Studies were conducted in mice, hamsters, sheep, and two species of nonhuman primates which demonstrate the adjuvant activity of a new metabolizable lipid emulsion with marginally immunogenic doses of Formalin-inactivated viral vaccines. The lipid base consists of highly refined peanut oil emulsified in aqueous vaccines with glycerol and lecithin. Hamsters and mice inoculated with lipid emulsion plus western or Venezuelan equine encephalitis vaccine were significantly more resistant than vaccinated controls to lethal homologous virus challenge. Sheep given one dose of lipid emulsion plus Rift Valley fever vaccine developed significantly higher antibody titers than control sheep receiving only vaccine. Cynomolgous monkeys inoculated with lipid emulsion plus Rift Valley fever vaccine developed 16-fold greater peak primary and 20-fold greater secondary antibody titers than those of vaccine controls. Similar lipid emulsion-Rift Valley fever studies in rhesus monkeys resulted in 37- and 300-fold increases in primary and secondary titers, respectively, compared with monkeys given vaccine alone. Neither the sequence of combining antigen with lipid nor the exact ratio of aqueous phase to lipid phase affected the survival of Venezuelan equine encephalitis-vaccinated mice challenged with homologous lethal virus. This lipid formulation has several advantages over other water-in-oil adjuvants for potential use in humans. The components are metabolizable or normal host constituents, it is easily emulsified with aqueous vaccines by gentle agitation, and it is relatively nonreactogenic in recipients.

There is an intensive search by numerous investigators to find safe and effective immune adjuvants for use with microbial vaccines. Freund complete or incomplete adjuvants are the classic adjuvants to which most others are compared, but their reactogenicity precludes clinical use in animals or humans (5, 23, 30). Within recent years, numerous adjuvants have appeared in the scientific literature which effectively potentiate the immune response to numerous antigens and several of these compounds exhibit minimal or no toxicity in recipients (19, 36). In the early 1980's, Di Luzio and co-workers described a radiolabeled corn oil emulsion for the measurement of reticuloendothelial function in humans and experimental animals (9; S. J. Riggi and N. R. Di Luzio, Fed. Proc. 21:279, 1963). This formulation, based on a slight modification of an emulsion described by Zellersmit (37), was shown by Ashworth et al. (1) to be localized almost exclusively within the reticuloendothelial system when given intravenously. This was in contrast to other lipid emulsions (LEs) and chylomicrons which were distributed both in hepatocytes and phagocytes (1, 8, 9). At about the same time, Hilleman and co-workers were developing a metabolizable LE adjuvant 65, whose major component was peanut oil (35). Hilleman's group demonstrated the adjuvanticity of this emulsion when used with influenza vaccine in both experimental animals and humans (16, 17, 34).

Because many adjuvants appear to function by their action on macrophages (4, 12, 14, 19, 24), we have utilized the purported adjuvanticity of peanut oil and the selective localization of Di Luzio's corn oil emulsion in macrophages to develop a new metabolizable LE for use with aqueous inactivated viral vaccines. The purpose of this report is to demonstrate the ability of this adjuvant formulation to potentiate both humoral and protective immunity in several experimental animal models with marginally immunogenic doses of nonreplicating viral antigens.

MATERIALS AND METHODS

Animals. Outbred male CD-1 mice (Charles River Breeding Labs, Wilmington, Mass.) or inbred male AKR/J mice (Jackson Laboratories, Bar Harbor,
solutions were made with sterile pyrogen-free saline, the challenge studies were done in mice and hamsters to establish a margin. Each vaccine, either undiluted or diluted to its original volume, was added to Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) in a 0.4 ml of aqueous saline combined with 2% heat-inactivated fetal bovine serum. The virus challenge dose was titrated by intraperitoneally inoculating serial 10-fold dilutions into nonvaccinated mice or hamsters and calculating the 50% lethal dose as previously described (28).

**Antibody determinations.** Individual animals were bled at intervals indicated in each experiment, and all sera were stored at -70°C until examined for neutralizing antibody. Plaque reduction neutralizing (PRN) antibody titrations to WEE (strain B-11), VEE (strain TC-83), and RVF (strain Zagmaz 501) viruses were done in triplicate on individual samples using vero cells grown in six-well plastic trays (10). Starting with an initial dilution of 1:8 for the WEE hamster or VEE mouse sera and 1:10 for the RVF sera, serial twofold dilutions were made in a microtiter plate and assayed in triplicate for plaques per well. The ability of each serum to neutralize virus was calculated as a percentage of uninfected control wells.

**Immunization and challenge studies.** Mice or hamsters were inoculated subcutaneously (s.c.) with marginally immunogenic doses of vaccine combined with LE or saline as shown in the tables of individual experiments. The volume of inoculum in each experiment was 0.1 ml. Inoculation of the hamster was done intramuscularly (i.m.) on days 0 and 28 with 0.1 ml of aseptically prepared saline containing 1% heat-inactivated fetal bovine serum. The greatest serum dilution giving 90% plaque reduction (PRN90) in the hamster and sheep studies or 50% plaque reduction (PRN50) in the monkey studies was selected as the endpoint. Endpoints below the initial dilution were assigned a value of one-half the initial dilution to calculate the geometric mean antibody titer.

**Antigena.** Formalin-inactivated Venezuelan equine encephalomyelitis (VEE) and western equine encephalitis (WEE) vaccines used in these studies have been previously described (23). The virus antigena was inactivated at 37°C and emulsified with divided Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) in a 1:1 (vol/vol) ratio and emulsified with an emulsion churning (Mulschum, Multi-Jet, Inc., Elmhurst, Ill.).

**Challenge virus.** The Walter Reed Army Institute of Research B-11 strain of WEE virus (27) was used to challenge hamsters, and the Trinidad strain of VEE virus (23) was used in the mouse challenge studies. Inocula were diluted before challenge with Hank's balanced salt solution containing 1% normal, heat-inactivated rabbit serum. The virus challenge dose was titrated by intraperitoneally inoculating serial 10-fold dilutions into nonvaccinated mice or hamsters (7) and calculating the 50% lethal dose as previously described (28).
Statistics. Chi-square analysis with Yate's correction was used to compare mortality data of test groups with their appropriate controls. One-way analysis of variance was used for intergroup comparisons of antibody data.

RESULTS

Enhancement of protection from VEE virus challenge. The ALB combined with marginally immunogenic doses of VEE vaccine enhanced the survival of both inbred and outbred mice challenged with homologous virus 14 or 21 days postvaccination. AKR/J mice given 0.15 ml of vaccine plus 0.15 g of ALB and challenged day 14 postvaccination had a higher survival rate (P < 0.05) compared with those given vaccine alone (Table 1). On day 35 postvaccination, there were 70% survivors in the LE group compared with only 15% survivors in the vaccine control group. The reciprocal geometric mean antibody titer on day 14 was 9 in the LE group and not detectable in the vaccine control group. In a second study, outbred mice vaccinated s.c. with 0.3 ml of VEE vaccine plus 0.5 g of LE (ALB emulsified with 0.5 ml saline) were more resistant (P < 0.05) to virus challenge on day 21 postvaccination than were mice given only vaccine (Table 2). The LE-plus-vaccine group had 88% survivors on day 42 postvaccination compared with 38% in the vaccine control group. The Freund complete adjuvant group, which received an equal amount of vaccine, had only a 6% survival rate, which was not significantly different from mice given vaccine alone. The survival of mice receiving vaccine in combination with LE was significantly (P < 0.05) higher in both studies when compared with vaccine alone.

Enhancement of resistance to WEE virus. Hamsters which were inoculated s.c. with 0.3 ml of WEE vaccine (either a 1:5 or 1:10 dilution) combined with 0.3 g of ALB produced detectable levels of antibody by day 14 postvaccination (Table 3). The 1:5 vaccine dilution group had a reciprocal geometric mean antibody titer of 16, whereas the 1:10 vaccine dilution group had a titer of 8. In contrast, serum from hamsters which received either dilution of vaccine without LE had no significant plaque reduction on day 14.

Similarly, the survival of hamsters receiving LE, combined with either dose of WEE vaccine, was higher than that of animals given only vaccine or saline. The 100% survival rate in the 1:5 vaccine dilution group and the 94% survival rate in the 1:10 dilution group were significantly (P < 0.001) greater than the survival in controls of only 19 and 6%, respectively. There were no survivors in hamsters receiving only LE or saline.

Effects of mixing sequence and lipid concentrations on adjuvanticity. In this study, the effects of lipid dosage and mixing sequence of vaccine with lipid on survival of vaccinated

Table 1. Effects of LE on survival of vaccinated AKR/J mice challenged on day 14 with 200 50% lethal doses of VEE virus

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Reciprocal geometric mean PRN* antibody (range) on day 35</th>
<th>% Survivors on day 35 (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccine-ALB*</td>
<td>9 (&lt;8-32)</td>
<td>70*</td>
</tr>
<tr>
<td>Saline-ALB</td>
<td>(&lt;8)</td>
<td>5</td>
</tr>
<tr>
<td>Vaccine controls</td>
<td>(&lt;8)</td>
<td>16</td>
</tr>
<tr>
<td>Saline controls</td>
<td>(&lt;8)</td>
<td>0</td>
</tr>
</tbody>
</table>

* All treatments given s.c. in a 0.2-ml total volume.

Table 2. Effects of LE on survival of vaccinated CD-1 mice challenged 21 days postvaccination with 425 50% lethal doses of VEE virus

<table>
<thead>
<tr>
<th>Treatment (s.c.)</th>
<th>% Survivors* on day 42 (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccine-LE*</td>
<td>88*</td>
</tr>
<tr>
<td>Vaccine controls</td>
<td>38</td>
</tr>
<tr>
<td>Vaccine - Freund complete adjuvant</td>
<td>6</td>
</tr>
<tr>
<td>Saline controls</td>
<td>6</td>
</tr>
</tbody>
</table>

* Antibody determinations not done.

Table 3. Adjuvant effects of LE on humoral antibody response and survival of hamsters challenged on day 14 postvaccination with 1,200 50% lethal doses of WEE (B-11) virus

<table>
<thead>
<tr>
<th>Treatment (g/hamster)</th>
<th>Reciprocal geometric mean PRN* (range) on day 14 (n = 4)</th>
<th>% Survivors on day 28 (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEE (1:5)</td>
<td>0.3</td>
<td>16 (8-64)</td>
</tr>
<tr>
<td>Saline</td>
<td>(&lt;8)</td>
<td>19</td>
</tr>
<tr>
<td>WEE (1:10)</td>
<td>0.3</td>
<td>8 (8-64)</td>
</tr>
<tr>
<td>Saline</td>
<td>(&lt;8)</td>
<td>6</td>
</tr>
<tr>
<td>Saline</td>
<td>0.3</td>
<td>(&lt;8)</td>
</tr>
<tr>
<td>Saline</td>
<td>0</td>
<td>(&lt;8)</td>
</tr>
</tbody>
</table>

* Not detectable (<8).

* P < 0.001 compared with the respective vaccine control group.
mice were examined. As shown in Table 4, either 0.2 ml of vaccine was added directly to the total dose of ALB and then diluted with saline, or conversely the saline was first added to the ALB and the resultant emulsion was combined with vaccine. Groups of 16 mice were immunized s.c. with the particular vaccine-LE combination as indicated (Table 4) and challenged on day 14 postvaccination with VEE virus.

The dose of LE given in combination with either dilution of vaccine had no significant effect on survival since the mortality rates between decreasing dosage groups were not statistically different. Furthermore, the sequence of adding the antigen to the ALB had no significant effect on survival. The groups which received the 1:4 vaccine dilution combined with a specific LE-vaccine mixture had lower survival rates in general when compared with the undilute vaccine-LE groups, but these differences were not statistically significant. In all but two groups which received LE plus vaccine, the percentage survival was significantly greater than the corresponding vaccine control group.

**Potentiation of antibody response to RVF vaccine.** The LE clearly enhanced both the primary and secondary antibody responses of cynomolgous monkeys immunized with RVF vaccine (Fig. 1). The peak primary antibody titer was 640 in the LE-treated group compared with 250 in the controls. The peak secondary titer of 11,760 in the LE group was nearly 20-fold greater than the peak secondary antibody titer of 536 in vaccine controls. In addition, the titers on day 14, after only one dose of LE-vaccine, were already higher than the highest titers in control monkeys, even after two doses of vaccine alone.

**Table 4. Effects of mixing sequence and lipid concentration on survival of vaccinated CD-1 mice challenged on day 14 postvaccination with 7 x 10^6 50% lethal doses of VEE virus**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lipid dose (g)</th>
<th>Ratio (aqueous:lipid)</th>
<th>% Survivors, day 35 (n = 16)</th>
<th>1:4 Vaccine dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEE-LE^1</td>
<td>0.2</td>
<td>1:1</td>
<td>75</td>
<td>69</td>
</tr>
<tr>
<td>VEE-LE^2</td>
<td>0.2</td>
<td>1:1</td>
<td>94</td>
<td>94</td>
</tr>
<tr>
<td>VEE-LE^3</td>
<td>0.1</td>
<td>2:1</td>
<td>94</td>
<td>81</td>
</tr>
<tr>
<td>VEE-LE^4</td>
<td>0.1</td>
<td>2:1</td>
<td>100</td>
<td>81</td>
</tr>
<tr>
<td>VEE-saline</td>
<td>0.05</td>
<td>4:1</td>
<td>94</td>
<td>78</td>
</tr>
<tr>
<td>Saline-LE</td>
<td>0.05</td>
<td>4:1</td>
<td>94</td>
<td>56</td>
</tr>
<tr>
<td>Saline</td>
<td>0.2</td>
<td>1</td>
<td>44</td>
<td>19</td>
</tr>
<tr>
<td>Saline</td>
<td>0.2</td>
<td>2</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

^1 Total dose of ALB mixed with 0.2 ml of vaccine and then saline added to yield 0.4 ml.

^2 Total dose of ALB mixed with saline to yield 0.2 ml and then combined with 0.2 ml of vaccine.

^3 P < 0.05 compared with VEE-saline.

40 in the controls. The peak secondary titer of 11,760 in the LE group was nearly 20-fold greater than the peak secondary antibody titer of 536 in vaccine controls. In addition, the titers on day 14, after only one dose of LE-vaccine, were already higher than the highest titers in control monkeys, even after two doses of vaccine alone.

The potentiation of neutralizing antibody responses to RVF induced by LE was even greater in rhesus monkeys (Fig. 2). The peak primary antibody response of 560 in the LE group was 37 times higher than the peak primary response of 15 in controls. The peak secondary antibody titer of 27,000 in the LE group was nearly 300-fold greater than the peak titer of 92 in controls. In addition, antibody titers on day 7, in animals given LE plus vaccine were higher than any titers observed during the entire study in monkeys given vaccine alone.

Serum neutralizing antibody titers in sheep given LE plus undiluted RVF vaccine were also significantly elevated compared to vaccine control sheep (Fig. 3). The peak antibody titer of 380 on day 21 in the LE group was more than threefold greater than the peak titer of 113 on day 14 in the vaccine control group. Moreover, antibody titers in the LE-treated sheep ranged from two- to eightfold higher than titers in vaccine controls throughout the duration of the study.

Antibody titers in sheep given 1.0 ml of 1:5 dilution of RVF vaccine s.c. combined with
with various Formalin-inactivated viral antigens in mice, hamsters, sheep, and two species of nonhuman primates. This adjuvant has several advantages over other known adjuvant compounds. It is formulated from the normal mammalian body constituents, glycerol and lecithin, and naturally occurring metabolizable vegetable oil, i.e., peanut oil. Each of these components is approved for parenteral use in humans; they have been shown to be safe through their long history of widespread pharmaceutical applications in humans and animals. Utilization of these specific ingredients may circumvent some of the adverse effects induced by other synthetic or natural adjuvants, as well as water-in-oil emulsions, which may contain attenuated microbial agents, nonmetabolizable or irritating oils, and potentially harmful emulsifying or stabilizing agents. In addition, aqueous antigens are easily emulsified in the ALB by gentle agitation, thereby obviating the need for emulsion churns and laborious techniques usually required to produce water-in-oil emulsions.

This adjuvant's mechanism of action is not entirely known. The repository deposition of antigens in body tissues with mineral or vegetable oils is generally recognized as providing for the sustained release of antigenic materials (12). A localized inflammatory response is produced at the deposition site which brings together the triad of immunocompetent host cells needed to induce and amplify the immune response (12, 16). This specific lipid mixture, when combined with an aqueous antigen, readily forms microscopic lipid droplets of a very heterogeneous size (9, 37). These lipid droplets could theoretically mimic lymph lipoproteins in that there is a phospholipid membrane surrounding a core of lipid or aqueous material (18). Consequently, it may be possible for these lipid droplets to be readily mobilized by the recipient and find their way to the host's lymphatic tissues and mononuclear phagocytes. This concept, as opposed to the repository effect, is supported by the finding that the amount of lipid required for adjuvant activity (Table 4) is not highly critical.

Antigens could be incorporated both onto the phospholipid membrane and within the lipid-aqueous core (11, 18, 22, 33). It is thought that the present lipid formulation is unique in that it has a tropism for macrophages (1, 9, 32). Consequently, it may facilitate the presentation of antigen to lymphoid cells (14, 29) by fusion of its membrane with that of the macrophage (11, 25) and, concomitantly, enhance the internalization of antigens with a subsequent increase in efficiency of processing these foreign substances (2, 30). However, the most important factor in the

**DISCUSSION**

The present studies have demonstrated the adjuvant activity of a novel metabolizable LE
adjuvanticity of the present formulation may reside in the specific phospholipids found in the soybean lecithin used to make the ALB. Other investigators have shown a close correlation between specific phospholipids in model membranes and their subsequent immunogenicity (8, 20).

The adjuvanticity of this LE formulation has only been examined with Formalin-inactivated viral antigens, and since we have measured only the humoral component of the immune response, the present adjuvant’s role in amplifying cellular immune responses is unknown.

It is important to recognize the difference in antibody responses seen in the monkey studies compared with those seen in the sheep and hamster studies. In addition to the species variability seen in the utilization of PRN<sub>o</sub> endpoints, as in both monkey studies, will usually give endpoints two to four times greater than PRN<sub>o</sub> endpoints. Regardless of the endpoints used, the relative increases remain constant, and the significant differences still remain. More importantly, all monkeys were immunized and boosted by the i.m. route, whereas, sheep and hamsters received only one inoculation by the s.c. route. The i.m. route of inoculation is the recommended method for injection of repository vaccines (36) and perhaps the most efficient route as well.

Preliminary examination of the reactogenicity of this adjuvant suggests that, in a relative sense, it is virtually nonreactogenic in recipients. Sequential histopathological observation of s.c. injection sites in mice through 21 days postvaccination reveals a minimal and transitory inflammatory response. At the end of 21 days, there are a few droplets of lipid surrounded by polymorphonuclear leukocytes, lymphocytes, and relatively few mononuclear cells. A significant granulomatous reaction was not observed. We closely observed the cutaneous injection sites of LE recipients and never observed any abscesses or unusual swellings.

The use of any adjuvant in humans, of course, will not only depend on its lack of acute toxicity or reactogenicity in recipients, but also on the demonstration of its long-term safety and failure to induce adverse autoimmune disease or neoplasia. The components of the present lipid adjuvant have all been individually used extensively in humans, but it would be some time before the combination could be approved for widespread clinical use as an adjuvant for humans. As an alternative, this lipid emulsion could feasibly be approved for use in veterinary vaccines within a short time span. Its widespread use in animal vaccines could contribute significantly to the long-term toxicity data needed for licensing for use in humans.

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LITERATURE CITED


of host defense mechanisms by pharmacological agents.


