

**From: MICROENVIRONMENTS IN THE LYMPHOID SYSTEM**  
**Edited by G.G.B. Klaus**  
**(Plenum Publishing Corporation, 1985)**

**EFFECT OF AVRIDINE ON ENTERIC ANTIGEN UPTAKE AND MUCOSAL IMMUNITY  
TO REOVIRUS (1/Lang)**

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**INTRODUCTION**

Antigen priming by a mucosal route has been shown to be superior to parenteral inoculation in eliciting a protective secretory IgA response<sup>1</sup>. Indeed, parenteral priming with non-replicating antigens often depresses or prevents specific IgA responses when that antigen is subsequently exposed to the mucosa<sup>2</sup>. Conversely, mucosal priming is often associated with induction of peripheral tolerance or reduced IgG levels and cell-mediated immunity to parenterally-administered antigen<sup>3-5</sup>. In addition, inactivated, attenuated, or toxoided antigens often fail to retain the essential immunogenic properties needed for an IgA response, and unmodified pathogens and toxins carry with them unacceptable toxicity. Therefore, studies focused on 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 depends upon its uptake and processing by mucosal lymphatic tissues. A number of hypotheses have been proposed, including one concerning specific receptors that facilitate binding of the antigen to mucosal surfaces, and the ability of the antigen to colonize the bowel lumen or invade the mucosa<sup>6</sup>. It is instructive that gastrointestinal pathogens, such as Salmonella, enteropathogenic E. coli, and Cholera toxin, normally have these properties, and convalescence often is associated with the presence of specific IgA in the secretions<sup>7</sup>. The present study presents new data indicating that the immunological adjuvant Avridine enhances secretory immunity when

delivered in combination with live and inactivated reovirus antigen by facilitating the uptake and retention of antigen in mucosal lymphatic tissues.

## MATERIALS AND METHODS

### Animals

Adult female Balb/c mice (NIH) were used for the immunization and radiolabel uptake studies. A/J, Balb/c(Jax), C<sub>57</sub>BL/Ka, DBA/2, CD<sub>F1</sub>, and C<sub>3</sub>H/HeJ strains were used for analysis of susceptibility to development of accessory Peyer's patches. All animals were housed in Biogard laminar flow units and fed conventional mouse chow.

### Antigens

Reovirus serotype 1/Lang were propagated on mouse L cells and purified as described previously<sup>8</sup>. The final concentration of viral particles was determined by optical density measurement at 260 nm. Infectious titers on mouse L cells were approximately 100-fold less than particle counts. Each mouse received  $1 \times 10^{10}$  particles of either live or UV-inactivated virus. 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. Avridine (previously called CP20-961) was provided by Dr. Ivan Otterness (Pfizer Central Research, Groton, Conn.). A 6-mg/ml emulsion was prepared by dissolving 42 mg Avridine in 0.3 ml absolute ethanol and 0.03 ml Tween 80. The mixture was agitated with 6.67 ml of Intralipid. Avridine was mixed 1:1 with antigen and sonicated prior to inoculation.

### Immunization procedures

Mice were anesthetized with methoxyflurane for direct intraduodenal (ID) injection via laparotomy<sup>9</sup>. Virus or Cholera toxin was mixed with Avridine in soybean oil emulsion (Intralipid; Cutter Laboratories, Berkley, Calif.) and sonicated for 10 seconds. The total injection volume was 0.1 ml. Groups of mice were sacrificed at 1, 3, 7, and 14 days. The entire small bowel, from duodenum through ileocecal 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 mucus 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.

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

### Radiolabel uptake

Reovirus 1/Lang were grown in the presence of 10mCi/ml of  $^3\text{H}$ -uridine to a specific activity of 600,000 DPM/ $10^{10}$  virions. Tritiated Avridine (specific activity 11.79 mCi/mg) was obtained as a gift from Drs. Keith Jensen and Sanford Figdor of Pfizer Central Research.  $^3\text{H}$ -reovirus, with or without unlabelled Avridine, was injected ID, and mice were sacrificed; the intestines, Peyer's patches, mesenteric lymph nodes, liver, spleen, and lungs were removed, weighed, and dissolved in scintillation cocktail. A Beckman scintillation counter was used to determine the DPM.

### Immunoassays

Antibody was measured in serum and intestinal secretions by enzyme-linked immunosorbent assay (ELISA)<sup>10</sup> and hemagglutination<sup>8</sup>. 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 reading at 405 nm ( $\times 10^{-3}$ ) of samples diluted 1/20, since OD correlated directly with titer.

## RESULTS

Eighty to ninety percent of the radioactivity associated with either virus or adjuvant was present in the feces collected between 6 and 10 hours after intraduodenal dosing. Association of  $^3\text{H}$ -virus or  $^3\text{H}$ -adjuvant with tissues reached a peak at 2 hours, through 48 hours, and gradually declined to barely detectable levels by 7 to 10 days after ID inoculation. The specific activities for  $^3\text{H}$ -virus,  $^3\text{H}$ -virus plus Avridine, and  $^3\text{H}$ -Avridine alone at 2 hours are representative of the uptake pattern found through 48 hours (table 1). These data have been corrected for differences in the DPM/mg of  $^3\text{H}$ -reovirus compared to  $^3\text{H}$ -Avridine. Total tissue activity compared to 24.9% for reovirus.

There was virtually no difference between the DPM/mg tissue for  $^3\text{H}$ -virus and  $^3\text{H}$ -virus plus Avridine with respect to uptake by small bowel minus Peyer's patches. In contrast, uptake of virus in Peyer's patch was significantly enhanced when virus was associated with Avridine. Since comparable specific activities were obtained with labelled Avridine alone in Peyer's patch, and there was no difference between the values for intestine and Peyer's patch in the  $^3\text{H}$ -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 cell wall. By the seventh day after inoculation, specific activities of 24.7 and 30.3 DPM/mg, respectively, were found in Peyer's patches of mice receiving  $^3\text{H}$ -virus plus Avridine or  $^3\text{H}$ -Avridine alone. In that time period some of the activity had migrated to the mesenteric lymph node, but no further. Radioautographs of Peyer's patches indicated that most of the retained Avridine was sequestered in mononuclear cells under the follicle-associated epithelium.

Intraduodenal immunization with reovirus, live or UV-inactivated, and Cholera toxin resulted in measureable antibody by 7 days after a single inoculation for each antigen except inactivated reovirus. Presence of Avridine in the ID-injected inoculum enhanced the reovirus and Cholera toxin secretory immune responses 2- to 4-fold in this time period (table 2). 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. In contrast, reovirus-specific hemagglutinin titers were reduced when the intraduodenal inoculum contained Avridine (table 3).

TABLE 1  
 UPTAKE AND DISTRIBUTION OF  $^3\text{H}$ -U LABELED REOVIRUS  
 Specific activity (DPM/mg tissue  $\pm$  SEM)

Organ	$^3\text{H}$ -reovirus	$^3\text{H}$ -reovirus+Avridine	$^3\text{H}$ -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	101.1 $\pm$ 8	88.8 $\pm$ 4	2.9 $\pm$ 1

Table 2  
 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 $10^{10}$ pparticles (p)	0	67	0	100
UV-ReoV $10^{10}$ p + Avridine	160	220	0	100
Avridine alone	0	0	0	0
Cholera toxin	0	0	0	0

Cholera toxin antibody	Cholera toxin antibody			
	Day 7	Day 14	Day 7	Day 14
	Cholera toxin 10 mcg	41	187	15
C. Toxin 10 mcg + Avridine	165	754	9	12
Avridine alone	0	0	0	0
Reovirus $10^8$ PFU	0	0	0	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 by 1/20.

TABLE 3

REDUCTION IN SERUM HA TITERS AFTER INTRADUODENAL PRIMING WITH  
 LIVE REOVIRUS (1/LANG) AND AVRIDINE

Vaccine composition ID inoculation (n=3)	Serum hemagglutination Geometric mean titers ( $\times 10^{-1}$ )		
	day 7	day 14	day 28
	Reovirus ( $10^{10}$ P)	30	1210
Reovirus ( $10^{10}$ P) + Avridine (0.3 mg)	38	762	605
Avridine alone (0.3 mg)	<10	<10	<10
Untreated	<10	<10	<10

The response of mucosa-associated lymphatic tissues to enteric immunization with reovirus, reovirus and Avridine, or Avridine alone is shown in table 4. Surprisingly, there were no changes in the mesenteric lymph nodes. In contrast, the spleen and Peyer's patches were the major sites of tissue reaction. All tissues were maintained at uniform hydration in buffered 10% formalin. There were three age-matched Balb/c(NIH) mice per group. The spleens enlarged reproducibly 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. Morphometric analysis of the splenic enlargement indicated that the periarteriolar lymphatic sheath (PALS) increased 4 and 6 times its mass between the first and third days following ID inoculation with reovirus and Avridine (table 5). By day 7 the PALS of both the reovirus + Avridine- and reovirus-treated mice were of comparable size, 2.5 times the untreated controls. Germinal centers showed progressive increase in mass till 7 and 14 days after ID priming. On day 7 the follicles in the reovirus + Avridine-treated spleens were 2.5 times the mass of the follicles in the reovirus control. A surprising finding was that red pulp compartments, where extramedullary hematopoiesis (EMH) occurs, were markedly enlarged when Avridine was given ID without antigen, but showed no appreciable changes if antigen was included. All tissues were maintained at uniform hydration in buffered 10% formalin. There were three age-matched Balb/c(NIH) mice per group.

TABLE 4  
RESPONSE OF LYMPHATIC TISSUES TO ID IMMUNIZATION

Treatment	Day	Wet weight in mg + -1SD				Number PP
		Spleen	Lymph node	Peyer's Patches		
Untreated	0	97.3 + 9	53.7 + 1	43.7 + 1	7.3 + 1	
ReoV + Avridine	3	164.1 + 3	57.9 + 1	68.3 + 3	8.7 + 1	
ReoV + Avridine	7	126.1 + 1	61.1 + 9	74.2 + 2	9.7 + 0	
ReoV + Avridine	14	130.0 + 2	60.0 + 7	67.8 + 4	12.3 + 1	
ReoV alone	3	148.6 + 3	53.9 + 3	58.1 + 5	8.0 + 2	
ReoV alone	7	123.5 + 5	55.9 + 2	53.9 + 6	8.3 + 1	
ReoV alone	14	113.8 + 9	50.1 + 5	75.8 + 9	11.3 + 0	
Avridine alone	3	112.3 + 9	63.1 + 3	61.4 + 7	7.5 + 1	
Avridine alone	7	126.6 + 8	61.9 + 6	54.6 + 1	6.3 + 1	
Avridine alone	14	120.8 + 5	79.9 + 8	52.7 + 9	6.8 + 1	

TABLE 5

## MORPHOMETRY OF SPLENIC LYMPHOID COMPARTMENTS

Mass of Lymphatic tissue (mg)					
Treatment ID	Day	GC	PALS	MZ	EMH
Untreated	0	1.94	14.69	14.68	6.81
ReoV + Avridine	1	2.09	58.47	24.74	2.50
ReoV + Avridine	3	4.43	86.49	37.25	4.10
ReoV + Avridine	7	8.87	39.53	28.76	10.13
ReoV + Avridine	14	8.71	42.78	27.44	9.90
ReoV alone	7	3.58	35.46	35.95	10.37
Avridine alone	7	3.67	21.02	19.25	31.66

The mass of lymphoid tissue was calculated from the wet weights using percent volumes determined on histological sections via planimetry. GC=Germinal Centers, PALS=Periarteriolar lymphatic sheath, MZ=Marginal zone, and EMH=Extramedullary hematopoietic compartments in red pulp.

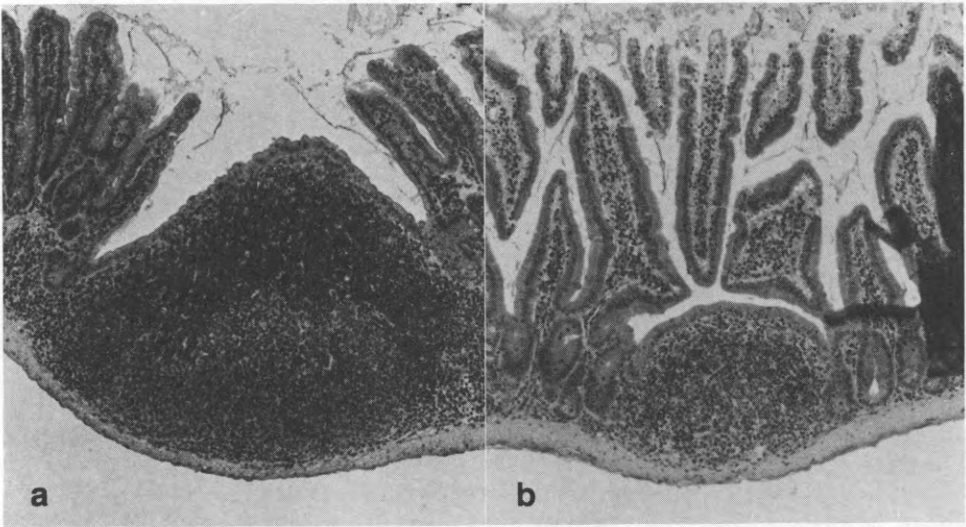


Figure 1. Accessory Peyer's patches are more than just isolated mucosal follicles since they possess specialized follicle associated epithelium (arrows) including structures that resemble M-cells, Germinal Centers (GC), and interfollicular areas containing high endothelial venules (TDA).

## DISCUSSION

Secretory IgA plays an important role in host resistance against many intestinal diseases by impairing toxin binding, bacterial colonization of the mucus 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<sup>1</sup>. With the exception of intraperitoneal priming, inoculation of non-replicating protein antigens by other parenteral routes may cause depression of subsequent secretory IgA responses<sup>2</sup>. 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 priming<sup>3-5</sup>. This is an especially important concern with regard to potential use of genetically-engineered polypeptide or subunit vaccines, since replicating/invasive viral or microbial antigens effectively cross-prime for mucosal and peripheral immunity<sup>1,6-8</sup>, but inactivated, synthetic or toxoided antigens do not cross-prime.

An important feature of effective mucosal immunogen is the ability of these agents to bind to mucosal epithelium or at least the M-cells of Peyer's patches<sup>11-14</sup>. Others are examining the utility of bacterial pili or the B subunit of Cholera toxin 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 Avidine as a candidate mucosal adjuvant because previous studies suggested that Avidine might insert in epithelial cell membranes, possibly via 18 carbon lipid tails, while electrostatically binding antigen to the hydrophilic propanediamine end<sup>15,16</sup>. In effect, Avidine might substitute for an antigen receptor on the M-cell. A receptor-like interaction between reovirus (1/Lang) and Peyer's patch M-cells has been observed using live virus<sup>13</sup>, but the ability of UV-inactivated reovirus to bind these specialized cells has never been demonstrated. UV-inactivated reovirus is ineffective in priming for an intestinal IgA response after ID or oral inoculation but will lead to specific reduction of delayed hypersensitivity or tolerance<sup>5</sup>. While the receptor hypothesis cannot be proven by these studies, the observation that Avidine enhances uptake and retention of labelled antigen in Peyer's patches lends some support to the idea. We have recently reported that an IgA-isotypic indirect plaque-forming-cell response can be initiated following a single ID inoculation with SRBC and Avidine<sup>14</sup>. A specific receptor for SRBC is not likely to exist on follicle-associated epithelium since chronic exposure to SRBC in drinking water is required for an IgA-SRBC response in the absence of Avidine. This

would imply that Avridine might substitute for the absent receptor,

Concentration of antigen in a tissue that clearly has a functional role in generating a clonal repertoire of IgA-committed B-cell precursors which react with environmental microbial antigens<sup>9,17</sup>, certainly must contribute to the selective enhancement of antigen-specific IgA responses over other isotypes when Avridine is administered ID. Since mucosal lymphatic tissues are also centers of priming for peripheral tolerance<sup>3-5</sup> it is not surprising that the use of Avridine with ID-administered antigen results in enhancement of the peripheral depression of IgG simultaneous to enhancement of a secretory IgA response.

Avridine was originally developed as a topical interferon inducer<sup>15</sup> but was more potent as an adjuvant when given parenterally<sup>16</sup>. However, the ability of Avridine to stimulate interferon secretion may be responsible for some of the adjuvant effects, since interferons effect surface expression of important molecules on accessory cells such as immune response-associated (Ia) antigens<sup>18,19</sup>. Avridine accelerates lymphoid cell traffic into lymph nodes while slowing the traffic through the paracortex<sup>16</sup>. It stimulates the proliferation and release into efferent lymph of monocytes and Ia-antigen positive dendritic cells<sup>20</sup> which present antigens (in the context of self), thereby causing helper T-cells to proliferate<sup>21</sup>.

By itself, Avridine is not a polyclonal B-cell activator; but, in the presence of antigen, proliferation of clonally-selected B-cells may be enhanced. Studies by Pierce and Sacci<sup>22</sup> indicate that Avridine enhances mucosal B-cell memory in rats. The histological nature of Pierce's antibody-forming cell assay would favor such an interpretation, since a secondary response would be required before a significant increase in the number of visually-scored specific IgA-containing B-cells could be seen in the intestine<sup>23</sup>. The enhancement of specific IgA secretion we observed following a single priming inoculation may have resulted from either increased commitment of B-cells to specific IgA secretion or increased secretion by fewer cells. In addition, there is likely to be specific s-IgA present in intestinal secretions that had been contributed by plasma cells located in other tissues. It is now well-recognized that s-IgA is scavenged from the circulating blood in the liver and re-secreted into bile that flows into the proximal small intestine<sup>24</sup>. Clonal expansion of IgA-committed B-cells in Peyer's patches may result in migration and lodging of antigen-specific IgA-plasma cells in mucosal sites all over the body<sup>25</sup>.

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. Development of additional germinal follicles in the intestine might be related to the enhancement of immunologic memory, since germinal follicles function to maintain B-cell memory. Although the development of accessory Peyer's patches in Balb/c(NIH) mice may be a genetic peculiarity, the phenomenon correlates with the susceptibility of this strain to develop IgA-secreting plasmacytomas after IP inoculation with mineral oil. Balb/c(NIH) mice are susceptible and Balb/c(JAX) mice are resistant to plasmacytoma induction. A more thorough genetic analysis will be necessary before this phenomenon is explained.

In conclusion, Avridine enhances mucosal immunity to reovirus antigens following intraduodenal inoculation while simultaneously enhancing suppression of serum hemagglutination titers. Mucosal priming with Avridine and antigen has a trophic effect on lymphatic tissues associated with sites of initiation and maturation of mucosal immune responses; e.g, it has been demonstrated that B-cells that have been primed in mucosal lymphatic tissues must lodge in the spleen for up to 7 days before they are mature enough to lodge in the lamina propria of the intestine and differentiate into IgA secreting plasma cells<sup>9,17</sup>. These effects correlate with the ability of Avridine to enhance the uptake and retention of ID-inoculated antigens in mucosal lymphatic tissues, and indicate a possible mechanism by which Avridine might be useful in enhancing secretory immune responses to a variety of enteric vaccines.

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