

Adjuvant Effects of the Lipid Amine CP-20,961

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Leukocyte migration into the injection sites and regional lymph nodes of inbred mice was studied by morphologic and autoradiographic techniques following s.c. inoculation of inactivated Venezuelan equine encephalitis virus vaccine (VEE) or vaccine combined with a biodegradable lipid vehicle containing CP-20,961. Adjuvanticity was demonstrated by the ability of these vaccine combinations to elevate plaque neutralizing titers and enhance protection from lethal virus challenge. The results indicated that vaccine combined with CP-20,961 in soybean oil lipid emulsion (Intralipid) induced transient acute inflammation in the injection site which was followed by lymphoid and monocytic infiltrates. By the fourteenth day no free lipid remained; however, lipid-laden macrophages were found at the site and epithelioid cells containing brown lipid degradation products were located in the subcapsular sinus and in crescents near germinal follicles of regional lymph nodes. Rapid expansion of the paracortex resulting from accelerated entry of recirculating T and B lymphocytes occurred in a dose dependent fashion 6 to 48 hr after s.c. inoculation of vaccine containing graded doses of CP-20,961. Only modest cellular accumulation was seen in nodes draining aqueous vaccine, or vaccine mixed with the lipid vehicle. Despite continued elevation of the rate of lymphocyte entry into these nodes they returned to nearly normal size by day 3 because of massive efflux of lymphocytes via efferent lymphatics. Proliferative activity and continued cellular influx in the paracortex resulted in a secondary peak in lymph node size by day 14. The magnitude of the latter node enlargement also varied according to the dose of CP-20,961. Thus CP-20,961 appears to behave as an immunologic adjuvant by enhancing cellular traffic through Ag depots and by potentiating the expansion of immunoreactive clones.

INTRODUCTION

Complete Freund's adjuvant is widely used to increase Ab formation (15) and cellular reactivity (45, 46) in experimental animals, but its application to vaccines for human use has been prohibited largely due to unacceptable acute and chronic toxicity (12, 18), and due to the risk of induction of autoallergic phenomena (14, 28, 29, 33, 38). Current interest in producing safe and effective vaccines directed against viral diseases which affect the health and economy of developing countries (29) has led to the development of several compounds (9, 23, 24, 27, 30, 32, 36) which might be used to potentiate marginal vaccines, alter the character of the immunologic response, or permit more widespread vaccination by reducing the required unit dose of Ag. One of these candidate adjuvants N, N-Dioctadecyl-N,

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N-bis (2-hydroxyethyl) propanediamine (CP-20,961) was originally developed as a topical interferon-inducer (23, 37). However, this lipid amine has been shown to enhance both humoral and cell-mediated immune responses to sheep erythrocytes, and the magnitude of immunopotentiality was equivalent or greater than that seen with complete Freund's adjuvant (35).

The ability of CP-20,961 to enhance antiviral immunity when used in conjunction with a dilute inactivated vaccine has not been previously demonstrated.

In the present studies we have examined the effects of CP-20,961 in an emulsion of aqueous vaccine and biodegradable lipid vehicle on cellular phenomena in the injection site and regional lymph nodes in addition to documenting the adjuvant effects of this lipid amine on antiviral immunity. The results indicate that CP-20,961 behaves as a potent adjuvant by producing prolonged increases in lymphoid cell traffic through Ag depots in the skin and regional lymph nodes while evoking minimal acute inflammatory changes in the injection site.

MATERIALS AND METHODS

Animals. CD-1 (Swiss) mice (Charles River) and BALB/C mice (Jackson Laboratories) 8-12 weeks of age were used for histologic and infectious challenge studies, while highly inbred BALB/C mice were used for lymphocyte traffic studies.

Agents. Formalin-inactivated Venezuelan equine encephalitis virus (VEE), MNLBR-109, Lot C-84-1 (Merrill National Laboratories) was used as the aqueous vaccine, diluted 1:5 in saline and administered in 0.1 ml aliquots. CP-20,961 (Lot #6480-156-2, a gift from Dr. Ivan Otterness, Pfizer Central Research, Groton, CT), was dissolved in 0.3 ml absolute ethanol, and added with or without Tween 80 to soybean oil lipid emulsion (Intralipid, Cutter Laboratories, Berkeley, CA) containing 10% soybean oil, 1.2% egg yolk phospholipids and 2.25% glycerin. The soybean oil fatty acids include linoleic 54%, oleic 26%, palmitic 9% and linolenic 8%. Complete Freund's adjuvant (Difco, Detroit, MI) was used as a water-in-oil emulsion. The following agents were administered as 0.2 ml doses: VEE in saline, VEE plus Intralipid, Intralipid plus saline, VEE plus 0.01, 0.1, or 1.0 mg doses of CP-20,961 in Intralipid, and VEE in complete Freund's adjuvant.

Experimental Design. Groups of 38 mice were inoculated s.c. with vaccine and/or adjuvant-vehicle combinations into the lymphatic drainage beds of the right inguinal lymph nodes. At sequential time intervals, 3 mice from each group were sacrificed and the abdominal walls containing the injection sites and regional lymph nodes were excised, fixed in formalin, and processed for histologic studies. During tissue processing the length, width, and thickness of each lymph node was measured with an ocular micrometer and the node volume was calculated.

Lymphocyte Traffic Studies. BALB/C mice were euthanized by cervical dislocation and the mesenteric, inguinal, axillary, submandibular and cervical lymph nodes were excised, immersed in cold RPMI 1640 and gently teased to release lymphocytes into suspension. The lymphocyte suspension was filtered free of debris and dead cells by passage over nylon wool at 4 C, washed, and resuspended in RPMI 1640 containing penicillin and streptomycin and 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY) to equal a concentration of 1×10^8 cells per ml. ^3H -uridine (New England Nuclear, Boston, MA, specific activity 21 Ci/mM) was added to yield a final concentration of 10 $\mu\text{Ci/ml}$ and the suspension

was incubated at 37C for 1 hr. The cells were washed twice, resuspended in RPMI 1640 containing 2% fetal calf serum and 5×10^7 labeled lymphocytes were injected into the tail vein of each recipient. The mice were sacrificed at 10, 30, 90 and 180 min after infusion and the regional and contralateral inguinal lymph nodes were removed and placed in cold 10% phosphate buffered formalin. The fixed lymph nodes were embedded in paraffin and sectioned at 4.0 μm thickness. Five sequential sections for each node were dipped in Kodak NTB-2 liquid emulsion and exposed for 8 weeks at 4C in light-tight boxes. After developing and fixing the slides were stained through the emulsion with hematoxylin and eosin and scanned by light microscopy. Labeled lymphocytes in the paracortex were counted through ten high powered fields in each node. The slope of the accumulating labeled cells equaled the rate of emigration or the number of lymphocytes per HPF per min.

Antibody Measurements. Three mice removed from each group were bled on the day of challenge to assess Ab levels. VEE plaque reduction neutralization titers were performed as previously described (25).

Infectious Challenge. Groups of 20 mice remaining from the above studies were challenged on the 14th day postvaccination by s.c. inoculation with 1000 MIP-LD₅₀ of virulent (Trinidad strain) VEE virus. Protection was indicated by better than 50% survival by day 35 postvaccination.

RESULTS

Local Injection Site. Neutrophil diapedesis into the s.c. fascial plane containing the injection mass was seen on the first day following injections of vaccine in Intralipid with CP-20,961, but not with Intralipid alone, or vaccine alone. This inflammatory infiltrate did not contain fibrin exudate as would be seen following inoculation of Freund's complete adjuvant. The intensity of inflammation appeared to be greater with higher doses of CP-20,961 but these differences could not be considered significant.

On days 2 and 3 following inoculation the neutrophils underwent necrosis and their cytoplasmic contents were released into the slightly edematous connective tissue (Fig. 1a). Monocytes and lymphocytes were first seen at the local site on day 3. Fibroblastic and angioblastic proliferation was also seen as granulation tissue penetrated the depot of necrotic neutrophils and lipid injection mass. By day 7 the lesion consisted almost entirely of fibrovascular connective tissue infiltrated with lymphocytes and monocytes, but in some specimens patches of amorphous basophilic material marked the sites where neutrophil exudate had been. Macrophages with foamy cytoplasm were seen at the borders of this lesion and in the draining lymphatics.

On days 14 and 21 postinoculation the size of the lesion appeared to be smaller and lymphocytes, foamy macrophages, and organized connective tissue remained at the injection site (Fig. 1b & 1c). Characteristic epithelioid cell granulomas were not seen. In one of the specimens lymphocytes were seen infiltrating the prickle cell layer of the acanthotic, hyperkeratotic squamous epithelium immediately overlying the injection site.

Regional Lymph Nodes. The inguinal lymph nodes draining injections of aqueous VEE vaccine enlarged gradually over a 3-day period, plateaued and receded by day 28. Between days 3 and 7 proliferative activity, indicated by the presence of scattered large immunoblastic lymphocytes, was seen within the paracortex but

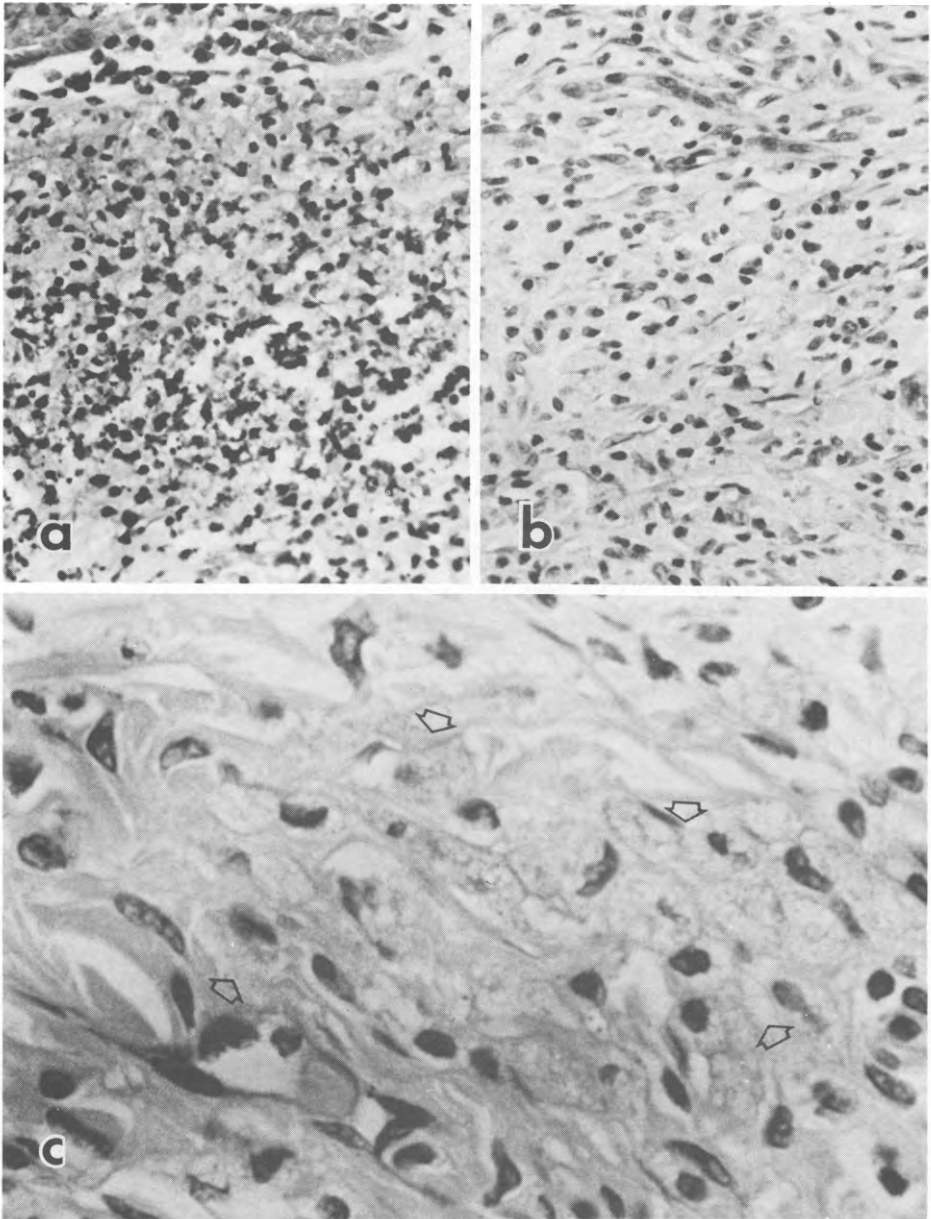


Fig. 1. (a-c) Illustrate changes in the local injection site. (a) Karyorrhexis of neutrophils at injection site 2 days after injection of CP-20,961, VEE, and Intralipid. (b) Organized connective tissue with monocytes and lymphocytes in local site 14 days after injection. (c) High magnification of 14 day lesion showing foamy macrophages and lymphocytes (arrows).

only segments of the cortical lobule were involved with these reactive nodules. In contrast, lymph nodes draining inoculations of vaccine in Intralipid, and vaccine in Intralipid containing CP-20,961 exhibited a biphasic early and late cycle of enlargement (Fig. 2).

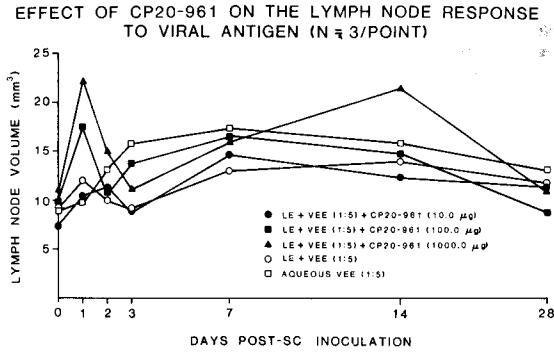


Fig. 2. Time course study of lymph node volume changes induced by the listed agents. Soybean oil lipid emulsion is (LE) and Inactivated Venezuelan equine encephalitis virus is (VEE).

The initial volume increase of these nodes occurred rapidly, appeared quantitatively related to the dose of CP-20,961, and peaked by 24 hr after inoculation. Edema was not associated with this early enlargement although there was vascular congestion and distention of high endothelial venules in these nodes. The paracortex was markedly expanded by tightly packed small lymphocytes. Planimetry documented that the cortex increased from 50 to 80% of the total node volume during this initial 48-hr period (Fig. 3). The large number of emigrating lymphocytes which were fixed enroute across the walls of the high endothelial venules and which also formed dense cuffs surrounding these vessels suggested that this cortical enlargement was due to a change in cell traffic rather than proliferation. Dense plugs of lymphocytes were seen filling intermediary sinuses in nodes excised 2 days after inoculation. By the third day these plugs were gone and the paracortex seemed to lose some of its density as large basophilic immunoblasts with prominent nucleoli appeared.

The later phase of node enlargement occurred gradually, also seemed related in magnitude to the dose of CP-20,961, and peaked 7 to 14 days after inoculation. The

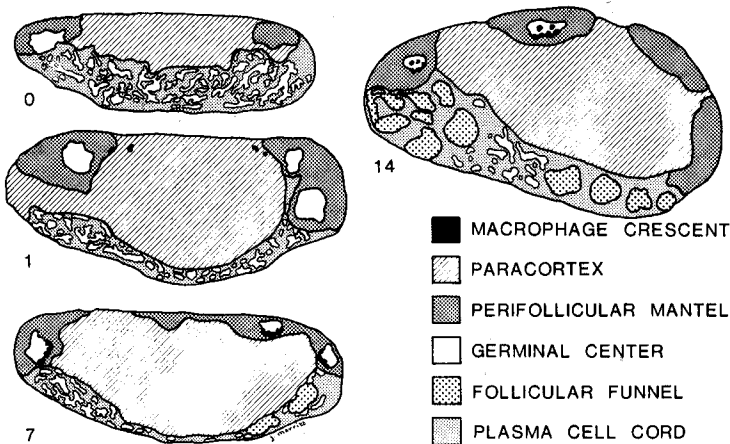


Fig. 3. Diagrammatic tracings of regional lymph nodes draining vaccine in Intralipid plus 0.01 mg. CP-20,961 which show the parenchymal changes quantitated by planimetry 0, 1, 7, and 14 days postinoculation.

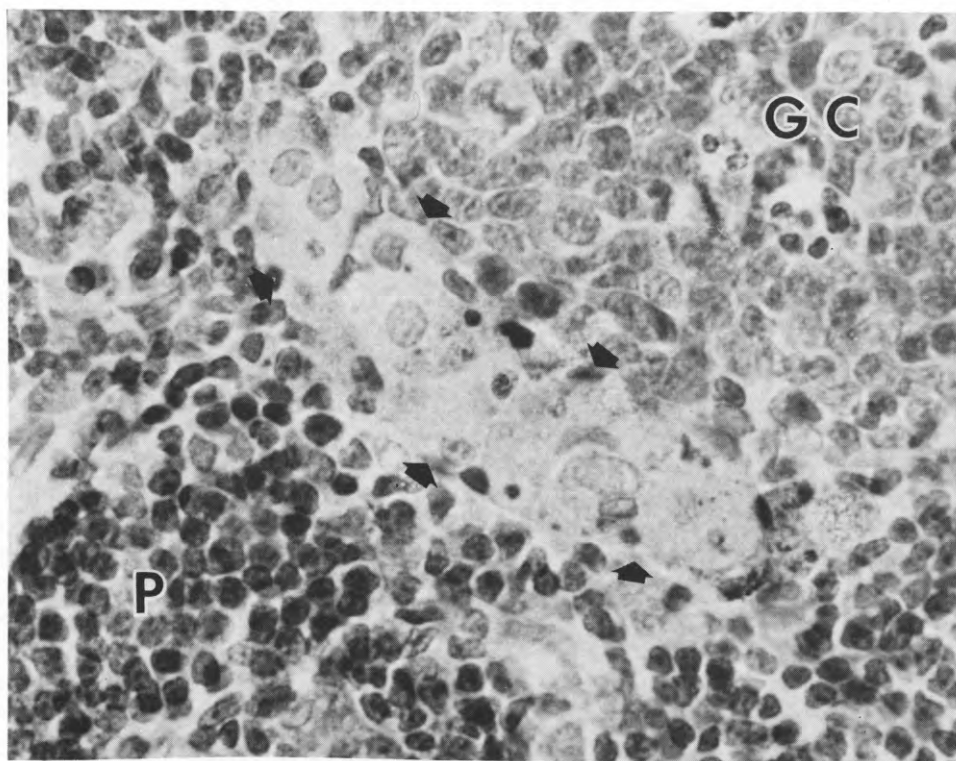


Fig. 4. Crescent of brown-pigmented epithelioid macrophages (arrows) separate germinal center (G.C.) from paracortex (P) and perifollicular lymphocyte mantle.

paracortex dominated the lymph node structure through day 14 and was still enlarged by large numbers of small lymphocytes and immunoblastic cells, but the planimetric ratio of cortical to medullary area was reduced due to expansion of the follicular funnels and plasma cell cords in the medulla by proliferating Ab forming cell precursors. Thus proliferation of immunoblastic cells in the presence of continued influx of lymphocytes was responsible for the second phase of node enlargement.

While germinal follicles seemed more prominent 7 and 14 days after inoculation with vaccine containing CP-20,961, there were no quantitative increases in the number or distribution of these structures. Tissue macrophages which may have traveled from the injection site were unexpectedly found associated with the deep cortical surfaces of germinal follicles. The source of these macrophages could be deduced since Intralipid produces a brown pigment when it is degraded by macrophages, and the macrophages which were found in afferent lymphatics, subcapsular sinuses and forming crescents near germinal follicles 7 days after inoculation bore large amounts of this brown pigment (Fig. 4). By day 14 some of these brown macrophages had entered the follicles.

Small clusters of epithelioid cells were found scattered through the paracortex

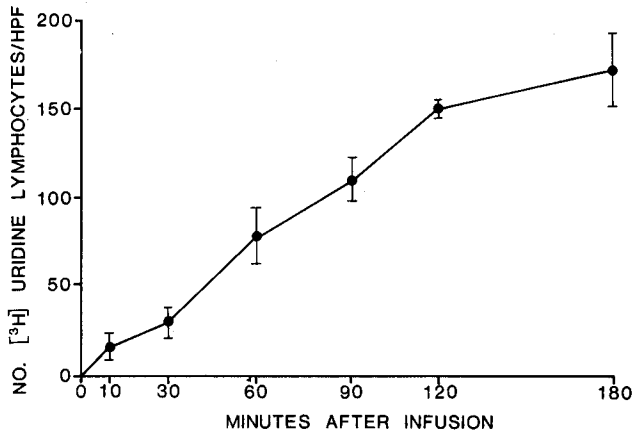


Fig. 5. Linear uptake of i. v. infused ^3H -uridine-labeled lymphocytes quantitated by autoradiography.

and intermediary sinuses of lymph nodes excised 14 to 28 days after inoculation with vaccine containing Intralipid and CP-20,961. While these structures clearly resembled granulomas, they lacked the clear lipid droplets, foreign body giant cells, and the acute inflammation and scarring associated with the lymphadenopathy induced by complete Freund's adjuvant. These granulomas induced by CP-20,961 did not cause distortion of the lymph node architecture and had completely resolved in specimens examined 60 to 100 days after inoculation.

Radiolabel Lymphocyte Traffic. Less than 1% of the injected radioactivity was recoverable in the regional and contralateral lymph nodes within the 180 min following i. v. infusion of 5×10^7 ^3H -uridine-labeled lymphocytes, but the increased sensitivity of light microscopic counts from radioautographs compensated for the low yield of whole tissue radioactivity. Lymph nodes containing 30 to 50 labeled cells per high powered field often produced counts barely above background when the remainder of the lymph node was extracted and counted in a scintillation counter. The accumulation of labeled lymphocytes in the paracortices of untreated lymph nodes was nearly linear through 130 min after infusion (Fig. 5). The slope of this line revealed the normal rate of emigration to be 0.96 to 1.35 labeled lymphocytes per high powered field per min. The rate appeared to fall off slightly by 180 min possibly because fewer labeled cells were available in the circulation to migrate into lymph nodes. Later, cells leaving the spleen enriched the blood levels of labeled cells and the rate returned to normal (4).

The rates of lymphocyte migration into lymph nodes draining injections of VEE in Intralipid plus CP-20,961 or VEE in complete Freund's adjuvant were consistently higher than the rates of migration induced by aqueous VEE (Table 1). Indeed, the higher doses of CP-20,961 produced more lymphocyte traffic than complete Freund's adjuvant. Increases in the rate of lymphocyte emigration were clearly related to the dose of CP-20,961 through 48 hr after inoculation, while at later time periods all of the doses produced comparable enhancement of cell traffic.

Transfused ^3H -uridine-labeled lymphocytes entered the lymph node parenchyma

TABLE 1
Effect of CP-20,961 on the Rate of Lymphocyte Emigration from the Blood
Radiographic Traffic Rate^a

Days After Inoculation	Aqueous VEE	VEE + Intralipid + Dose of CP-20,961			VEE CFA	Contralateral Nodes
		0.01 mg	0.1 mg	1.0 mg		
1	1.39 ± 0.23	2.00 ± 0.31	2.68 ± 0.28	4.57 ± 0.16	1.78 ± 0.21	1.35 ± 0.09
2	1.89 ± 0.19	2.46 ± 0.43	4.21 ± 0.12	5.81 ± 0.60	2.65 ± 0.34	1.29 ± 0.29
3	1.52 ± 0.16	2.62 ± 0.06	5.10 ± 0.11	5.52 ± 0.25	3.00 ± 0.18	1.41 ± 0.30
7	1.36 ± 0.34	3.86 ± 0.36	5.50 ± 0.36	4.37 ± 0.21	2.20 ± 0.40	1.36 ± 0.41
14	1.35 ± 0.12	3.48 ± 0.24	4.85 ± 0.26	3.65 ± 0.58	3.52 ± 0.24	1.30 ± 0.20
28	1.32 ± 0.30	3.14 ± 0.20	3.53 ± 0.29	2.82 ± 0.41	2.80 ± 0.36	1.40 ± 0.38

^aRate = No. ³H-V Lymphocytes/HPP/Min ($\bar{X} \pm SE$).

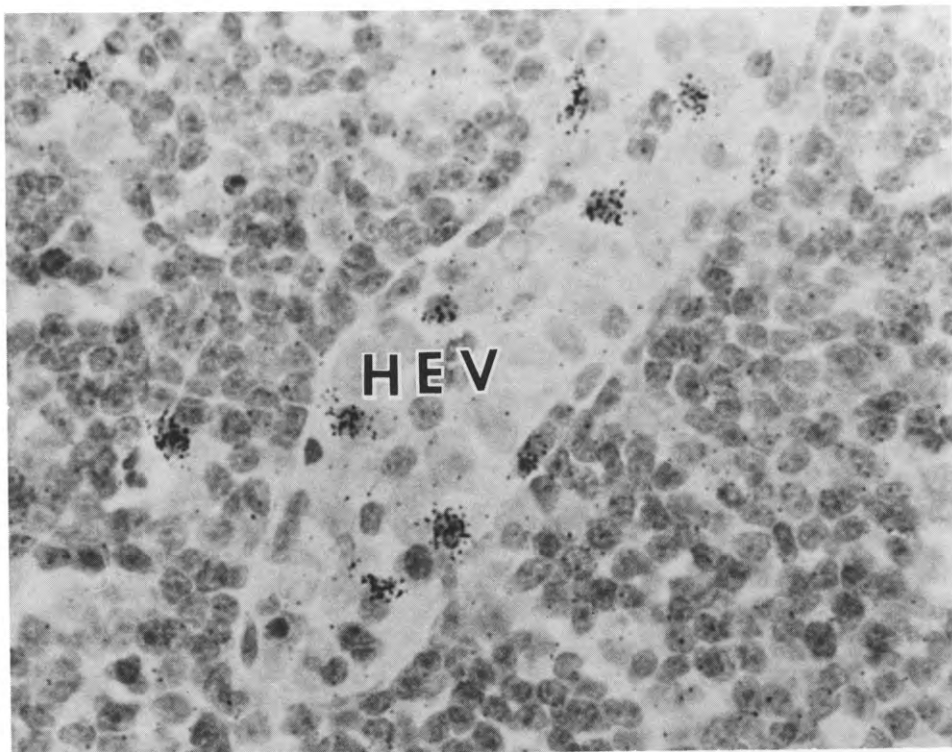


Fig. 6. Autoradiograph of lymph node paracortex showing high endothelial venule (HEV) site of entry of labeled circulating lymphocytes.

by crossing the walls of high endothelial venules which lay in the paracortex (Fig. 6). Labeled cells accumulated in the wall, reticular cell sheath and the interstitial tissue surrounding these vessels. The observation that T cells incorporate more ^3H -uridine than B cells (26, 41) was utilized to examine changes in the proportion of T cells to B cells in adjuvant-Ag draining lymph nodes. Heavy grain lymphocytes (T cells) comprised 69 to 75 percent of the paracortical cells during all time intervals after i.v. infusion, while only 40 to 50 percent of the emigrating cells were T cells in lymph nodes draining VEE-CP-20,961-Intralipid injections. Since there was a significant increase in total migrating lymphocytes this observation indicated increased selection of B cells.

Some labeled cells usually those with lower grain counts, migrated up to the edge of germinal centers, while others crossed the cortical interstitium and entered intermediary sinuses by passing between lymphatic endothelial cells. In lymph nodes draining injections of vaccine containing CP-20,961 substantial numbers of transfused labeled cells were found at the injection site, in afferent lymphatics and in the subcapsular sinuses of regional lymph nodes examined after 7 days post-inoculation. This observation suggested that a significant traffic of circulating lymphocytes was established at the injection site and provided the regional nodes with an additional source of immunocompetent cells.

Antiviral Immunity and Protection. On day 14 after vaccination groups of mice

TABLE 2.
Adjuvant Effects of Subcutaneous CP-20,961 on Antibody
Response and Survival of Vaccinated Mice Challenged on
Day 14 Postvaccination with 1,000 MIP-LD₅₀ VEE (Trinidad) Virus

Vehicle Group (Lipid Base)	Adjuvant Dose CP-20,961 (mg/Mouse)	Vaccine ^a	GEO \bar{X} SN Ab (Range) (N=3)	% Survival Day 35 (N=20)
Intralipid	0.01	+	25 (8-64)	90 ^c
	0.1	+	25 (<8-128)	90 ^c
	1.0	+	80 (32-256)	85 ^c
Intralipid Tween 80	0.01	+	128 (128) ^b	95 ^c
	0.1	+	128 (64-256)	90 ^c
	1.0	+	40 (16-128)	100 ^c
Intralipid	0	+	25 (16-32)	55
	0.1	-	0	0
	0	-	0	0
CFA	0	+	80 (40-160)	75
None	0	+	5 (<8-8)	50
None	0	0	0	0

^a0.1 ml of VEE Vaccine, diluted 1:5 with saline and combined with an equal volume of adjuvant or diluent.

^bMouse died during bleeding.

^cSignificance $P < 0.005$.

were bled for Ab determination and the remaining animals were challenged with virulent VEE (Trinidad strain) virus. The results indicated that Ab formation and protection from lethal challenge was significantly enhanced by vaccines containing CP-20,961 in Intralipid (Table 2). It appears that the minimal and maximal doses of CP-20,961 were equally effective in enhancing immunity to VEE, and all were slightly but not significantly more effective than CFA. Aqueous vaccine and vaccine suspended in Intralipid yielded comparable protective effects while vaccine in Intralipid produced slightly more Ab. The interferon inducing effects of Intralipid or CP-20,961 in Intralipid failed to protect mice challenged 14 days postinoculation.

DISCUSSION

The induction of immunity requires an intricate series of cooperative and regulatory cellular interactions among classes of T and B cells and macrophages (13). *In vivo* immune responses to non-replicating vaccine Ag most likely begin within the regional lymph nodes where specialized phagocytic cells bind and display the Ag (1) to recirculating and lodging T and B lymphocytes which enter the node by crossing the walls of specialized venules in the paracortex (5, 20, 21). Since migrating lymphocytes comprise a diverse immunoreactive repertoire, the traffic of lymphocytes through lymphatic tissues provides a natural environment for clonal selection (10, 20). The process of coordinating the cellular interactions necessary for induction of immunity is amplified by nonspecific and Ag-specific alterations in lymphocyte traffic through lymph nodes draining Ag inoculation sites (7, 11, 16, 43). Not all Ags are capable of accelerating lymphocyte traffic, and the ability of certain

Ag to promote lymphocyte emigration appears to correlate with the immunogenicity (11) and adjuvanticity (17) of the Ag.

Inactivated Venezuelan equine encephalitis virus vaccine (VEE) is a safe and effective immunogen. In the present studies VEE was used as a 1:5 dilution which was marginally immunogenic in mice, produced low level Ab and only 50 percent protection from lethal challenge. The synthetic lipid amine (CP-20,961) increased neutralizing Ab production and protective immunity as well if not better than complete Freund's adjuvant (CFA). Concerns about local reactogenicity (12, 18) were dispelled as CP-20,961 emulsified in biodegradable soybean oil emulsion (Intralipid) produced only mild acute inflammation which cleared completely, and the lipid depot was dispersed and carried away by foamy macrophages within the time period of this study. Important questions about the ability of CP-20,961 to evoke chronic residua such as thyroiditis (33), orchitis (14), encephalomyelitis (28) and arthritis (38) associated with the effects of CFA remain to be resolved, however.

The mechanisms of adjuvant action of CP-20,961 resembled those of CFA since paracortical reactivity predominated throughout the lymph node response (2, 17, 22, 31, 44, 48) and there was increased traffic of recirculating and lodging T and B lymphocytes through the regional lymph nodes (17) and injection site (47).

It is necessary to make a distinction between "lymphocyte trapping" which has been proposed as an adjuvant effect (17) and increased lymphocyte traffic. In this study we measured the rate of entry of circulating lymphocytes while studies dealing with "trapping" measured the total accumulation in the regional node at 24 or 48 hr compared to the accumulation in the contralateral node (17). Trapping studies may fail to record an increased traffic if the efflux of lymphocytes suddenly equals or exceeds the influx.

These conditions were met in the present study when CP-20,961 induced a biphasic lymph node enlargement which occurred as the rate of lymphocyte influx was still increasing. This suggested that the lymphocytes which accumulated through 48 hr were released into the efferent lymph. This biphasic sequestration and dumping of circulating lymphocytes is well documented in studies of the effect of Ag on the efferent lymph of sheep, and is associated with hemodynamic alterations (16, 22).

Increased traffic through adjuvant-Ag draining lymph nodes in this study was not confined to entry via specialized blood vessels. Fourteen days following vaccination, significant numbers of lymphocytes entered the regional node via afferent lymphatics originating in the injection site. This phenomenon also occurs following injections of CFA and is associated with the appearance of new blood vessels which morphologically resemble lymph node high endothelial venules (47). The induction of new vessels which support lymphocyte traffic has also been described in delayed hypersensitivity lesions (39) and in lymph nodes draining skin allografts (7). Although we have not specifically documented the proliferation of these specialized venules in CP-20,961 sites through electron microscopy, nonspecific esterase stains (8) or microvascular perfusion techniques (6), new vessels with plump endothelium and migratory lymphocytes were seen by light microscopy.

The shift in classes of lymphocytes entering lymph nodes draining Ag-adjuvants toward nearly equal proportions of B and T lymphocytes may have been influenced

by traffic from the injection site or may reflect recruitment of B cells by Ag (40). Absence of negative feedback control of migratory B cells may also explain the increased traffic. White (47) has seen crescents of epithelioid cells obliterating the surface of germinal follicles following CFA injections in chickens which is similar to the crescents described in this study. He proposed that the presence of this barrier of macrophages shielding the germinal centers prevents the negative feedback control of Ab production by immunoglobulins or immunoregulatory cells. An alternative explanation for the association of these epithelioid cell crescents with enhanced and persisting immunity might be the increased availability of Ag presenting macrophage surfaces to recently emigrated T and B lymphocytes.

The ability of CP-20,961 to evoke epithelioid cell granulomata in the regional lymph nodes resembled the effects of CFA, but the CP-20,961-induced clusters of epithelioid cells were not associated with the extensive inflammation, fibrosis, and architectural destruction seen following injections with CFA (19).

The lipid amine CP-20,961 is one of a series of new immunopotentiating agents (9, 23, 24, 27, 30, 32, 36) which might someday be used in combination with vaccines for agricultural or public health use. In cases where live virus vaccines, or whole inactivated vaccines are for some reason unsuitable and synthetic or weakly immunogenic subunit vaccines must be used (34) combination of these vaccines with CP-20,961 in lipid emulsion or on liposomes (3) might produce the desired result.

In conclusion, CP-20,961 in Intralipid enhances antiviral immunity without causing extensive local reactions and appears to act through a mechanism similar to complete Freund's adjuvant.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Ivan Otterness for providing the CP-20,961, Jonathon T. Warren for technical assistance and Mary Culler and Diane Hill for typing the manuscript.

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