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MULTIPLE EFFECTS OF IMMUNOLOGICAL ADJUVANTS ON LYMPHATIC MICROENVIRONMENTS

I. ROLE OF IMMUNOLOGICALLY-RELEVANT ANGIOGENESIS IN THE MECHANISMS OF ACTION OF CFA, MDP AND AVRIDINE

ANDERSON A. O.

Laboratory of Respiratory and Mucosal Immunity, Aerobiology Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701, U.S.A.

Summary: *Complete Freund's adjuvant (CFA), muramyl dipeptide (MDP) in oil, and the lipoidal amine Avridine, exhibit comparable adjuvant activities with regard to cellular and humoral immune responses to viral antigens when administered parenterally with antigen. The present study correlates the ability of the above agents to produce alterations in the lymphatic microenvironments of the local injection site and regional lymph node with enhancement of immunity. These adjuvants increase lymphoid and mononuclear cell traffic in the local site and regional lymph nodes, initially by effecting blood flow and transvascular chemotaxis, and subsequently by inducing angiogenesis and differentiation of specialized high-endothelial venules. The development of lymphatic tissue in the local injection site corresponds chronologically with the appearance of precursors of monocytes, interdigitating dendritic cells (IDC), fibroblastic reticular cells (FRC), and vascular endothelial cells in thoracic duct lymph. Thus effective adjuvants are capable of causing systemic alterations to lymphatic microenvironments through mobilization of various accessory cell types which may "metastasize" to resolving sites of inflammation. In addition, culture supernatants from these adherent cell types are effective* in vitro *as stimuli for lymphocyte chemotaxis, lymphocyte activation and endothelial cell proliferation. In conclusion, immunological adjuvants appear to be exceedingly complex in their mechanisms for enhancing the functions of the lymphatic system.*

Introduction

Immunological adjuvants are an ever-present component of experiments in cellular or molecular immunology, production of antibodies, and induction of autoimmune disease models, yet very little is known about how adjuvants work *in vivo*. Complete Freund's adjuvant will never be used in medical or veterinary vaccines because of the persistent inflammatory and tissue-destructive toxic effects, yet CFA is the most potent immunological adjuvant for enhancing both cellular and humoral immunity (1). Recently interest in development of adjuvants and immunomodulators has increased as concerns about persistent viral infections (2), virally-induced oncogenes (2), and viral aetiologies of endocrine malfunctions (3) and autoimmune diseases (4) lead the medical and veterinary professions to consider inactivated, purified-subunit, or genetically engineered polypeptide vaccines. Many of these non-replicating viral vaccines lack immunogenicity, some result in protection only for infectious

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challenges administered via the route of original vaccination (5), while others result in tolerance or immunosuppression. In addition, interest in adjuvants as agents that can change the nature of an immune response (6), or favour a specific immunoglobulin isotype, has developed (7).

Although the toxicity of CFA has inhibited large-scale interest in adjuvant development, a number of synthetic analogues of CFA (the muramyl dipeptides) (8) and novel lipoidal amines (9) have been developed which appear to have many of the positive attributes of CFA and few of the toxicities. In screening candidate adjuvants for potential vaccine use over the past 6 years, I observed that the effective agents produced similar alterations of microvasculature and mononuclear cell populations in local injection sites and regional lymphatic tissues.

The present report will summarize many of these published and unpublished observations in the hope that they will elucidate an *in vivo* mechanism of immunopotentiation that involves the participation of multiple cell types and multiple organ systems.

Materials and methods

Animals. The majority of these studies were carried out in Lewis rats (Microbiological Assoc., Walkersville, Md.), weighing 180–200 g, that were housed in environmentally-controlled facilities and fed conventional rat feed *ad libitum.*

Antigens. Formalin-inactivated Venezuelan equine encephalitis (VEE) vaccine (C-84) (MNLBR 109, lot# C-84-1, Merrell-National Drug Co., Swiftwater, Pa.) was used; the viral titre in filtered chick embryo culture fluid before inactivation was 9.5–10.0 $\log_{10}$/ml. Infectious challenge studies were carried out using the Trinidad strain of VEE virus.

Adjuvants. Complete Freund's adjuvant (Difco, Detroit, MI) was prepared by emulsification with aqueous antigen using a double-hubbed needle and two syringes. Muramyl dipeptide was a gift of Dr. Louis Chedid of the Pasteur Institute, Paris, France, and Avridine (previously called CP-20, 961) was a gift of Dr. Ivan Otterness of Pfizer Central Research, Groton, Conn. Both synthetic adjuvants were administered either in mineral oil or biodegradable soybean oil lipid emulsion (Intralipid, Cutter Labs. Berkeley, Calif).

Immunization procedures. All vaccine mixtures were administered under pentabarbital anaesthesia to the lymphatic drainage beds in the right lower abdomen which empty into the superficial inguinal lymph node (Fig. 1). This permitted ready access to both the injection site and the regional lymph node.

Immunoassays. The humoral immune response to VEE was quantitated by viral plaque neutralization on Vero cells infected with Trinidad-strain VEE as previously reported (10). Data are reported as geometric mean titres ($x10^{-1}$).

Tissue responses. The subcutaneous injection site and regional lymph node were excised from euthanized animals in contiguity with the skin of the abdominal wall, pinned to cork board and fixed in 10% buffered formalin for routine histology. The nature of the inflammatory response in the local site and alterations in lymphoid compartments of the regional and contralateral lymph nodes was determined by light microscopy of 1-μ plastic sections stained with toluidine blue. In like experiments the lymph nodes were excised and weighed.

Microvascular responses. The microvasculature of the injection site and the regional and contralateral lymph nodes were delineated by arterial perfusion with 1% Alcian blue dye (Eastman Kodak, Rochester, N.Y.) in normal saline (11) followed by fixation in 2% glutaraldehyde in phosphate buffer, clearing in absolute DMSO, and examination by light microscopy. The microvasculature was traced by projecting temporary microscope slides. Specific structures were excised from respective slices, embedded in Epon and identified by electron microscopy.

Radioautography. Adjuvant-inoculated rats were placed in Bollman cages and continuously infused with saline containing 3H-thymidine (specific

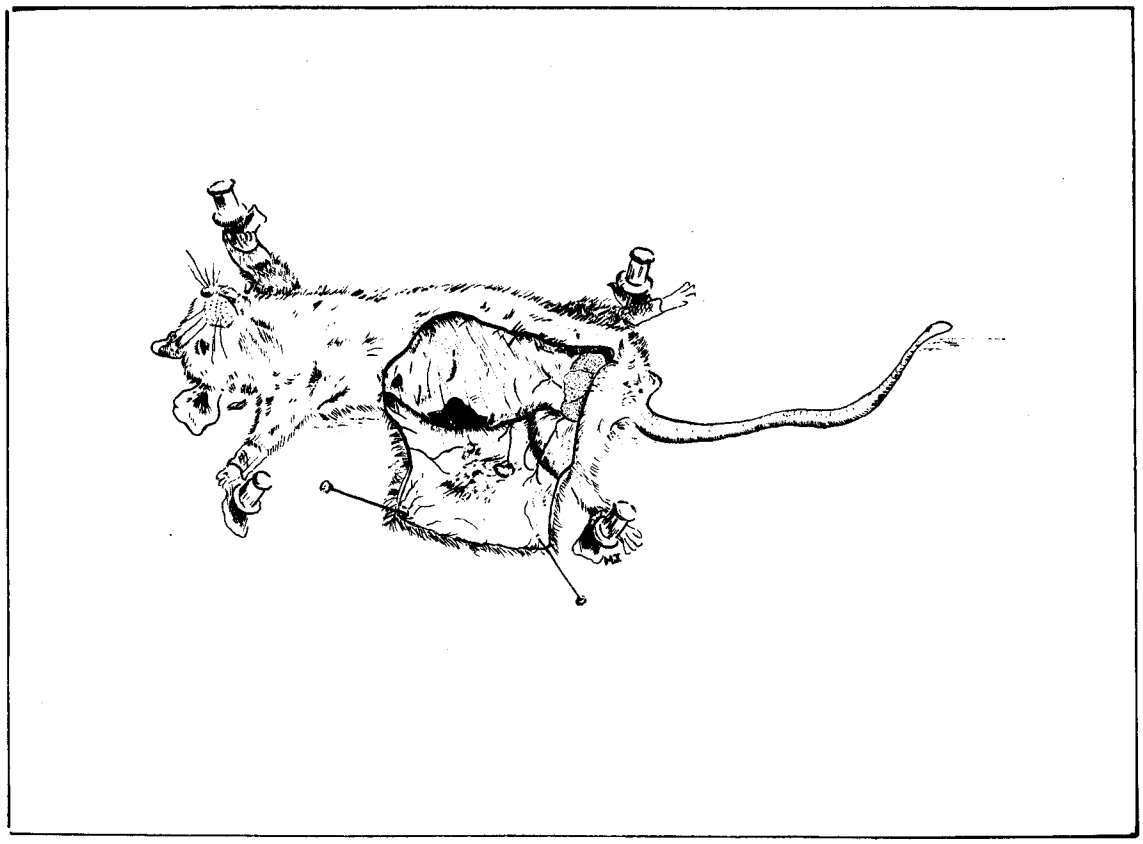

Fig. 1 Line drawing showing the location of the superficial inguinal lymph node with respect to the site of SC inoculation with test adjuvant.

activity, 15.8 Ci/mole; New England Nuclear, Boston, Mass.) at doses of 0.5 microCi/g per 24 h for 72 h, and the rats were euthanized. The local injection site and the regional and contralateral lymph nodes were excised, fixed in formalin and histological sections prepared. The slides were dipped in NTB-2 liquid emulsion (Eastman Kodak, Rockester, N.Y.), exposed for 5–8 weeks, developed and stained through the emulsion. The radioautographs were examined by light microscopy for the presence of silver grains over the nuclei of vascular endothelial cells. The labelling index equals the percent of all endothelial cells of a given type bearing 8 or more grains, divided by the total number of endothelial cells examined.

Lymphocyte traffic. Bollman fistulas were surgically implanted in Lewis rats, and populations of small lymphocytes enriched for a recirculating phenotype were collected from thoracic duct lymph (TDL) (15). Aliquots of 3×10^8 viable TDL were labelled *in vitro* for 30 min, using 3H-uridine at 10 mCi/ml, washed and injected i.v. Traffic was enumerated by radioautography as previously reported (10).

Results

The effects of s.c. inoculation of CFA were studied sequentially in 80 female Lewis rats by histologic, morphometric, vascular perfusion and radiokinetic techniques. The studies with MDP and Avridine were virtually the same, any differences being mentioned where appropriate.

The local injection site

On day 1 the initial local response to the deposit of CFA was acute fibrinopurulent inflammation with accompanied vascular hyperaemia. Polymorphonuclear leukocytes (PMN) and fibrin exudate surrounded the oil droplets when CFA or MDP were inoculated in mineral oil (Fig. 2). Avridine in soybean lipid was also associated with acute inflammation, but there were no oil droplets and the material spread diffusely in the dermal connective tissue. Between days 2 and 3 the acute inflammation intensified in the periphery of the depot as the PMN's degranulated adjacent to the oil containing CFA or MDP in oil and the connective tissue. In contrast, Avridine did not aggregate into an oil droplet and the acute inflammation began to recede by day 3. Fibrovascular proliferation penetrated the injection site between day 3 and 7 for CFA, MDP and Avridine, with the exception that the vascular infiltration did not penetrate the oil droplet containing CFA or MDP but remained confined to the fibrin matrix. The proportion of lymphoid cells and monocytes in these lesions increased progressively between days 3 and 14; however, foci of acute inflammation persisted near depots of CFA or MDP in oil. By day 7 the Avridine depot consisted entirely of fibrovascular connective tissue infiltrated with lymphocytes, monocytes and foam cells. Granulomas containing epithelioid cells and multinucleated giant cells were present in the periphery of the CFA and MDP in oil depots by days 14 and 21 after inoculation. Aggregates of lymphocytes surrounding mononuclear cells were present in all three sites. The oil droplets containing CFA or MDP were progressively walled off by connective tissue and by day 56 the oil appeared to be contained within a dense connective tissue sack. Large aggregates of small lymphocytes including germinal follicles were present adjacent to the sequestered oil. At the Avridine injection site characteristic tuberculoid granulomas were never seen although the foamy lipid-containing macrophages persisted there through 28 days. Smaller and more diffuse aggregates of lymphocytes were also present in the Avridine depot at these later time points.

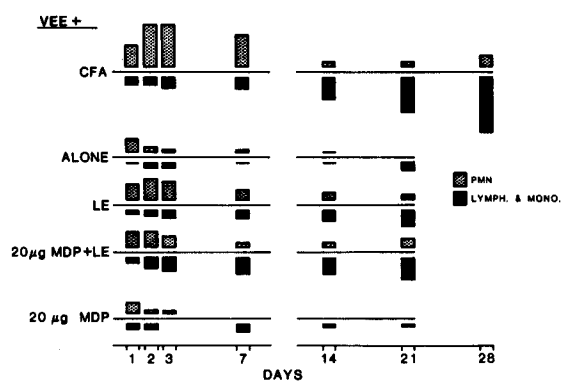

Fig. 2 The relative proportions of neutrophils (stippled) to mononuclear cells (solid) found in the local injection sites is shown for MDP and CFA over a 28-day time-course. CFA = Complete Freund's adjuvant; LE = Soybean lipid emulsion; MDP = Muramyl dipeptide.

Perfusion of the microvasculature with Alcian blue dye clearly delineated all vessels because the dye is soluble until it precipitates on the acidic glycoproteins on endothelial cell surfaces (11). On day 1 following inoculation of CFA, only dilatation of existing vessels was seen. Between day 1 and day 3 the vessels adjacent to the adjuvant depot became more tortuous, and branching "corkscrew-like" small vessels penetrated the fibrin matrix around oil droplets (Fig. 3). New vessel growth progressively increased through day 14, when some organization of the vessels was apparent. By day 21, vessels forming irregular hairpin loops were present. Some of these structures bore the lumen-within-lumen

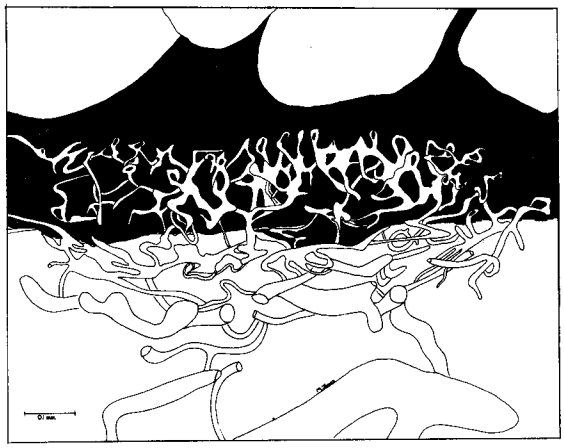

Fig. 3 Camera lucida tracing of the angiogenic response in the wall of a CFA deposit. The oil droplets are above, the crosshatch represents fibrin exudate and the tortuous looping vessels within the fibrin are all newly formed by days 3–7 after inoculation.

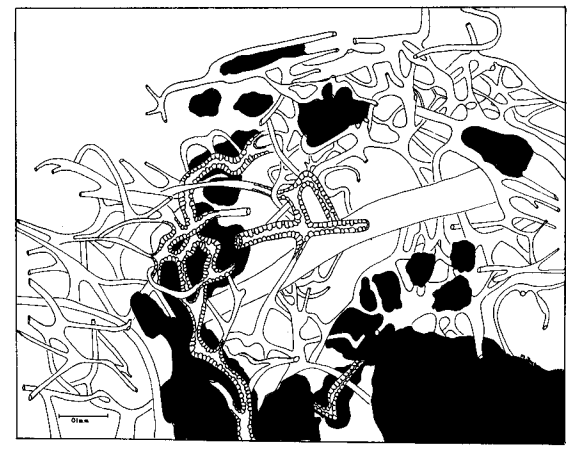

Fig. 4 Aggregates of small lymphoid cells (crosshatch), visible at 14 days after CFA inoculation, surrounding the vessels within the adjuvant depot that resemble HEV.

appearance of high-endothelial venules (HEV) usually found in lymphatic tissues (Fig. 4). No new vessel growth was found after day 28 but the unusual HEV-like vessels remained at least until the end of the study on day 56 after injection.

Radioautography of histological sections of the adjuvant depot indicated that 3H-thymidine was incorporated into small vessel endothelial cells between days 3 and 7 with maximal uptake present on day 7. Between days 14 and 21, endothelial cells in the HEV-like structures were also labelled. Following intravenous infusion of 3H-uridine-labelled TDL lymphocytes the first appearance of large-scale traffic of circulating lymphocytes into the area of adjuvant depot was between days 14 and 21.

The regional lymph node

Following subcutaneous inoculation of CFA, the regional lymph node underwent progressive enlargement which peaked on day 7 at nearly 3 times the weight of the contralateral node. The largest incremental increase occurred on day 1 when the node doubled its size, primarily due to the appearance of increased numbers of small lymphocytes in the deep cortex (Table I). At this time the lymphocyte migration index (LMI = lymphocytes in HEV wall /

Table I *Effects of CFA on the regional lymph node.*

Days past	Node wt (mg)	Cortex mass (mg)	Follicle mass (mg)	LMI L/En	Traffic cpm_R/cpm_C	HEV length microns
0	18	9.0	0.4	0.9	0.9	613
1	38	21.2	0.3	1.4	1.9	618
3	41	26.2	1.2	1.7	3.0	1199
07	53	37.0	1.2	1.4	2.2	1269
14	47	30.5	1.6	1.5	2.9	1251
28	38	22.8	2.2	1.5	1.6	2145
56	34	19.0	1.7	1.3	1.2	1740

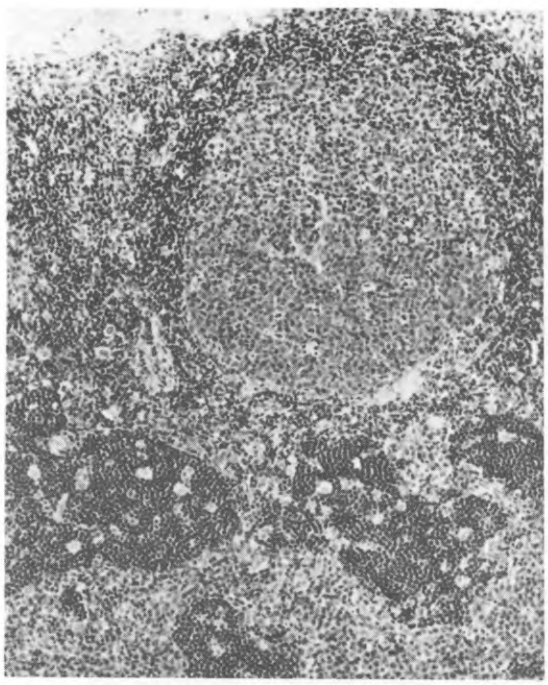

Fig. 5 Histological section of the superficial cortex of an adjuvant-draining lymph node. A follicle is present along with tightly-packed clusters of small lymphocytes within paracortical intermediary sinuses.

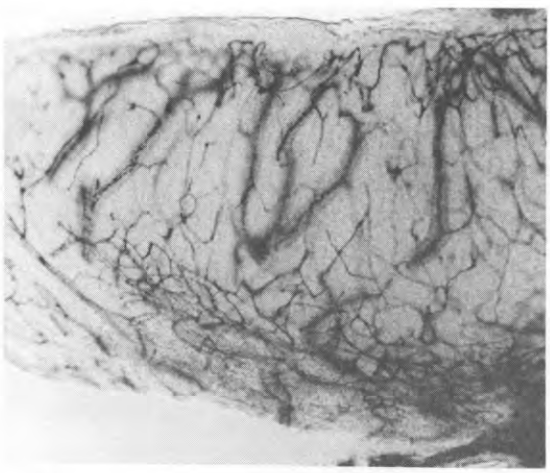

Fig. 6 Section 200 microns thick of an Alcian-blue perfused control lymph node. Note the lumen-within-lumen appearance of the HEVs.

Fig. 7 Camera lucida tracing of the section shown in Fig. 6, since the artist can refocus at different focal planes the distribution of HEV is complete.

total HEV endothelial cells) increased to a peak on day 3, remaining elevated throughout the study. On days 3–7 the intermediary lymphatic sinuses were plugged and distended with tight aggregates of small lymphocytes (Fig. 5). The mass of the germinal follicles began to increase by day 3 and peaked 28 days after injection. Oil droplets and granulomas containing multinucleated giant cells were present in the cortex by 21 days after CFA inoculation. Granulomas containing giant cells were present in regional nodes draining MDP-in-oil injection sites, but only sarcoid-like granulomas were induced by Avridine.

Vascular perfusion with Alcian blue clearly delineated the lymph node vasculature, especially high-endothelial venules. The microvasculature of the contralateral lymph node is shown in Fig. 6, and in Fig. 7 as a tracing. The HEV are vertically oriented and bear short and infrequent side branches. The initial vascular changes in the regional node draining

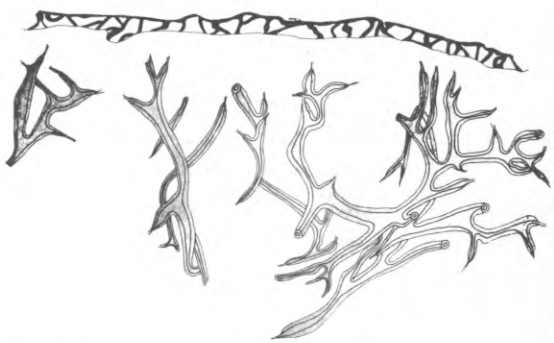

Fig. 8 Camera lucida tracing of a lymph node draining an adjuvant depot at 21 days. Note the increased length and arborization of the HEV compared to those in Fig. 7.

an adjuvant depot (CFA, MDP or Avridine) include capillary dilatation on days 1 and 2, venous dilatation on day 2, and evidence of increased permeability and extravasation between days 2 and 3. Between days 3 and 7 there is clear-cut evidence of increased length and arborization of HEV in the regional node (Table I). By day 14 the HEV have formed an interlacing plexus that runs throughout the deep cortex (Figure 8). The HEV remain prominent through day 56.

Radioautography following 3H-thymidine infusion indicated incorporation by HEV endothelial cells in side-branches and at proximal junctions with post-capillary venules lined with flat endothelium (Fig. 9). The labelling index for HEV endothelial cells was 26.2% in the regional lymph nodes as compared to 3.5% in the contralateral controls in rats continuously infused with 3H-thymidine between days 3 and 7 after injection of CFA. Traffic of 3H-uridine-labelled lymphocytes increased to a peak of 3.03 times control values by day 3 after injection and remained markedly elevated at least through day 14 (Table I). Virtually all the labelled lymphocytes were found near HEV and within the paracortex until day 14, when labelled cells began to enter the regional nodes via afferent lymph draining the adjuvant depot. Avridine and MDP produced dose-dependent increases in lymphocyte traffic into the regional lymph node cortex (Figs. 10 and 11).

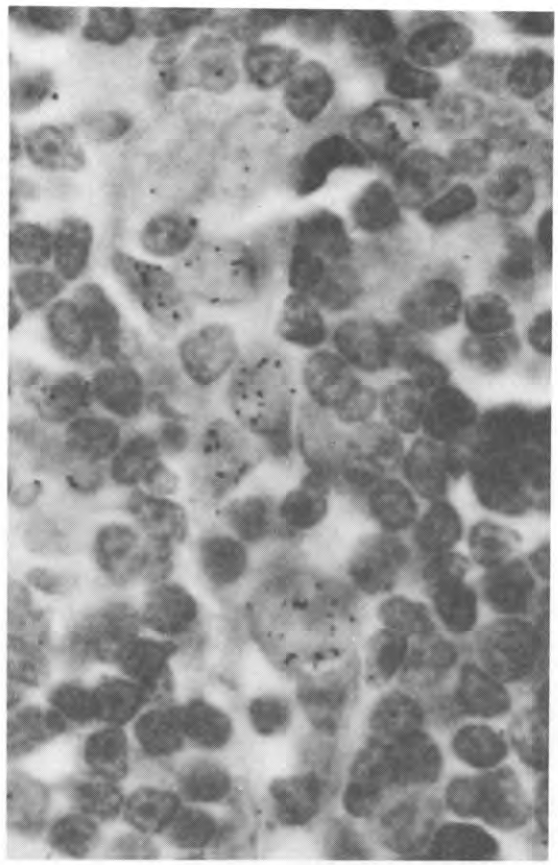

Fig. 9 Radioautograph of an HEV from a CFA draining lymph node in an animal who received 3H-thymidine. Note the silver grains deployed only over the endothelial cell nuclei.

Effect of adjuvant on antibody response

CFA and MDP in aqueous and mineral oil vehicles were compared, the results with Avridine having been reported elsewhere (10). Complete Freund's adjuvant (100 mcg/rat) significantly enhanced the primary plaque reduction neutralization titre to VEE when inoculated with the antigen (Fig. 12). The titre is increased on secondary boost with VEE vaccine. In contrast, aqueous MDP does not appear to poten-

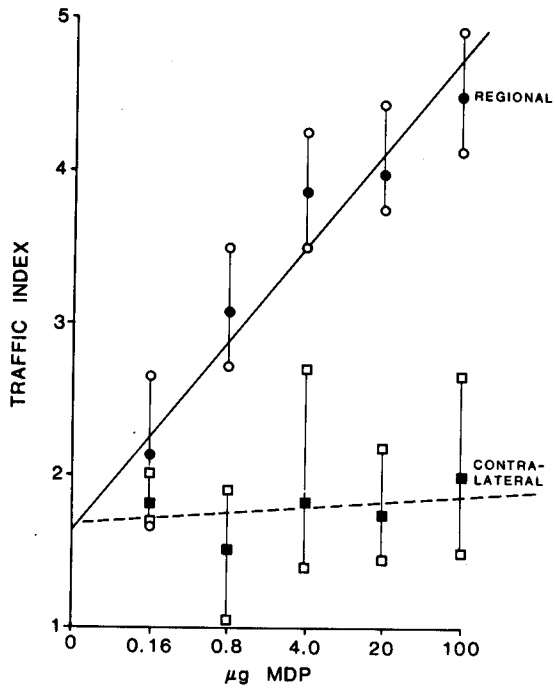

Fig. 10 Dose-related increase in lymphocyte traffic into regional lymph nodes draining sites of inoculation with doses of MDP.

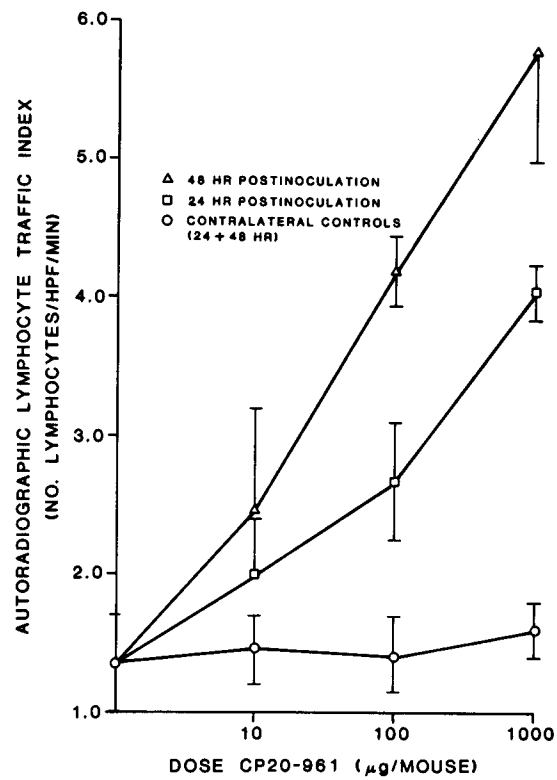

Fig. 11 Dose-related increase in lymphocyte traffic into regional lymph nodes draining sites of inoculation with Avridine (CP-20, 961), Means ± S.D.; n = 3.

tiate the primary response to VEE. However, a second inoculation of antigen resulted in titre increases that were comparable to those elicited in the CFA-treated rats. The potentiation of the secondary anti-VEE response elicited by priming with MDP-VEE appeared to be related to the dose of MDP used. When MDP was administered in a water-in-oil emulsion there appeared to be a dose-dependent suppression of the primary response (Fig. 13). Booster inoculation with antigen resulted in almost complete recovery of the immunopotentiation, however.

Discussion

It is clear from these studies that the principal effect of CFA and related adjuvants on the local injection site and regional lymph nodes is an alteration of the lymphatic microenvironments in such a way as to favour increased lymphocyte traffic, cellular cooperation (12), and increased activities of accessory cell types (13). Since lymphatic tissues are dependent upon lymphatic circulation and emigration of circulating lymphoid and mononuclear cells from the blood (14), the alterations in the microvasculature of the local site and regional lymph nodes described here must substantially contribute to the so-called adjuvant effects of CFA, MDP and Avridine. Early changes in blood flow to both sites

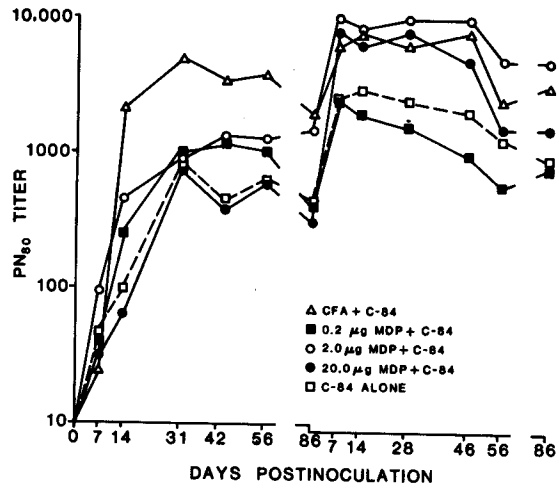

Fig. 12 VEE-specific viral plaque-neutralizing titres stimulated by aqueous MDP and CFA.

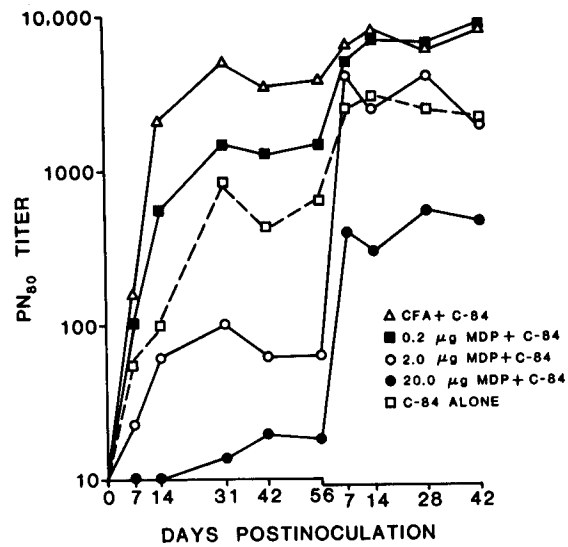

Fig. 13 VEE titre data for MDP as a water-in-oil emulsion.

appears to correlate with inflammation and the release of inflammatory mediators including interleukin-1 (IL-1). Induced release of IL-1 has been described for CFA and MDP (15). The ability of adjuvants to stimulate angiogenesis is not unusual, since adjuvants are known to activate macrophages which then release, among other biologically-active molecules, factors that induce angiogenesis (16) and stimulate proliferation of vascular endothelial cells and fibroblasts *in vitro*. However, the development of HEV vessels in the local site and regional lymph nodes is not ordinary angiogenesis. HEV appear to be critically important to the biology of recirculating lymphocytes since recirculation terminates in their absence (17). HEV express on their luminal surfaces receptor complexes that facilitate the selective attachment (18) and signal transmural emigration of blood lymphocytes into lymphatic tissues (19). Interruption of this recognition event will result in the inability of immunoregulatory and cooperative cell types to interact with antigen-presenting cells and with each other, because the most effective site for these interactions is within lymphatic tissues (20).

Avridine is a mild interferon inducer (9); MDP and CFA may also have effects on interferon release. The changes in lymphocyte traffic across HEV into the regional lymph nodes resemble the alterations in lymphocyte recirculation produced by pure interferons (21). It is not known whether interferon slows traffic through the node directly; by stimulating release of other cytokines; or by causing expression of important surface receptors (22) that will effect emigration and transit of recirculating lymphocytes. In addition, gamma-interferon has been shown to induce release mononuclear/myeloid cell colony stimulating factor (CSF-1)(23).

Release of colony CSF-1 is correlated with an increased output of small monocytes and precursors of dendritic cells in the thoracic duct lymph of CFA- or Avridine-treated rats (13) and with increased extramedullary haematopoiesis in spleens of mice treated with Avridine by the intraduodenal route (7). Increased availability of accessory cell types in

the circulation must lead to local development of ancillary lymphatic tissues which can assist in antigen presentation and the expansion of immunoreactive lymphocyte clones. Perhaps one of the effects of adjuvants that results in long-term immune enhancement is this ability to produce trophic effects on existing lymphatic tissues, and possibly to induce development of new lymphatic tissues in sites of antigen concentration (10).

The inability of MDP to potentiate a primary immune response is worthy of comment, because it suggests a cellular function of this small molecule that may be critically important to lymphocyte memory. The dose-dependent suppression of the PN/80% response to VEE induced by MDP was completely reversed in the potentiated secondary response. This could be explained if MDP had the ability to programme the proliferative response of the antigen-stimulated lymphocytes to turn out more memory cells and fewer effector cells. It is not known whether a similar suppression of a primary response is seen for T-cell phenomena such as cellular cytotoxicity, but the problem would be worth exploring.

In conclusion,* immunological adjuvants are exceedingly complex in their action, and attempts to simplify their mechanisms often fail because adjuvants work in intact organisms that integrate multiple organ systems to carry out nearly all bodily functions including initiation of an effective immune response. While alterations in the microvasculature are important to the "adjuvant effect", we presume that all the other cellular and molecular effects of adjuvants are also operating.

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* The views of the author do not purport to reflect the positions of the Department of the Army or the Department of Defense, U.S.A. (Para. 4-3, AR 360-5).

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