

EFFECT OF ORALLY ADMINISTERED AVRIDINE ON ENTERIC ANTIGEN UPTAKE AND MUCOSAL IMMUNITY

ANDERSON A.O., MACDONALD T.T.¹, RUBIN D.H.²

Laboratory of Respiratory and Mucosal Immunity, Aerobiology Division, USAMRIID, Fort Detrick, Maryland, 21701.

1) Department of Microbiology, Jefferson Medical College, Philadelphia

2) Department of Medicine, The University of Pennsylvania School of Medicine, Philadelphia, U.S.A.

Summary: *The lipoidal amine N, N-dioctadecyl N', N'-bis (2-hydroxyethyl) propanediamine (Avridine) was used to potentiate mucosal immunity in mice against reovirus (1/Lang), cholera toxin, and sheep erythrocytes following direct intraduodenal inoculation. Two- to four-fold increases in secretory IgA specific for reovirus and cholera toxin measure by ELISA or IgA/IgG indirect plaque-forming cells specific for SRBC were found by 7 to 14 days after priming, as compared to responses elicited in controls that did not receive adjuvant. Since secretory immunity is dependent on antigen processing by mucosal lymphatic tissues, we examined the uptake of labelled antigen using 3H-uridine-labelled reovirus and unlabelled Avridine or unlabelled antigen and 3H-Avridine. The tissues surveyed included: intestine, Peyer's patch, mesenteric lymph nodes, liver, spleen and lung. The results indicated that Avridine enhances uptake, localization and retention of antigen in Peyer's patches. Avridine also appeared to slow the degradation of antigen since labelled virus that was not associated with adjuvant rapidly lost its radioactivity at time points when adjuvant-treated virus was still detectable in Payer's patches. Thus Avridine may have general utility for oral vaccination against various mucosal pathogens and toxins through its ability to enhance antigen processing and presentation by mucosal lymphatic tissues.*

Introduction

Antigen priming by a mucosal route has been shown to be superior to parenteral inoculation in eliciting a protective secretory IgA response (1). Indeed, parenteral priming with nonreplicating antigens often depresses or prevents specific IgA responses when that antigen is subsequently

exposed to the mucosal (2,3). Conversely, mucosal priming is often associated with induction of peripheral tolerance or reduced IgG levels and cell-mediated immunity to parenterally-administered antigen (4-6). Moreover inactivated, attenuated, or toxoided antigens often fail to retail the essential immunogenic properties needed for an IgA response, and unmodified pathogens and toxins

Address for reprints: Dr A.O. Anderson, Laboratory of Respiratory and Mucosal Immunity, Aerobiology Division, USAMRIID, Fort Detrick, MD 21701, U.S.A.

carry with them unacceptable toxicity. Therefore studies aiming at the development of immunological adjuvants that can facilitate mucosal priming may simplify these difficulties, especially in cases where it is desirable to use polypeptide subunit vaccines produced by gene cloning. A number of studies have proposed that the ability of an antigen to elicit a secretory response is a function of its ability to be retained in mucosal lymphatic tissues. This could be due to specific receptors that facilitate binding of the antigen to mucosal surfaces, or the ability of the antigen to colonize the bowel lumen or invade the mucosa (7-10). It is instructive that gastrointestinal pathogens such as Salmonella (7), enterotoxigenic *E.coli* (8), reoviruses (11) and cholera toxin (9) normally have these properties, and convalescence is often associated with the presence of specific IgA in the secretions. The present study presents new data indicating that the immunological adjuvant Avridine enhances secretory immunity when delivered in combination with antigen by facilitating the uptake and retention of antigen in mucosal lymphatic tissues.

Material and methods

Animals. Adult female Balb/c mice (NIH) were used for the immunization and radiolabel uptake studies, and Balb/c mice from NIH and Jackson laboratories were used for enumeration of accessory Peyer's patches. All animals were housed in Biogard laminar-flow units and fed conventional mouse pellets.

Antigens. Reovirus serotype 1/Lang were propagated on mouse L cells and purified as described previously (11). The final concentration of viral particles was 3×10^{12} particles/ml as determined by optical density measurement at 260nm (11). Infectious titres of inoculated virus assayed on mouse L cells were approximately 100 times less than particle counts. Half the virus was inactivated by exposure to UV light for 45 min. Each

mouse received 1×10^{10} particles of either live or inactivated virus. The purified cholera toxin was lot 09-72 prepared by R.A. Finkelstein and provided by Juliett Fuhrman of the University of Pennsylvania. Each mouse received a 10-microgram dose. Purified sheep erythrocytes (SRBC) were administered in doses of 2×10^8 cells per mouse, initially by oral, intraduodenal (i.d.), or intraperitoneal (i.p.) routes, and subsequently by i.p. injection 6 days prior to harvesting the spleens for plaque assay. Avridine (previously called CP20-961) was provided by Ivan Otterness (Pfizer Central Research, Groton, Conn.) as a dry powder. To make a 6-mg/ml emulsion, 42 mg Avridine was dissolved in 0.3 ml absolute ethanol; 0.03 ml Tween 80 was added and the mixture was agitated with 6.7 ml of Intralipid (Cutter, Berkeley, Calif.). Avridine was mixed 1:1 with antigen and sonicated prior to inoculation.

Immunization procedures. Mice were anaesthetized with methoxyflurane and then immunized by direct i.d. injection through a small right upper quadrant laparotomy (12). The respective antigen was mixed with an equal volume of 6 mg/ml Avridine in soybean oil emulsion (Intralipid: Cutter laboratories, Berkeley, Calif.) and injected via 30-gauge needle and tuberculin syringe. The intestine was returned to the abdominal cavity and the wound was closed with silk sutures. Groups of mice were sacrificed at 1, 3, 7 and 14 days. The entire small bowel from duodenum through ileocaecal valve was removed, flushed with ice-cold saline and threaded onto a black glass rod for rapid assessment of the size and number of Peyer's patches. In other experiments the Peyer's patches were individually excised and weighed. The small bowel was opened along the mesenteric border and scraped with a scalpel blade to remove the mucous membrane. This material, containing epithelial cells mucus and other secretions, was sonicated and frozen for antibody measurements. Excised Peyer's patches, mesenteric lymph nodes, and spleens were prepared for histological examination.

Radiolabel uptake. Reovirus 1/Lang was grown

in the presence of 10 mCi/ml of ^3H -uridine to a specific activity of 600,000 DPM/ 10^{10} virions. Tritiated Avridine was obtained from Pfizer Central Research (specific activity 11.79 mCi/mg.). Labelled reovirus with or without unlabelled Avridine was injected i.d. as described above, and at sequential time intervals mice were sacrificed and the intestines, Peyer's patches, mesenteric lymph nodes, liver, spleen, and lungs were removed, weighed and sonicated in the presence of 1 ml of Tissuesolve. The identical procedure was carried out for labelled Avridine alone or with unlabelled virus. A Beckman scintillation counter was used to determine the DPM.

Immunoassays. Antibody in serum and in intestinal secretions was measured by enzyme-linked immunosorbent assay (ELISA) (13), and by haemagglutination (11) using previously published methods. Enzyme-labelled affinity-purified rabbit antisera specific for alpha or gamma heavy chains were used to score the response. Values are expressed as the optical density (OD) reading at 405 nm ($\times 10^{-3}$) of samples diluted 1/20, OD having been shown to be directly correlated with titre. An isotype-specific indirect erythrocyte plaque-forming cell assay (14) was performed on spleen cell suspensions from mice that had been primed by i.d. or i.p. inoculation of 4×10^8 SRBC followed by i.p. inoculation with SRBC 6 days before being plaqued. Splens from three mice per group were pooled.

Results

Eighty to ninety percent of the radioactivity associated with either virus or adjuvant was present in the faeces collected between 6 and 10 h after intraduodenal dosing. Association of ^3H -virus or ^3H -adjuvant with tissues reached a peak at 2 h, plateaued through 48 h and gradually declined to barely detectable levels by 7 to 10 days after inoculation. The specific activities for ^3H -virus, ^3H -virus plus Avridine and ^3H -Avridine alone at 2 h are representative of the uptake pattern found through

Table 1 Uptake and distribution of ^3H -U labelled reovirus.

Organ	Specific activity (DPM/mg tissue \pm SEM)		
	^3H -reovirus	^3H -reovirus + Avridine	^3H -Avridine
Intestine	75.0 \pm 9	75.1 \pm 6	111.7 \pm 7
Peyer's patch	78.5 \pm 6	219.1 \pm 11	294.0 \pm 14
M Lymph node	68.9 \pm 6	45.7 \pm 2	6.2 \pm 3
Liver	162.0 \pm 13	80.6 \pm 5	7.2 \pm 2
Lung	75.8 \pm 8	73.6 \pm 4	1.6 \pm 0
Spleen	101.8 \pm 8	88.8 \pm 4	2.9 \pm 1

These figures have been corrected for differences in the DPM/mg of ^3H -reovirus compared to ^3H -Avridine. Total tissue recovery of labelled adjuvant was less than 10.7% of the injected activity compared to 24.9% for reovirus.

48 h (Table 1). There was virtually no difference between the DPM/mg tissue for ^3H -virus and ^3H -virus plus Avridine with respect to uptake by small bowel minus Peyer's patches. In contrast, uptake of ^3H -virus in Peyer's patch was significantly enhanced when virus was associated with Avridine. Since comparable specific activities were obtained for Peyer's patch with ^3H -Avridine and there was no difference between the values for intestine and Peyer's patch in the ^3H -virus group, it is concluded that Avridine enhances uptake and retention of antigen in mucosal lymphatic tissue. Very little free Avridine passes beyond the intestinal wall, and the amount of ^3H -virus in liver and spleen is reduced when the virus was associated with Avridine. By the 7th day after inoculation, specific activities of 24.7 and 30.3 DPM/mg respectively were found in Peyer's patches of mice receiving ^3H -virus plus avridine or ^3H -Avridine alone. In that time period some of the activity had migrated to the mesenteric lymph node but no farther. Radioautography of cryostat sections of Peyer's patch, and electron microscopic evidence of Avridine liposome-like structures in phagosomes of macrophages located immediately beneath the dome epithelium, indicated that most or all of the retained Avridine and/or antigen was sequestered in macrophages of mucosal lymphatic tissue (Fig. 1).

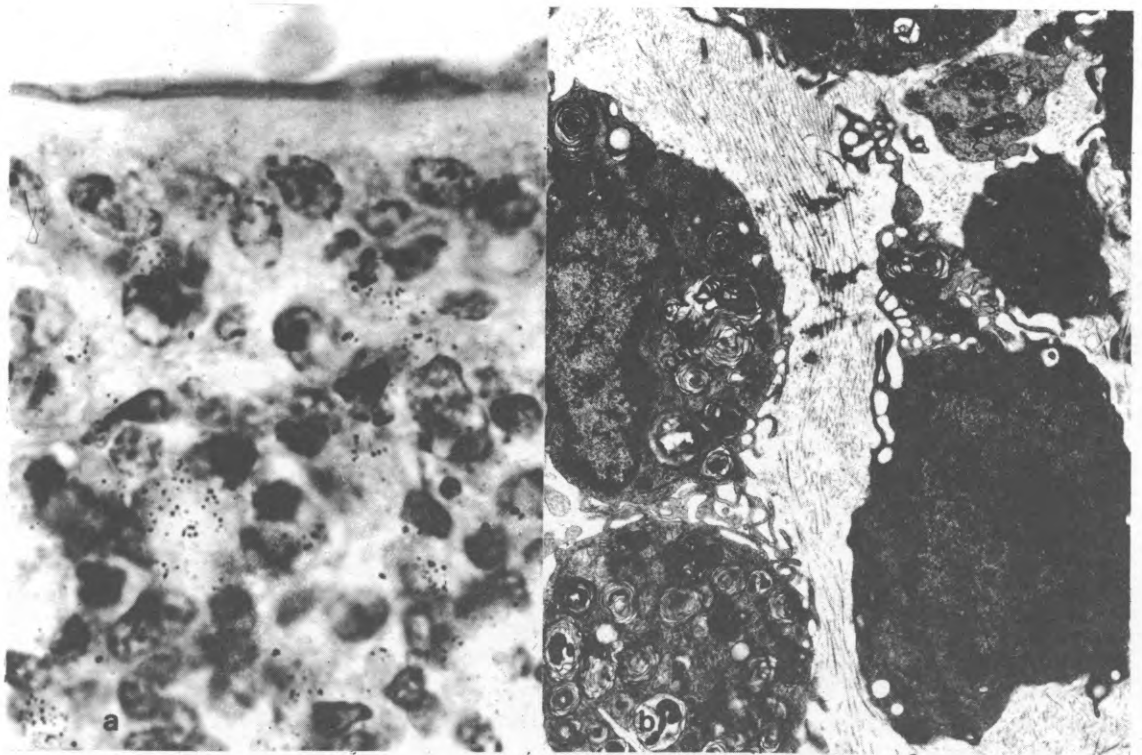


Fig. 1 Sections of the dome region of a Peyer's patch from an antigen-Avridine-treated mouse; a. cryostat radioautograph; b. electron micrograph. a. Note the silver grains overlying a large mononuclear cell just beneath the epithelium. b. The preceding cell bears a strong resemblance to a macrophage when examined ultrastructurally. Note the multilamellar membrane whorls in phagolysosomes that resemble Avridine liposomes.

Intraduodenal immunization with reovirus, cholera toxin and SRBC resulted in measurable antibody by 7 days after a single inoculation for each antigen except SRBC. Presence of Avridine in the ID-injected inoculum enhanced the reovirus and cholera toxin secretory immune responses by two to four times during this period (Table II). In the case of SRBC there was no IgA-PFC response when Avridine was not present in the inoculum that was given i.d. Avridine enhanced the IgG anti-SRBC response when it was given i.p. with SRBC, but a markedly reduced or no IgG response to SRBC was elicited on i.p. boost following i.d. priming with

Avridine SRBC (Table III). The immunopotentiating effect of avridine depended upon its being administered with the antigen, and Avridine alone failed to prime for any of the above antigens.

The response of mucosa-associated lymphatic tissues to enteric immunization with antigen, antigen and Avridine or Avridine alone was similar for the three antigens. The results for the tissue response to reovirus are shown in Table IV. Surprisingly the mesenteric lymph nodes were not different from each other at any time point following priming. In contrast, the spleen and Peyer's patches appeared to be the major sites of antigen-induced tissue

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Table II Specific antibody in intestinal secretions following intraduodenal immunization.

Vaccine, composition	Reovirus antibody ELISA units*			
	IgA		IgG	
	Day 7	Day 14	Day 7	Day 14
Reovirus 10 ⁸ PFU	588	234	0	15
Reovirus 10 ⁸ PFU + Avridine	1352	849	78	0
UV-ReoV 10 ¹⁰ P†	0	67	0	0
UV-ReoV 10 ¹⁰ P + Avridine	160	220	0	100
Avridine alone	0	0	0	0
Cholera toxin 10 mcg	0	0	0	0
	Cholera toxin antibody*			
	IgA		IgG	
	Day 7	Day 14	Day 7	Day 14
Cholera toxin 10 mcg	41	187	15	0
C. toxin 10 mcg + avridine	165	754	9	12
Avridine alone	0	0	0	0
Reovirus 10 ⁸ PFU	0	0	9	0

* As determined by ELISA using alpha and gamma heavy-chain specific antisera. * Values expressed as the mean optical density reading at 405 nm ($\times 10^{-3}$) of duplicate tests per sample diluted 1/20. † P = Viral particle determined by ODat 260 NM; 10¹⁰ P = 10⁸ PFU.

Table III Effect of avridine on isotype-specific indirect plaque-forming cell response to sheep erythrocytes (SRBC).

Site of primary immunization	SRBC-PFC/spleen	
	IgA	IgG
Parenteral		
4 \times 10 ⁸ SRBC IP	ND	21,000
4 \times 10 ⁸ IP + Avridine	ND	81,250
Avridine alone	ND	0
Intraduodenal	IgA	IgG
4 \times 10 ⁸ SRBC ID	0	0
4 \times 10 ⁸ ID + Avridine	500	0
Avridine alone	0	0
Oral	IgA	IgG
1 \times 10 ⁹ SRBC PO	ND	87,500
1 \times 10 ⁹ PO + Avridine	ND	18,250
Avridine alone	ND	0

Each point represents counts from three mice which were given a booster injection i.p. at 7 days and the spleens were harvested for plaque assay 6 days after the booster.

Table IV Response of lymphatic tissues to i.d. immunization.

Treatment	Day	Spleen (mg)	Lymph node (mg)	Peyer's patches (mg)	Number PP
Untreated	0	97.3 \pm 9	53.7 \pm 1	43.7 \pm 1	7.3 \pm 1
ReoV + Avridine	3	164.1 \pm 3	57.9 \pm 1	68.3 \pm 6	8.7 \pm 1
ReoV + Avridine	7	126.7 \pm 1	61.1 \pm 9	74.2 \pm 2	9.7 \pm 0
ReoV + Avridine	14	130.0 \pm 2	60.0 \pm 7	67.8 \pm 4	12.3 \pm 1
ReoV alone	3	148.6 \pm 3	53.9 \pm 3	58.1 \pm 5	8.0 \pm 2
ReoV alone	7	123.5 \pm 5	55.9 \pm 2	53.9 \pm 6	8.3 \pm 1
ReoV alone	14	113.8 \pm 9	50.1 \pm 5	75.8 \pm 9	11.3 \pm 0
Avridine alone	3	112.3 \pm 9	63.1 \pm 3	61.4 \sim 1	7.5 \pm 1
Avridine alone	7	126.6 \pm 8	61.9 \pm 6	54.6 \pm 1	6.3 \pm 1
Avridine alone	14	120.8 \pm 5	79.9 \pm 8	52.7 \approx 1	6.8 \pm 1

All tissues were maintained at uniform hydration in buffered 10% formalin. There were three age-matched Balb/c (NIH) mice per group.

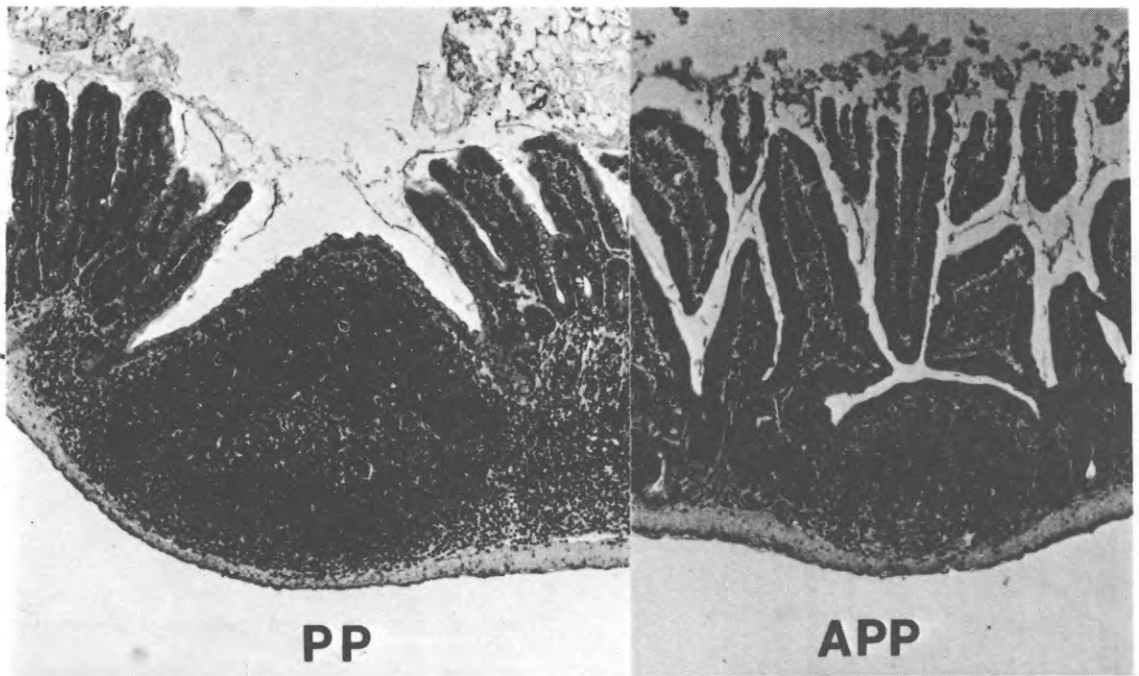


Fig. 2 Section of accessory Peyer's patches (APP) for comparison with true Peyer's patches (PP). Accessory Peyer's patches are more than just isolated mucosal follicles since they possess specialized follicle-associated epithelium (arrows), M-cells, germinal centres, and interfollicular areas containing high endothelial venules.

changes. The spleens were reproducibly enlarged by the third day after priming when reovirus or reovirus plus Avridine was given. Very slight splenic enlargement was seen later in the response when Avridine was given alone. Peyer's patches enlarged to nearly twice their mass between 7 and 14 days after priming with Avridine and/or reovirus, but not after priming with Avridine alone. When the number of Peyer's patches was examined either by simply counting the excised patches or by using the black glass rod, it was clear that in the Balb/c (NIH) mice we were using the number of Peyer's patches had increased over the 14 days of the study (Table IV). The increase in number was in the form of tiny 1-3-follicle patches that were randomly located along the bowel wall rather than in the antimesenteric

location customarily seen of Peyer's patches. The accessory patches had the histological appearance of Peyer's patches (Fig. 2) and contained dome epithelium rich with interepithelial lymphoid cells, follicles and parafollicular areas containing high endothelial venules. Development of these accessory Peyer's patches was found in NIH-derived Balb/c mice but not in Balb/c mice from Jackson laboratories. The numbers of APP in untreated Balb/c J mice were already maximal and mucosal stimulation failed to produce further increase.

Discussion

Secretory IgA plays an important role in host

resistance against many intestinal diseases by impairing toxin binding and bacterial colonization of the mucous membranes, and by preventing reinfection by the same virus. The efficacy of this process has been well established by the use of oral immunization with live-attenuated viruses such as the Sabin poliovaccine, which prevents both reinfection and establishment of a carrier state (1). With the exception of intraperitoneal priming, inoculation of non-replication protein antigens by other parenteral routes may cause depression of subsequent secretory IgA responses (2,3,15). In addition, inactivated, or toxoided antigens may not only fail to induce protective mucosal immunity but can also result in suppression of peripheral immunity following oral immunization (4-6). This is an especially important concern with regard to the potential use of genetically-engineered polypeptide or subunit vaccines, since replicating/invasive viral or microbial antigens effectively cross-prime for mucosal and peripheral immunity. An important feature of effective mucosal immunogens appears to be the ability of these agents to bind to mucosal epithelium or at least to the M-cells of Peyer's patches (7,9-18). Others have examined the utility of bacterial pili (8) or the B subunit of cholera toxin (12), that have a natural ability to bind to mucosal surfaces, as carriers for mucosally-applied antigens.

In the present study we used the lipoidal amine Avridine as a candidate mucosal adjuvant because previous studies indicated that Avridine might insert into epithelial cell membranes, possibly via 18-carbon lipid tails, while electrostatically binding antigen to the hydrophilic propanediamine end (13). In effect Avridine might substitute for an antigen receptor on the M-cell (16-22). While this receptor hypothesis cannot be proven by these studies, the observation that Avridine enhances uptake and retention of labelled antigen in Peyer's patches lends some support to the idea. The SRBC response is important because there is no natural receptor for binding antigen to mucosal epithelium, and successful priming would imply the ability of Avridine to

substitute for that receptor. It is interesting to note that UV-inactivated reovirus also failed to elicit a response in the absence of Avridine whereas live reovirus is an effective IgA-inducing immunogen. Cholera toxin binds to GM1 gangliosides in epithelial cells and does not depend upon Avridine for priming, although avridine enhances the toxin-specific s-IgA response.

Sequestration of antigen in a tissue that clearly has a functional role in priming a clonal repertoire of IgA-committed B-cell precursors to be reactive against critical environmental antigens (7,16,23) must contribute to the selective enhancement of antigen-specific IgA responses over other isotypes when Avridine is administered i.d. Having the antigen in the tissue obviates any requirement for chronic exposure or colonization of the gut lumen (7-23). Since mucosal lymphatic tissues are also centres of priming for peripheral tolerance (4-6) it is not surprising that use of Avridine with ID administered antigen results in an enhancement of the peripheral depression of IgG simultaneous with enhancement of a secretory IgA response.

Assistance in the transport of antigen from the environment via follicle-associated epithelium, and sequestration of antigen in mucosal lymphatic tissue, represent only a fraction of the functions that might be mediated by Avridine. Avridine was originally developed as a topical interferon inducer (19) but was more potent as an adjuvant when given parenterally (20,21). However, the ability of Avridine to stimulate interferon secretion may be responsible for some of the adjuvant effects, since interferons have the ability to affect expression of immune-response-associated (Ia) antigens on accessory cells (24-25). Avridine accelerates lymphoid cell traffic into lymph nodes while slowing the traffic through the paracortex (21). It stimulates the proliferation and release into efferent lymph of monocytes and Ia-antigen-positive dendritic cells (26) which stimulate proliferation of helper T-lymphocytes (27). By itself Avridine is not a polyclonal B-cell activator, but in the presence of

antigen the proliferation of clonally-selected B-cells may be enhanced. Studies by Pierce and Sacci (28) indicate that Avridine enhances mucosal B-cell memory in rats. The histological nature of Pierce's antibody-forming cell assay (19) would favour such a conclusion, since a secondary response would be required before a significant increase in the number of antigen-specific IgA-containing B-cells could be seen in the lamina propria. The enhancement of specific IgA secretion that we found to follow a single priming inoculation may have resulted either from increased commitment of B-cells to specific IgA secretion or from increased secretion by a smaller number of cells. In addition, there is likely to be specific s-IgA present in intestinal secretions that had been produced by plasma cells located in other tissues but scavenged from the blood in the liver and secreted into bile (30). The biliary IgA scavenging system would therefore release into the intestine the products of progeny of B-cells that had been primed in Peyer's patches but had lodged in non-intestinal mucosal sites belonging to the "common mucosal system" (31).

The ability of Avridine to enlarge the lymphoid compartments of the spleen and Peyer's patches, and to induce development of accessory mucosal lymphatic tissue in Balb/c (NIH) mice, indicates that local sequestration of adjuvant results in systemic effects that are trophic for mucosal lymphatic tissue. Although the development of accessory Peyer's patches in Balb/c (NIH) may be a genetic peculiarity, the phenomenon correlates with the predilection of this strain of mouse to develop plasma-cell tumours of the IgA isotype after long-term exposure to i.p. mineral oil (32). Balb/c mice from the NIH colony are susceptible to plasma-cell tumour induction by mineral oil, but Balb/c mice from Jackson laboratories are resistant. A more thorough genetic analysis of the conditions leading to the development of accessory Peyer's patches will be necessary before this phenomenon is explained.

In conclusion,* the present studies have demonstrated that Avridine enhances mucosal

immunity to a variety of antigens when administered enterically; the capacity of mucosal priming to induce tolerance or peripheral suppression is also enhanced by trophic effect on lymphatic tissues associated with sites responsible for initiation and maturation of mucosal immune responses. These effects correlate with the ability of Avridine to enhance the uptake and retention of enterically-introduced antigens in mucosa-associated lymphatic tissues. If the safety and efficacy of the oral or enteric use of Avridine is assured, this drug is likely to become a valuable means of enhancing the immunogenicity of veterinary and human vaccines directed against enteropathogenic bacteria, viruses and toxins.

* The views of the authors 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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