Enhanced cellular response in mice treated with a Brucella antigen-liposome mixture

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Abstract: The capacity of liposomes constituted by dycetyl-phosphate (0.009 mM), cholesterol (0.017 mM), lecithin (0.003 mM), and myristic (0.1 mM), stearic (0.1 mM), or oleic acid (0.1 mM) to modify the lymphocyte response to Brucella melitensis antigens in mice was studied. Mice treated with antigens mixed with liposomes containing myristic, stearic or oleic acid had higher antibody titres than mice given antigen suspended in a saline solution. Liposomes alone, without Brucella antigens, resulted in increased 3H-thymidine incorporation by lymphocytes both in vivo and in vitro. The addition of polyclonal activators (LPS and ConA) caused a further increase of 3H-thymidine uptake. Moreover, spleen lymphocytes from mice inoculated with Brucella antigens mixed with the liposomes had a significantly lower population of B lymphocytes (10%), and a notable increase in the Tc lymphocytes (20%). Autoradiography of sections of popliteal ganglia of treated mice showed that the radioactivity was concentrated mainly in the membrane structures of the cell.

Key words: Liposome; Brucella melitensis antigen, Cellular response

Introduction

Antigen processing is a critical step in antigen recognition by helper T cells. There are distinct antigen processing pathways for native and endogenous antigens. When spleen cells from mice infected with influenza virus are stimulated in vitro, the type of T cell generated depends on the secondary stimulus [1]: restimulation with virally infected cells results in CD8+ class I-restricted T cells, while restimulation with UV-inactivated virus results in CD4+ class II-restricted T cells. Thus, the antigen dramatically affects the T cell response. In most studies, peptides failed to prime cytotoxic T-lymphocytes (CTL) in vivo, despite their ability to do so in vitro [1]. By coupling the peptide to a synthetic lipid, Deres et al. [2] obtained CTL in vivo that were specific for a peptide derived from influenza virus nucleoprotein.

Many liposome-encapsulated substances show a characteristic pattern of interaction with cells [3,4] and it is possible to hypothesize that antigen vehicled by liposomes could modify the endogenous or exogenous track response of the interested cells.

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Galdiero et al. previously described the role of the composition of liposome alone on the cells involved in the immune response [5]. In this study liposomes with adjuvant property were selected [6]. We have examined the capacity of liposomes consisting of dicetyl-phosphate (0.009 mM), cholesterol (0.017 mM), lecithin (0.003 mM) and myristic (0.1 mM), stearic (0.1 mM) or oleic acid (0.1 mM) to affect the lymphocyte response to *Brucella* challenge.

**Materials and Methods**

**Animals**

Three-week-old Swiss mice weighing 20–25 g kept on a “70-K Mignini diet” (Petragnani-Assisi, Perugia, Italy) were used. The animals were maintained in a controlled room (20 ± 2°C with automatic 12 h cycles of lighting). They had free access to water.

**Liposome preparation**

The liposomes were prepared as described by Wassef and Riving [7]. Dicetyl-phosphate (0.009 mM), cholesterol (0.017 mM), lecithin (0.003 mM), and myristic (0.1 mM), stearic (0.1 mM) or oleic (0.1 mM) acids (Sigma) were mixed in a pear-shaped flask with (2 ml) chloroform. The mixture was dried in a rotary evaporator (Heidolph-200) for 1 h under vacuum. The desiccated lipids were resuspended by adding RPMI 1640 (Labtek Laboratories, Eurobio, Paris, France) and a small quantity (approx. 70–100 μl) of 0.5 mm glass beads. The lipid suspension was flushed with nitrogen, after which the flask was sealed and vortexed repeatedly at top speed. This process caused the formation of multilamellar vesicles with a diameter of 40–100 nm as seen in an electron microscope (Zeiss EM 109, Germany). The liposomes suspended in 1 ml volume in RPMI 1640 were added to 100 μg of *Brucella* lyophilizate.

**Preparation of Brucella antigens**

*Brucella melitensis* strain H38 phase S were grown at 37°C for 48 h on trypticase soy agar plates supplemented with 0.1% (w/v) yeast extract. The purity and phase S were verified according to a standard procedure [8]. To avoid human infection, cells were inactivated by heat (1 h at 65°C). Cells were broken in a cooled Braun-type disintegrator for 30 min. Crude cell-wall fractions were recovered by centrifugation at 53,000 × g for 1.5 h at 4°C. The pellet was suspended in saline at a concentration of protein 30–40 mg ml⁻¹, washed three times and finally resuspended in distilled water and lyophilized. The protein assays were performed following the methods of Lowry [9].

**Immunization**

Groups of 30 mice were given three intramuscular injections at intervals of a week as follows: Group 1, saline solution; Groups 2, 3 and 4, liposomes constituted by dicetyl-phosphate, cholesterol, lecithin, and myristic acid (2) or stearic acid (3) or oleic acid (4) without *Brucella* antigens; Group 5, *Brucella* antigens suspended in physiological solution; Groups 6, 7 and 8, *Brucella* antigen-mixed liposomes constituted by dicetyl-phosphate, cholesterol, lecithin, and myristic acid (6) or stearic acid (7) or oleic acid (8).

**Antibody response**

The serum of each mouse was assayed for antibody activity by solid phase ELISA against antigens diluted five-fold in glycine-buffer saline (GBS) (0.17 M NaCl, 0.1 M glycine, 6 mM NaN₃, pH 9.2) in water [10]. The antigens used to coat the plates consisted of crude cell wall fractions. Sera were serially diluted in GBS-EDTA-Tween (GBS plus 50 mM EDTA and 0.1% Tween 80, final pH 9.2). Binding of the antibodies was visualized with peroxidase-conjugated rabbit antimouse antiserum (Sigma Immuno Chemicals) diluted in GBS-EDTA-Tween containing 2% fetal calf serum. Excess reagents between the different incubations were removed by five washings with NaCl-Tween, O-Phenylenediamine (0.4%, w/v) and 2 mM H₂O₂ in citrate phosphate buffer (0.051 M Na₂HPO₄, 0.024 M citric acid, pH 5) were used to visualize peroxidase activity. The titers were estimated as the highest dilution giving a difference of more than twice the mean of the corresponding blank values. The absorbance
was measured at a wavelength of 492 nm and a reference of 620 nm on a Behring ELISA plates reader.

Lymphocyte counting and typing

Circulating lymphocytes were counted in a Burker chamber according to conventional procedure. For typing, lymphocytes were concentrated at $2 \times 10^6$ cell ml$^{-1}$ and the lymphocyte clones were examined with a fluorescence activated cell sorter (Ortho Cytofluorograph Model 2150) after reaction with mAbs anti-mouse Thy 1.2, anti-mouse Lyt-2 and anti-mouse LT3T4 (Becton-Dickinson). Briefly, $1 \times 10^6$ lymphocytes were incubated on ice for 30 min with a 1 in 10 dilution of mAb anti-mouse Lyt-2 fluorescein conjugate for T$_c$ lymphocytes, mAb anti-mouse LT3T4 for T$_h$ lymphocytes and anti-mouse I-A$^d$ for B lymphocytes. They were washed three times with phosphate buffered saline. For B lymphocytes, fluorescein-conjugated goat F(ab)$_2$ anti-mouse IgG (H and L chains) (DuPont Co.) was added to the cell suspension after which the mixture was incubated for an additional 30 min. After three washings, the cells were examined by flow cytometry using FACS IV (Becton Dickinson).

Mitogen-induced spleen cell proliferation

Spleen cells were prepared from mice spleens, which had been aseptically removed, by pushing the minced tissues through a very fine metallic mesh, washed three times in RPMI 1640 (Labtek Laboratories, Eurobio, Paris, France). Ammonium chloride (0.17 M) was added to lyse red blood cells, and, after two washings with RPMI 1640, the lymphocytes were isolated on ‘Milieu de separation des lymphocytes’ (Eurobio, Paris, France) at $600 \times g$ for 30 min at room temperature. The isolated lymphocytes were washed, re-suspended in RPMI 1640 at a concentration of $3 \times 10^6$ cells ml$^{-1}$ and checked by flow cytometry analysis (FACS IV, Becton Dickinson); 90 ± 4% of the isolated cells were lymphocytes. Aliquots of 100 $\mu l$ placed in multiwell plates with 1.2 $\mu g$ ConA or 2 $\mu g$ LPS per well were incubated in 5% CO$_2$ at 37°C for 72 h. Six hours before termination of incubation $^3$H-thymidine (0.5 $\mu$Ci; specific activity 5 Ci mmol$^{-1}$; Amersham, UK) was added to each culture. The lipopolysaccharide extract used was from Escherichia coli 0128:B12 (Sigma). All cultures were harvested with a multiple automated sample harvester (Skatron cell Harvester-Biochem, Italy) onto glass fibre filters. The filters were dried and placed in vials that were filled with 3 ml of scintillation cocktail (Ready Gel Beckman) and counted in a Beckman spectrometer with a standard error of $\epsilon = 0.5\%$. All determinations were carried out in triplicate.

Electron microscopic autoradiography

Localization of labelled liposomes in popliteal lymph nodes of mouse was studied by electron microscopic autoradiography. In this experiment only liposomes prepared by mixing dicetyl-phosphate, cholesterol, lecithin and $^3$H-labelled myristic acid were used. [9,10(\textsuperscript{3H})] myristic acid was obtained from the Radiochemical Centre (Amersham International plc, UK). The specific activity of labelled liposomes used in the experiment was approximately 160 $\mu$Ci mg$^{-1}$. The popliteal lymph nodes were removed and quickly immersed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH = 7.2), at 4°C. Thereafter, the samples were washed for 30 min in three changes of 0.1 M cacodylate buffer and post-fixed in 1% aqueous solution of OsO$_4$ for 90 min at 4°C. After repeated washings, the samples were dehydrated through a graded series of ethanol and propylene oxide, and embedded in Epon. Ultrathin sections were cut by a diamond knife in an LKB Ultrotome Nova ultramicrotome. The sections were placed up on collodion-coated 200-mesh microscope nickel grids and radioautographed using a wire loop containing a film of Ilford L4 Nuclear Research Emulsion diluted 1:3. Autoradiographs were stored in light-proof boxes containing silica gel, at a temperature of 4°C for about 2 months. After exposure, the radioautograms were developed in freshly prepared Ilford Phenisol diluted 1:4 with distilled water for 3 min at 20°C. Subsequently the specimens were fixed and washed for 30 min with distilled water. All sections were systematically double-stained with aqueous uranyl acetate and lead citrate according
to standard procedures, and examined with a Zeiss EM 109 electron microscope.

**Statistical analysis**
Statistical significance of the difference between each test and the respective control was examined by Student’s *t*-test, with significance determined at the 5% and 1% levels.

**Results**

**Antibody response**
As shown in Fig. 1, the mice given *Brucella* antigens suspended in saline solution (group 5) had an anti-*Brucella* titre of 1/1024 seven days after treatment, whilst in mice given *Brucella* antigen mixed with liposomes containing myristic acid (group 6) or stearic acid (group 7) they had a titre about 4-fold higher. The titre increase was 2-fold higher with liposomes containing oleic acid.

**Table 1**

<table>
<thead>
<tr>
<th>Added substances</th>
<th>Lymphocytes</th>
<th>Lymphocytes + LPS (2 Ìg/well)</th>
<th>Lymphocytes + ConA (1.2 Ìg/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline Solution (1)</td>
<td>450±21</td>
<td>3620±60</td>
<td>4300±65</td>
</tr>
<tr>
<td>Liposomes with myristic acid (2)</td>
<td>2210±47</td>
<td>5860±76</td>
<td>7240±85</td>
</tr>
<tr>
<td>Liposomes with stearic acid (3)</td>
<td>2100±46</td>
<td>5700±75</td>
<td>7100±84</td>
</tr>
<tr>
<td>Liposomes with oleic acid (4)</td>
<td>2050±45</td>
<td>5670±75</td>
<td>7180±85</td>
</tr>
<tr>
<td><em>Brucella</em> antigens in saline (5)</td>
<td>1020±32</td>
<td>2410±49</td>
<td>4160±64</td>
</tr>
<tr>
<td><em>Brucella</em> antigens mixed liposomes with myristic acid (6)</td>
<td>2900±54</td>
<td>2700±52</td>
<td>7100±84</td>
</tr>
<tr>
<td><em>Brucella</em> antigens mixed liposomes with stearic acid (7)</td>
<td>3000±54</td>
<td>3100±55</td>
<td>8000±89</td>
</tr>
<tr>
<td><em>Brucella</em> antigens mixed liposomes with oleic acid (8)</td>
<td>2800±53</td>
<td>2900±54</td>
<td>7200±89</td>
</tr>
</tbody>
</table>

* The data are the average of 3 experiments ± S.D.  
** cpn/1.5×10⁶ cells.

![Graph showing reciprocal antibody titre](https://academic.oup.com/femspd/article-abstract/10/3-4/235/609757)
Mice inoculated with only liposomes (groups 2, 3 and 4) or with saline solution did not show anti-Brucella antibody.

Control groups of mice treated with lecithin, cholesterol and dicetyl-phosphate did not show any difference compared to untreated controls. Another control group treated with each fatty acid also did not show significant difference to controls.

**Cell response**

The cell response was evaluated from in vitro incorporation of $^3$H-thymidine by spleen cells from the eight groups of animals studied. The liposomes that contained fatty acid, myristic acid, stearic acid or oleic acid alone, without *Brucella* antigen caused an increase in $^3$H-thymidine incorporation both in vivo and in vitro. In vitro (Table 1), spleen lymphocytes responded to the addition in culture of liposomes containing 0.1 mM fatty acid/10$^6$ cells with a remarkable stimulation. Liposomes containing less than 0.05 mM fatty acid were without effect, while liposomes containing 0.2 mM fatty acid were toxic. The individual liposome components did not exert a stimulatory effect (data not shown). Subsequent stimulation with the polyclonal activators, LPS (2 $\mu$g/well) and ConA (1.2 $\mu$g/well), further enhanced $^3$H-thymidine incorporation by circulating lymphocytes. LPS increased stimulation by 165% with respect to liposome alone. Stimulation with ConA caused an even greater increase (227%). Similar results were obtained with spleen lymphocytes from animals treated in vivo.

Cells from non-treated animals stimulated in vitro with *Brucella* antigens suspended in saline did not show a remarkable increase of $^3$H-thymidine incorporation after stimulation with LPS or ConA compared with controls. When the antigen was added to liposomes the incorporation increase was similar to that obtained with liposomes alone. On the contrary, the data reported in Table 2, show a totally different response to in vitro stimuli was obtained in the groups of mice.

**Table 2**

$^3$H-thymidine incorporation in circulating lymphocytes (cpm/1.5X10$^6$ cells) from groups of mice treated with liposomes and *Brucella* cell wall fragments and stimulated with LPS from *E. coli* 0:128:B12 or with ConA *

<table>
<thead>
<tr>
<th>Group of treated mice with</th>
<th>Lymphocytes</th>
<th>Lymphocytes + LPS (2 $\mu$g/well)</th>
<th>Lymphocytes + ConA (1.2 $\mu$g/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline Solution (1)</td>
<td>450 * * ± 21</td>
<td>3500 ± 59</td>
<td>4420 ± 66</td>
</tr>
<tr>
<td>Liposomes with myristic acid (2)</td>
<td>2210 ± 47</td>
<td>5860 ± 76</td>
<td>7240 ± 85</td>
</tr>
<tr>
<td>Liposomes with stearic acid (3)</td>
<td>2100 ± 46</td>
<td>5600 ± 75</td>
<td>7000 ± 84</td>
</tr>
<tr>
<td>Liposomes with oleic acid (4)</td>
<td>2000 ± 45</td>
<td>5400 ± 73</td>
<td>7100 ± 84</td>
</tr>
<tr>
<td><em>Brucella</em> antigens in saline (5)</td>
<td>4160 ± 64</td>
<td>7880 ± 88</td>
<td>7050 ± 84</td>
</tr>
<tr>
<td><em>Brucella</em> antigens mixed liposomes with myristic acid (6)</td>
<td>10080 ± 100</td>
<td>14180 ± 119</td>
<td>25690 ± 160</td>
</tr>
<tr>
<td><em>Brucella</em> antigens mixed liposomes with stearic acid (7)</td>
<td>10000 ± 100</td>
<td>14000 ± 117</td>
<td>25500 ± 160</td>
</tr>
<tr>
<td><em>Brucella</em> antigens mixed liposomes with oleic acid (8)</td>
<td>10100 ± 100</td>
<td>14200 ± 119</td>
<td>25700 ± 160</td>
</tr>
</tbody>
</table>

* The data are the average of 3 experiments ± S.D.

** * cpmp/1.5X10$^6$ cells.
primed in vivo with *Brucella* antigens suspended in saline solution (group 5) or mixed with liposomes (groups 6, 7 and 8). Groups 6, 7 and 8 showed an increase in lymphocyte stimulation of about 140% compared with group 5. Further stimulation with lipopolysaccharide increased the incorporation to about 80%. Stimulation with ConA was even more effective, and the level of lymphocyte stimulation in groups 6, 7 and 8 was 270% higher than in group 5.

Because of the different response of lymphocytes from treated animals to in vitro stimulation with lipopolysaccharide or with ConA, we typed, after in vitro stimulation, the spleen lymphocytes in the eight groups (Table 3). Immunization with *Brucella* antigens in saline solution (group 5) did not cause a significant increase in B lymphocytes, while immunization with *Brucella* antigens mixed with liposomes (groups 6, 7 and 8) resulted in a significant per cent decrease in B lymphocytes with per cent increase in Tc lymphocytes.

**Ultrastructural localization of radiolabelled liposomes in popliteal lymph node**

Labelled liposomes were injected intramuscularly into the animal's hind leg, and ultrastructural studies were conducted on the popliteal lymph node. The sinusoidal macrophages are the first cells to be involved in, through phagocytosis, to elimination of particles from the lymphatic stream.

Autoradiographs of popliteal lymph nodes showed numerous silver grains around germinal centres and over macrophages of the medulla and superficial cortex. All the lymphoid cells were uniformly labelled. Silver grains were also detected on follicular dendritic cells, which trap and deliver immune complexes to B cells [11]. In particular, silver grains were present on the nuclear membrane and on the heterochromatin of lymphoidal cells (Fig. 2).

**Discussion**

It is well known that the 'meeting' between the antigen and immune cells is the most important step in the immune process. Various attempts have been made to obtain efficient artificial protection by manipulation of the antigen [2], the antigen vehicle [12] and the immune cells [13]. We have tried to modulate the immune process percentage of lymphocyte clones obtained from eight groups of mice immunized with liposomes and *Brucella* cell wall fragments *.

<table>
<thead>
<tr>
<th>Group of treated mice with</th>
<th>B 1 lymphocytes (I-A&lt;sup&gt;+&lt;/sup&gt;) %</th>
<th>T&lt;sub&gt;4&lt;/sub&gt; Lymphocytes (L3T4) %</th>
<th>T&lt;sub&gt;8&lt;/sub&gt; Lymphocytes (Lyt-2) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline Solution (1)</td>
<td>42 ± 6</td>
<td>38 ± 6</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>Liposomes with myristic acid (2)</td>
<td>41 ± 6</td>
<td>37 ± 6</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>Liposomes with stearic acid (3)</td>
<td>45 ± 6</td>
<td>36 ± 6</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>Liposomes with oleic acid (4)</td>
<td>42 ± 6</td>
<td>35 ± 6</td>
<td>20 ± 4</td>
</tr>
<tr>
<td><em>Brucella</em> antigens in saline (5)</td>
<td>44 ± 7</td>
<td>40 ± 6</td>
<td>16 ± 4</td>
</tr>
<tr>
<td><em>Brucella</em> antigens mixed liposomes with myristic acid (6)</td>
<td>38 ± 6</td>
<td>27 ± 5</td>
<td>35 ± 6</td>
</tr>
<tr>
<td><em>Brucella</em> antigens mixed liposomes with stearic acid (7)</td>
<td>37 ± 6</td>
<td>28 ± 5</td>
<td>34 ± 6</td>
</tr>
<tr>
<td><em>Brucella</em> antigens mixed liposomes with oleic acid (8)</td>
<td>39 ± 6</td>
<td>26 ± 5</td>
<td>36 ± 6</td>
</tr>
</tbody>
</table>

* The results are the means of 3 experiments ± S.D.
using *Brucella* antigens mixed with liposomes. Liposomes are lipid vesicles that form spontaneously upon addition of an aqueous solution to a dry lipid film [3]. Liposomes have been used to deliver chemotherapeutic agents, immunomodulators, and antifungal agents in vitro [14, 15] and in vivo in animals and humans [16–18]. Liposomes favour endocytosis and the incorporation of lipids in the membrane [5]. It has been suggested that changes in the physical state of the membrane lipids caused by alterations in fatty acid composition could modulate membrane-mediated functions [19–24].

We have previously tried to manipulate the functional response of polymorphonucleates, lymphocytes and macrophages by treating with phospholipids that have different polar groups and different isomerisms with saturated and unsaturated fatty acids from C₁₂ to C₂₀ [5, 6]. In these, and the present study, we used liposomes that act as adjuvants, and that stimulate spleen lymphocytes both in vitro and in vivo. The latter effect is enhanced by LPS and ConA. Treatment with antigens mixed with liposomes remarkably increases the cell response in vivo.

Protective antigens against *Brucella* infection were found in both soluble and insoluble complex fractions extracted from the *Brucella* cell wall [25, 26]. We used *Brucella* antigens obtained from suspension of *Brucella* broken with ultrasound. *Brucella* antigens mixed with liposomes containing myristic or stearic acid, primed in vivo, act mainly on the T<sub>c</sub> lymphocyte population. A total increase of lymphocyte population in circulation and in germinative centres resulted after immunization; however, with the liposome treatment a prevalent percentage of lymphocyte T<sub>c</sub> was obtained.

Liposomes containing oleic acid had a similar effect, albeit to a lesser extent. Exposure to *Brucella* is known to trigger an immune response that can be transferred by either immune sera [27–31] or immune spleen cells [29, 32, 33]. Treatment of animals with the *Brucella* antigen-liposome mixture favours a cell-mediated response. Our results show a large increase in ³H-thymidine incorporation by lymphocytes primed in vivo and stimulated in vitro with ConA compared with those stimulated with LPS; by non-primed lymphocytes in vitro, an increase was observed only with ConA and LPS; the slight stimulation observed in non-primed lymphocytes with *Brucella* antigens in saline solution was probably due to contamination by components of the *Brucella* outer membrane (lipopolysaccharide and membrane proteins) in our preparations.

We do not know the mechanisms underlying in vivo priming by liposome-mixed *Brucella* antigens. Liposomes could mediate the introduction and internalization of the antigen components in the pathway of the membrane structures thereby favouring major histocompatibility complex class I presentation.

This is supported by the localization of labelled liposomal constituents in the membrane structures of cells from the popliteal ganglion of treated mice. The penetration of liposomal constituents into heterochromatic nuclear areas needs further consideration. However, it is known that phospholipids are present in the nucleus, and variations in the qualitative and quantitative composition of the chromatin-associated phospholipids have been described in different cell
types and in varying metabolic conditions [34–39]. Furthermore, in recent studies it has been observed that phospholipids are localized in the nuclear domains involved in the synthesis, maturation and transport of ribonucleoproteins [40].

Taken together, our results suggest that the antigen-liposome mixture may prove useful in vaccine design.

References


22 Wilson, G., Rose, S.P. and Fox, C.F. (1970) The effect of...


