INFECTION AND IMMUNITY, June 1980, p. 937-943
0019-9567/80/03-0937/07$02.00/0

Vol. 28, No. 3

Adjuvant Activity of a Novel Metabolizable Lipid Emulsion with Inactivated Viral Vaccines

J. A. REYNOLDS,† D. G. HARRINGTON, C. L. CRABBS, C. J. PETERS, AND N. R. DI LUZIO

United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701,[1] and Tulane University School of Medicine, Department of Physiology, New Orleans, Louisiana 20112[2]

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 1960'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, 1962). This formulation, based upon a slight modification of an emulsion described by Zilversmit (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 coworkers 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,

† Present address: Pfizer Central Research, Groton, CT 06340.

Maine) weighing 20 to 25 g and male golden Syrian hamsters (Charles River Breeding Labs) weighing 80 to 90 g were housed in steel, closed-bottom cages with hardwood chip bedding. All rodents were fed a commercial diet (Purina Rodent Chow, Ralston-Purina Co., St. Louis, Mo.) and given water ad libitum. The monkeys used were healthy, mature, laboratory-conditioned cynomolgous monkeys (*Macaca fascicularis*) or rhesus monkeys (*Macaca mulatta*) of either sex which were seronegative for Rift Valley fever (RVF) virus neutralizing antibody. All monkeys were housed individually in suspended metal cages and fed a commercial monkey diet (Purina High-Protein Monkey Chow, 5045) twice daily and provided water ad libitum. The monkey diet was supplemented three times per week with fresh fruit. All animals were kept in rooms with a constant temperature (22°C) and a 12-h light-dark cycle. Mixed breed, laboratory-conditioned, adult sheep of either sex were kept in a 20-acre pasture during warmer months. During the winter, they were kept in a large corral with access to a barn and fed baled legume-timothy hay and water ad libitum. Their diet was supplemented daily with goat chow (Ralston Purina).

Adjuvants. (i) LE. White glycerin, USP (Fisher Scientific Co., Fair Lawn, N.J.) was passed through a 0.2-μm filter unit (no. 450-0020, Nalge Co., Rochester, N.Y.). Measuring materials on a weight basis, 1 part soybean lecithin (Centrolex-F, Central Soya, Ft. Wayne, Ind.) was dissolved in 10 parts glycerol by gentle heating (60°C) on a hot plate and stirring with a sterile Teflon magnetic bar. The glycerol and lecithin mixture was transferred to a sterile blender cup (250 ml) on a high-speed blender base (Waring CB-6, Dynamics Corporation of America, New Hartford, Conn.). Refined peanut oil, USP (lot 28-105, Welch, Holme and Clark, Co., Harrison, N.J.), was also passed through a 0.2-μm filter unit. While blending at moderate speed, 10 parts peanut oil was slowly added to the other components in the blender from a sterile separatory funnel. The resulting anhydrous lipid base (ALB) was apportioned into sterile, rubber-topped vaccine vials when still warm from mixing. The LE was made by mixing aqueous vaccine or pyrogen-free saline with the ALB as indicated in the specific experiments. Complete emulsification was facilitated by vigorous shaking of the vial for a brief period.

(ii) Freund complete adjuvant. Aqueous vaccine, diluted as indicated in the individual experiments, was added to Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) in a 1:1 (vol/vol) ratio and emulsified with an emulsion churn (Mulsichurn, Mulsi-Jet, Inc., Elmhurst, Ill.).

Antigens. Formalin-inactivated Venezuelan equine encephalomyelitis (VEE) and western equine encephalitis (WEE) vaccines used in these studies have been previously described (3, 5). Lyophilized, Formalin-inactivated RVF vaccine (NDBR 103, National Drug Co., Philadelphia, Pa.) was rehydrated with sterile water to its original volume. Antigen extinction assays were done in mice and hamsters to establish a marginally immunogenic dose for each lot of vaccine used in the challenge studies (6). All subsequent vaccine dilutions were made with sterile pyrogen-free saline.

Challenge viruses. 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 Hanks 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).

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 Zagazig 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 HBSS containing 2% heat-inactivated fetal bovine serum. The greatest serum dilution giving 80% plaque reduction (PRN_{80}) in the hamster and sheep studies or 50% plaque reduction (PRN_{50}) 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 group geometric mean antibody titer.

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 kept constant by substituting 1 ml of saline for each gram of ALB. Rodents were challenged with homologous virulent virus on either day 14 or 21 postimmunization and were observed daily for mortality. Percentage survival was calculated on day 21 postchallenge. Serum for WEE and VEE antibody determinations was collected from hamsters and mice by exsanguinating four animals from each group on day 14 postimmunization, just before challenging the remaining animals.

Antibody response studies to RVF vaccine. The ability of LE to potentiate the antibody responses to RVF virus was assessed in two species of monkeys and in sheep. Five cynomolgous monkeys were inoculated intramuscularly (i.m.) on days 0 and 28 with 0.4 ml of aqueous RVF vaccine combined with 0.4 g of ALB, and four control monkeys received only vaccine plus 0.4 ml of saline on the same days. Similarly, five rhesus monkeys per group were vaccinated i.m. with 0.5 ml of vaccine combined with either 0.4 g of ALB or 0.4 ml of saline on days 0 and 28. All monkeys were bled for antibody determination at approximately weekly intervals for 10 weeks.

Four sheep per treatment group were inoculated s.c. with one dose of ALB plus vaccine containing 1.0 ml of RVF vaccine, either undiluted or diluted 1:5 in saline combined with 1 g of ALB. Control sheep received only vaccine, either undiluted or diluted 1:5 and combined with 1.0 ml of saline. Blood was drawn for antibody determinations at weekly intervals for 3 weeks and then at approximately monthly intervals for 6 months.

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*

Treatment[a]	Reciprocal geometric mean PRN_{80} antibody (range) on day 14 ($n = 4$)	% Survivors on day 35 ($n = 20$)
Vaccine[b]-ALB[c]	9 (<8–32)	70[d]
Saline-ALB	(<8)[e]	5
Vaccine controls	(<8)	15
Saline controls	(<8)	0

[a] All treatments given s.c. in a 0.3-ml total volume.
[b] A 0.15-ml amount of a 1:15 vaccine dilution.
[c] ALB (0.15 g) mixed with aqueous vaccine.
[d] $P < 0.05$.
[e] Not detectable (<8).

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

Treatment (s.c.)	% Survivors[a] on day 42 ($n = 16$)
Vaccine[b]-LE[c]	88[d]
Vaccine controls	38
Vaccine - Freund complete adjuvant (1:1)	6
Saline controls	6

[a] Antibody determinations not done.
[b] A 0.3-ml amount of a 1:4 vaccine dilution.
[c] ALB (0.5 g) in 0.5 ml of saline.
[d] $P < 0.05$.

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*

Treatment	ALB (g/hamster)	Reciprocal geometric mean PRN_{80} (range) on day 14 ($n = 4$)	% Survivors on day 28 ($n = 16$)
WEE (1:5)	0.3	16 (8–64)	100[b]
	Saline	(<8)[a]	19
WEE (1:10)	0.3	8 (8–64)	94[b]
	Saline	(<8)	6
Saline	0.3	(<8)	0
	0	(<8)	0

[a] Not detectable (<8).
[b] $P < 0.001$ 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

TABLE 4. *Effects of mixing sequence and lipid concentration on survival of vaccinated CD-1 mice challenged on day 14 postvaccination with 7×10^3 50% lethal doses of VEE virus*

Treatment[a]	Lipid dose (g/mouse)	Ratio (aqueous: lipid)	% Survivors, day 35 ($n = 16$)	
			Undiluted vaccine	1:4 Vaccine dilution
VEE-LE[b]	0.2	1:1	75	69[d]
VEE-LE[c]	0.2	1:1	94[d]	94[d]
VEE-LE[b]	0.1	2:1	94[d]	81[d]
VEE-LE[c]	0.1	2:1	100[d]	81[d]
VEE-LE[b]	0.05	4:1	94[d]	75[d]
VEE-LE[c]	0.05	4:1	94[d]	56
VEE-saline	0		44	19
Saline-LE	0.2		6	6
Saline controls	0		25	25

[a] A 0.4-ml total volume given s.c.
[b] Total dose of ALB mixed with 0.2 ml of vaccine and then saline added to yield 0.4 ml.
[c] Total dose of ALB mixed with saline to yield 0.2 ml and then combined with 0.2 ml of vaccine.
[d] $P < 0.05$ compared with VEE-saline.

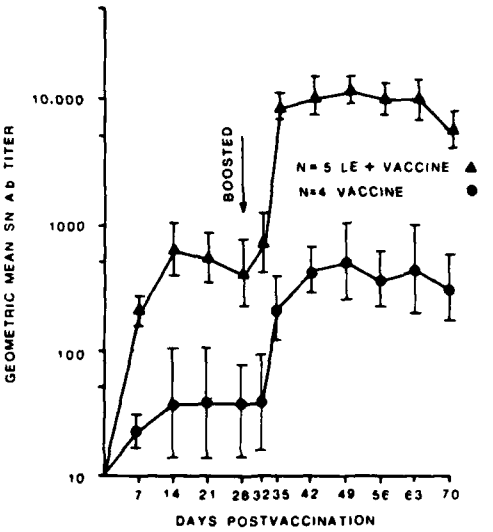

FIG. 1. *PRN_{50} antibody titers of cynomolgous monkeys inoculated i.m. on days 0 and 28 with 0.4 ml of RVF vaccine combined with either 0.4 g of ALB or 0.4 ml of saline.*

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 538 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 a 1:5 dilution of RVF vaccine s.c. combined with

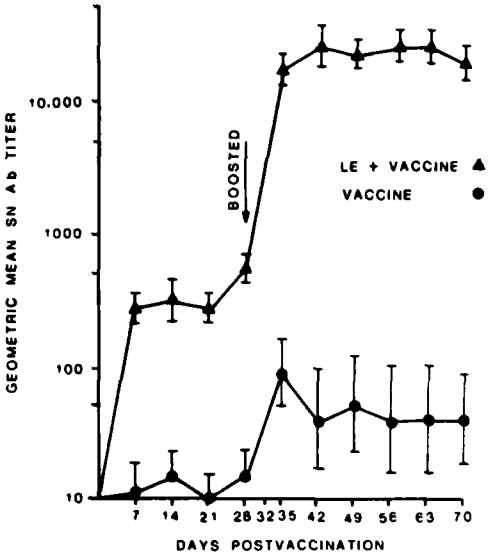

FIG. 2. *PRN_{50} antibody titers of rhesus monkeys inoculated i.m. on days 0 and 28 with 0.5 ml of RVF vaccine combined with either 0.4 g of ALB or 0.4 ml of saline (n = 5 per group).*

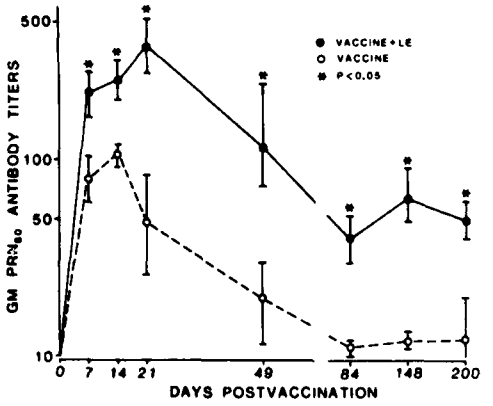

FIG. 3. *Antibody titers of sheep inoculated s.c. with one dose of 1.0 ml of RVF plus 1.0 g of ALB or vaccine alone (n = 4 per group).*

either 1 g of LE or 1 ml of saline were much lower than those receiving undilute vaccine. The peak antibody titer occurred on day 14 in both groups and was 57 in the adjuvant-treated group and 33 in the controls (data not shown). These differences were not statistically different.

DISCUSSION

The present studies have demonstrated the adjuvant activity of a novel metabolizable LE 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

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 in immune responses, the utilization of PRN_{50} endpoints, as in both monkey studies, will usually give endpoints two to four times greater than PRN_{80} 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.

ACKNOWLEDGMENTS

The excellent technical assistance of James R. Brown, Rhonda Lambert, Leif Hoffman, and Linda Stewart is gratefully acknowledged. We also thank Bill Prosise, Central Soya, Inc., Ft. Wayne, Ind., for supplying the lecithin and Barbara Kline for her expedient typing of the manuscript.

LITERATURE CITED

1. **Ashworth, C. T., N. R. Di Luzio, and S. J. Riggi.** 1963. A morphologic study of the effect of reticuloendothelial stimulation upon hepatic removal of minute particles from the blood or rats. Exp. Mol. Pathol. **2**(Suppl. 1): 83-103.
2. **Ashworth, C. T., V. A. Stembridge, and E. Sanders.** 1960. Lipid absorption, transport and hepatic assimilation studied with electron microscopy. Am. J. Physiol. **198**:1326-1328.
3. **Bartelloni, P. J., R. W. McKinney, F. M. Calia, H. H. Ramsburg, and F. E. Cole, Jr.** 1971. Inactivated Western equine encephalomyelitis vaccine propagated in chick embryo cell culture. Clinical and serological evaluation in man. Am. J. Trop. Med. Hyg. **20**:146-149.
4. **Chedid, L., and E. Lederer.** 1978. Past, present and future of the synthetic immunoadjuvant MDP and its analogs. Biochem. Pharmacol. **27**:2183-2186.
5. **Cole, F. E., Jr., S. W. May, and G. A. Eddy.** 1974. Inactivated Venezuelan equine encephalomyelitis vaccine prepared from attenuated (TC-83 strain) virus. Appl. Microbiol. **27**:150-153.
6. **Cole, F. E., Jr., and R. W. McKinney.** 1969. Use of hamsters for potency assay of Eastern and Western equine encephalitis vaccines. Appl. Microbiol. **17**:927-928.
7. **Cole, F. E., Jr., C. E. Pedersen, Jr., and D. M. Robinson.** 1972. Early protection in hamsters immunized with attenuated Venezuelan equine encephalomyelitis vaccine. Appl. Microbiol. **24**:604-608.
8. **Dancey, G. F., T. Yasuda, and S. C. Kinsky.** 1978. Effect of liposomal model membrane composition on immunogenicity. J. Immunol. **120**:1109-1113.
9. **Di Luzio, N. R., and S. J. Riggi.** 1964. The development of a lipid emulsion for the measurement of reticuloendothelial function. RES J. Reticuloendothel. Soc. **1**: 136-149.
10. **Earley, E., P. H. Peralta, and K. M. Johnson.** 1967. A plaque neutralization method for arboviruses. Proc. Soc. Exp. Biol. Med. **125**:741-747.
11. **Fendler, J. H., and A. Romero.** 1977. Liposomes as drug carriers. Life Sci. **20**:1109-1120.
12. **Freund, J.** 1956. The mode of action of immunologic adjuvants. Adv. Tuberc. Res. **7**:130-148.
13. **Friedman, H.** 1978. Macrophages in immunity—concluding remarks and general summary. Fed. Proc. **37**:102-104.
14. **Frost, P., and E. M. Lance.** 1978. On the mechanism of action of adjuvants. Immunology **35**:63-68.
15. **Haywood, A. M.** 1975. 'Phagocytosis' of Sendai virus by model membranes. J. Gen. Virol. **29**:63-68.
16. **Hilleman, M. R.** 1966. Critical appraisal of emulsified oil adjuvants applied to viral vaccines. Prog. Med. Virol. **8**: 131-182.
17. **Hilleman, M. R., A. F. Woodhour, A. Friedman, R. E. Weibel, and J. Stokes, Jr.** 1972. The clinical application of adjuvant-65. Ann. Allergy **30**:152-158.
18. **Jain, M. K.** 1975. Role of cholesterol in biomembranes and related systems. Curr. Top. Membr. Transp. **6**:1-57.
19. **Jordan, G. W., and T. C. Merigan.** 1975. Enhancement

of host defense mechanisms by pharmacological agents. Annu. Rev. Pharmacol. 15:157–175.
20. **Kinsky, S. C., and R. A. Nicolotti.** 1977. Immunological properties of model membranes. Annu. Rev. Biochem. 46:49–67.
21. **Lane, F. C., and E. R. Unanue.** 1973. Immunologic events during Listeria monocytogenes infection in mice: adjuvanticity and immunogenicity of macrophage-bound antigens. J. Immunol. 110:829–834.
22. **Mooney, J. J., J. M. Dalrymple, C. R. Alving, and P. K. Russell.** 1975. Interaction of Sindbis virus with liposomal model membranes. J. Virol. 15:225–231.
23. **Murray, R., P. Cohen, and M. C. Hardegree.** 1972. Mineral oil adjuvants: biological and chemical studies. Ann. Allergy 30:146–151.
24. **Nelson, D. S. (ed.).** 1976. Immunobiology of the macrophage. Academic Press, Inc., New York.
25. **Ozato, K., H. K. Ziegler, and C. S. Henney.** 1978. Liposomes as model membrane systems for immune attack. I. Transfer of antigenic determinants to lymphocyte membranes after interactions with hapten-bearing liposomes. J. Immunol. 121:1376–1382.
26. **Randall, R., and J. W. Mills.** 1944. Fatal encephalitis in man due to the Venezuelan virus of equine encephalomyelitis in Trinidad. Science 99:225–226.
27. **Randall, R., J. W. Mills, and L. L. Engel.** 1947. The preparation and properties of a purified equine encephalomyelitis vaccine. J. Immunol. 55:41–52.
28. **Reed, L. J., and H. A. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. 27: 493–497.
29. **Rosenthal, A. S., M. A. Barcinski, and L. J. Rosenwasser.** 1978. Function of macrophages in genetic control of immune responsiveness. Fed. Proc. 37:79–85.
30. **Stuart-Harris, C. H.** 1969. Adjuvant influenza vaccines. Bull. WHO 41:617–621.
31. **Unanue, E. R., and J.-C. Cerottini.** 1970. The immunogenicity of antigen bound to the plasma membrane of macrophages. J. Exp. Med. 131:711–725.
32. **Waddell, W. R., R. P. Geyer, I. M. Saslaw, and F. J. Stare.** 1953. Normal disappearance curve of emulsified fat from the blood stream and some factors which influence it. Am. J. Physiol. 174:39–42.
33. **Wilson, T., D. Papahadjopoulos, and R. Taber.** 1977. Biological properties of poliovirus encapsulated in lipid vesicles: antibody resistance and infectivity in virus-resistant cells. Proc. Natl. Acad. Sci. U.S.A. 74:3471–3475.
34. **Woodhour, A. F., D. P. Metzgar, G. P. Lampson, R. A. Machlowitz, A. A. Tytell, and M. R. Hilleman.** 1966. New metabolizable immunologic adjuvant for human use. 4. Development of a highly purified influenza virus vaccine in adjuvant 65. Proc. Soc. Exp. Biol. Med. 123:778–782.
35. **Woodhour, A. F., D. P. Metzgar, T. B. Stim, A. A. Tytell, and M. R. Hilleman.** 1964. New metabolizable immunologic adjuvant for human use. I. Development and animal immune response. Proc. Soc. Exp. Biol. Med. 116:516–523.
36. **WHO Scientific Group.** 1976. Immunologic adjuvants. WHO Technical Report Series no. 595. World Health Organization, Geneva.
37. **Zilversmit, D. B., N. K. Salky, M. L. Trumbull, and E. L. McCandless.** 1956. The preparation and use of anhydrous fat emulsions for intravenous feeding and metabolic experiments. J. Lab. Clin. Med. 48:386–391.