit Listeria monocytogenes. Een ruw ribosomaal preparaat geïsoleerd uit L. monocytogenes L242, serotype 4b, werd met behulp van gel-filtratie gescheiden in cel-envelop fragmenten (fractie I) en gezuiverde ribosomen (fractie II). Door immunisatie met fractie I of fractie II in combinatie met het adjuvans DDA kon in BALB/c muizen bescherming tegen een dodelijke infectie met Listeria monocytogenes worden opgewekt. Bescherming kon niet worden geïnduceerd met fractie I of fractie II zonder DDA, of met DDA alleen. Ribosomaal RNA werd geïdentificeerd als de belangrijkste beschermende component in fractie II, omdat de beschermende activiteit van deze fractie werd vernietigd door behandeling met RNase, maar niet door behandeling met een eiwit-splitsend enzym (proteïnase K). Bovendien kon bescherming ook geïnduceerd worden met gezuiverd ribosomaal RNA, geïsoleerd uit fractie II. De beschermende activiteit van fractie I werd niet aangetast door behandeling met RNase of proteïnase K, of door behandeling met RNase gevolgd door behandeling met proteïnase K. Behandeling van muizen met fractie I plus DDA of RNA plus DDA induceerde niet alleen bescherming tegen L. monocytogenes serotype 4b, maar ook tegen L. monocytogenes serotype 3, Streptococcus pneumoniae en Pseudomonas aeruginosa.

Aangezien immuniteit tegen listeriose vaak gepaard gaat met een toestand van vertraagd-type overgevoeligheid (VTO) tegen Listeria-antigenen (te vergelijken met de Mantoux-reactie zoals gebruikt bij de controle op TBC-besmetting) werd ook onderzocht of VTO geïnduceerd of opgewekt kon worden met fractie I of RNA. Fractie I plus DDA induceerde VTO tegen dode Listeria. Bovendien kon VTO geïnduceerd door immunisatie met levende Listeria opgewekt worden met fractie I. Een VTO-reactie kon echter niet opgewekt of geïnduceerd worden met RNA. De VTO-inducerende eigenschappen van fractie I werden vernietigd door proteinase K-behandeling. Proteinase K-gevoelige eiwitten waren derhalve verantwoordelijk voor de inductie van VTO met fractie I, terwijl de bescherming geïnduceerd door fractie I klaarblijkelijk gebaseerd was op andere componenten dan eiwitten, of op eiwitten of eiwitfragmenten die ongevoelig waren voor proteinase K. Uit VTO-experimenten met eiwitfracties geïsoleerd uit fractie I werden aanwijzingen verkregen dat meerdere eiwitten een bijdrage leverden aan de inductie van VTO met fractie I. Eén van deze eiwitten (molecuulgewicht 20.400) werd gezuiverd.

Aangezien de beschermende componenten van fractie I niet konden worden geïdentificeerd, werden verdere experimenten beperkt tot opheldering van het mechanisme van de bescherming geïnduceerd door RNA plus DDA. Bescherming tegen L. monocytogenes kon niet alleen geïnduceerd worden met Listeria-RNA plus DDA, maar ook met heteroloog ribosomaal RNA (geïsoleerd uit P. aeruginosa) plus DDA of met synthetisch RNA (poly A:U) plus DDA. Op basis van deze waarneming werd de hypothese geformuleerd dat de bescherming geïnduceerd met Listeria-RNA plus DDA waarschijnlijk niet gebaseerd was op herkenning van Listeria-antigenen door Listeria-specifieke T-cellen. Deze hypothese werd onderzocht met behulp van cyclosporine A, waarmee de inductie van T-cel afhankelijke immuniteit tegen Listeria kan worden onderdrukt. Behandeling van muizen met cyclosporine A verhinderde de inductie van bescherming door immunisatie met levende Listeria, maar had geen effect op de inductie van

bescherming met RNA plus DDA, zodat het T-cel onafhankelijke karakter van de verhoogde weerstand geïnduceerd met RNA plus DDA kon worden bevestigd.

Na opwekking van een ontstekingsreactie in de buikholte door injectie van proteose pepton bleken zich in de buikholte van muizen behandeld met RNA plus DDA aanzienlijk meer polymorfonucleaire leukocyten (PMNs) en macrofagen op te hopen dan in de buikholte van muizen behandeld met DDA alleen. De bescherming geïnduceerd door RNA plus DDA was derhalve geassocieerd met een versterking van aspecifieke ontstekingsreacties. Een versterkte ontstekingsreactie (vooral van PMNs) werd ook waargenomen in muizen behandeld met poly A:U plus DDA.

Onbehandelde muizen konden tegen een dodelijke Listeria-infectie beschermd worden door injectie van ontstekings-PMNs of ontstekings-macrofagen geïsoleerd uit muizen behandeld met RNA plus DDA, maar niet door injectie van PMNs of macrofagen geïsoleerd uit muizen behandeld met DDA alleen. Zowel PMNs als macrofagen geïsoleerd uit muizen behandeld met RNA plus DDA leken derhalve geactiveerd te zijn ten opzichte van dezelfde cellen geïsoleerd uit muizen behandeld met DDA alleen.

In tegenstelling tot de bescherming tegen L. monocytogenes geïnduceerd door immunisatie met levende Listeria was de bescherming geïnduceerd met RNA plus DDA T-cel onafhankelijk en - althans gedeeltelijk - gebaseerd op activering van PMNs, cellen waarvan in het algemeen wordt aangenomen dat deze slechts van weinig belang zijn voor de eliminatie van intracellulaire parasieten zoals Listeria monocytogenes.

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CURRICULUM VITAE

Ad Antonissen werd op 22 april 1953 geboren te Oudenbosch.

Na het behalen van het diploma Gymnasium B in juni 1971 studeerde hij scheikunde aan de Rijksuniversiteit te Utrecht. Het kandidaatsexamen Sl werd afgelegd in juni 1975 en het doctoraal-examen (hoofdvak biochemie, bijvak immunologie) in oktober 1979. Van 15 oktober 1979 tot 1 januari 1981 was hij wetenschappelijk assistent bij de sectie experimentele immunologie van het laboratorium voor microbiologie (vakgroep besmettingsleer van de medische faculteit) te Utrecht alwaar onder leiding van Dr. H. van Dijk en Prof. Dr. J.M.N. Willers onderzoek werd verricht aan een RNase-gevoelig vaccin geïsoleerd uit Listeria monocytogenes.

Van 1 mei 1981 tot 1 mei 1985 was hij wetenschappelijk ambtenaar bij de capaciteitsgroep medische microbiologie van de Rijksuniversiteit Limburg, alwaar het in dit proefschrift beschreven onderzoek werd uitgevoerd.