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Jon L. Patterson
Major professor

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THE EFFECT OF RETINOL PALMITATE AND/OR PUTRESCINE ON SMALL INTESTINAL REGENERATION IN PIGLETS

By

Soegiarto

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ABSTRACT

THE EFFECT OF RETINYL PALMITATE AND/OR PUTRESCINE ON SMALL INTESTINAL REGENERATION IN PIGLETS

By

Soegiarto

This research examined the effect of daily oral administration of retinyl palmitate and putrescine alone or in combination on the clinical signs, lesions and intestinal mucosal epithelial regeneration in rotavirus-infected piglets. For the first 48 hours of life, 128 newborn piglets consumed colostrum. Starting on day 3, the piglets were divided into 4 groups of 32 piglets. Group RP was given retinyl palmitate, group P was given putrescine, group RPP was given retinyl palmitate and putrescine and group MO was given milk only. On day 5 half of the piglets in each group were infected with porcine rotavirus. The piglets were then euthanatized on day 6, 7, 8 and 13 (1, 2, 3 and 8 days postinoculation [DPI]). Treatment did not prevent or shorten the course of the disease, and did not affect the morphology of the intestinal lesions. Treatment did not have any significant effect on villous height, crypt depth, complexity index or goblet cell number in the small intestine. Infection increased the small intestinal mucosal complexity indices ($P<0.05$). Infection reduced the relative volume of the small intestinal mucosa ($P<0.01$). Treatment increased the mitotic index in the small intestinal crypt epithelium ($P<0.01$). Mucosal protein and RNA concentrations

were greater in piglets 8 DPI than in piglets 1 and 2 DPI, regardless of treatment group. The mucosal DNA concentration was decreased by infection status ($P<0.01$), but it was not affected by treatment or time. Liver vitamin A concentration was influenced by treatment and time but not by infection status. Piglets in groups given RP and RPP had higher liver vitamin A concentrations than piglets given P and MO ($P<0.01$). Piglets in groups given RP and RPP that were 8 DPI had higher concentrations of liver vitamin A than piglets 1 DPI ($P<0.01$). Putrescine tended to decrease the concentration of liver vitamin A in infected animals. Infected piglets given RPP had significantly lower vitamin A concentrations than infected piglets given RP ($P<0.01$).

Dedicated to my parents

Ibuk Soewarti and Bapak Tamid

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CDCD	:	Colostrum-deprived caesarean-derived
CF	:	Complement fixation
CRABP	:	Cellular retinoic acid-binding protein
CRBP	:	Cellular retinol-binding protein
DFMO	:	DL-α-Difluoromethylornithine
DMBA	:	dimethylbenzanthracene
DNA	:	Deoxyribonucleic acid
DPI	:	Day postinoculation
DU	:	Duodenum
ELISA	:	Enzyme linked immunoassay
FA	:	Fluorescent antibody
FABP	:	Fatty acid-binding protein
GD	:	Gel-diffusion
HRP	:	Horseradish peroxidase
HTC	:	Hepatoma cell culture
IEM	:	Immunoelectron microscopy
Ig	:	immunoglobulin
IU	:	International unit
LI	:	Lower ileum
LJ	:	Lower jejunum
MI	:	Mid intestine
mRNA	:	Messenger ribonucleic acid
MO	:	Milk only
NB	:	Naturally born
NSP	:	Nonstructural polypeptides
ODC	:	Ornithine decarboxylase
OSU	:	Ohio State University
P	:	Putrescine
PBS	:	Phosphate-buffered saline
PRN	:	Plaque reduction neutralization
RAR	:	Retinoic acid receptor
RBP	:	Retinol-binding protein
RNA	:	Ribonucleic acid
RP	:	Retinol palmitate
RPP	:	Retinol palmitate and putrescine combined
RXR	:	Retinoid X receptor
SAM-DC	:	S-adenosylmethionine decarboxylase
SDSU	:	South Dakota State University
TGF	:	Transforming growth factor
TTR	:	Transthyretin
UI	:	Upper ileum
UJ	:	Upper jejunum
VP	:	Viral polypeptides

INTRODUCTION

Small intestinal mucosal regeneration is essential for animals and human beings, either for continual physiological renewal or for recovery from intestinal injury. Intestinal injury with mucosal damage can be caused by various protozoal, bacterial and viral pathogens (Holland, 1990).

Major efforts to reduce intestinal damage have focused on immunization to prevent enteric diseases or the use of antibiotics and chemotherapeutic agents to treat the diseases. Vaccination against some intestinal diseases has proven to be beneficial, but has a limited protective effect in immunosuppressed individuals. Mishandling of vaccines may also contribute to vaccine failure or inadequate protection. In addition, the current concern with chemical residues has restricted antibiotic use in food animals. Therefore, the use of natural chemicals, such as vitamin A and/or putrescine, to prevent or to minimize intestinal mucosal damage would be extremely beneficial.

The scientific literature has suggested, but not conclusively proven, that vitamin A and putrescine may enhance intestinal epithelial regeneration. We hypothesized that the oral administration of vitamin A and/or putrescine in the diet would minimize the effect of rotavirus on the

enhancing mucosal regeneration. Rotavirus, like several other viral and protozoal enteric diseases, causes small intestinal damage which results in villous atrophy.

Various forms of vitamin A are known to have an effect on vision, reproduction and the maintenance and growth of epithelial cells. Most studies involved the use of vitamin A deficient animals. Vitamin A deficiency reduced cellular DNA, RNA and protein synthesis in the intestinal mucosa. Readministration of vitamin A in the animals restored cellular DNA, RNA and protein synthesis (Johnson *et al*, 1969, Zachman, 1967). Recently, vitamin A was reported to protect the small intestine of rats from morphological, biochemical and physical damage caused by methotrexate administration (Tsurui *et al*, 1990). Methotrexate, as an antitumor drug, inhibits mitosis of epithelial cells in small intestinal crypts, disrupts the steady state system of the epithelium and progressively reduces the size of crypts and villi (Jeynes and Altmann, 1978).

Polyamines, spermidine and spermine, and their diamine precursor, putrescine, are found in virtually all cells of eukaryotic organisms. Their cellular concentrations are highly regulated. They play an important role in cellular growth and differentiation, but their cellular functions at the molecular level are not known (Heby, 1981).

In the present study, vitamin A and/or putrescine were given to young piglets in an attempt to prevent or decrease

the effects of rotavirus infection. The objectives of this study were:

1) To determine if the clinical signs, lesions and rotavirus antigen expression in the small intestinal mucosa produced by rotavirus infection in piglets could be altered by the daily dietary administration of vitamin A and/or putrescine;

2) To determine the effect of the daily dietary administration of vitamin A and/or putrescine on the small intestinal mucosal morphometry of rotavirus-infected piglets;

3) To determine the effect of the daily dietary administration of vitamin A and/or putrescine on hepatic vitamin A, and small intestinal DNA, RNA and protein concentrations of rotavirus-infected piglets.

LITERATURE REVIEW

SMALL INTESTINAL MUCOSAL KINETICS

Mature intestinal mucosal cells on the tips of villi are continuously sloughed and replaced by new epithelial cells which migrate from the crypts by a complex mechanism. Apart from normal physiological processes, the sloughing or destruction of mature or immature intestinal epithelial cells may also be caused by such factors as weaning (Hampson, 1986 and Kenworthy, 1976), stress (Wang and Johnson, 1991), ionizing radiation (Thomassen, 1972; Kent and Moon, 1973) and starvation (Clarke, 1970b). Enterocyte destruction may also be caused by various infectious agents, including intestinal parasites such as coccidia (Fernando and McCraw, 1973), pathogenic bacteria such as *Serpulina hyodysenteriae* and *Campylobacter* species (Neef *et al*, 1994; Eaton *et al*, 1989), bacterial toxins such as *Clostridium perfringens* type C exotoxin (Bergeland, 1972) and *Escherichia coli* heat-stable enterotoxin b (Rose *et al*, 1987) and viral agents such as coronavirus (Thake, 1968) and rotavirus (Theil *et al*, 1978).

Organization of cell proliferation in the small intestine

Epithelial renewal systems in the small intestine are usually divided into proliferation, maturation and

functional compartments. In the small intestine, the functional compartment is found in the villus, whereas, both proliferation and maturation compartments are located in the crypt. In the maturation compartment, cells lose their proliferative capacity and acquire characteristics of mature functional cells (Leblond *et al*, 1967, Lesher, 1967). A steady flow of cells migrates from the proliferation compartment into the maturation compartment and then to the functional compartment in the villus. Subsequently cells will exfoliate from the tip of the villus. A second functional compartment or terminally differentiated cell compartment, referred to as the Paneth cell zone, is present in the base of the small intestinal crypt (Cheng and Bjerknes, 1980).

Mitosis in the lower portion of the proliferation compartment yields two new proliferating cells. Mitosis in the middle portion of the proliferation compartment, corresponding to the distance from the base, yields cells with a declining probability for proliferation. Mitosis in the top portion of the proliferation compartment yields terminally differentiated cells (Cheng and Bjerknes, 1980).

The small intestinal crypt contains Paneth cells, goblet cells, enteroendocrine cells, intra-epithelial lymphocytes and columnar epithelial cells. At least 4 of these cell types - Paneth, goblet, enteroendocrine and columnar epithelial cells - originate from a multipotential stem cell situated at or near the crypt base (Cheng,

1974a,b; Cheng and Leblond, 1974a,b,c). Paneth cells are absent in cats, dogs and pigs (Magee and Daley, 1986).

Most of the columnar epithelial, goblet and enteroendocrine cells differentiating from the stem cells migrate in an upward direction to populate the villi. All of the Paneth cells, some columnar epithelial and a few goblet and enteroendocrine cells migrate downward into the stem cell zone at the base of the crypt (Cheng and Bjerknes, 1980).

Factors affecting cell proliferation in the small intestine

Multiple factors, such as species, animal age, nutrition, hormones, intestinal flora and the degree of epithelial damage, influence intestinal mucosal regeneration. These factors affect each other, but the underlying mechanism leading to an increase or a decrease in the number of cells proliferating in the crypts is still unknown.

Species and age of animal

The effect of species and age on small intestinal epithelial cell proliferation has been studied by several investigators. In albino rats, the rate of epithelial cell production in the small intestine is approximately 36 cells per crypt per hour (Clarke, 1970a). The replacement time, starting with the synthesis of DNA in the crypt until the cell reaches the tip of the villus, varies depending upon the age of the animal, the species and the region of the

intestine. Moon and Skartvedt (1975) showed that the replacement time of small intestinal mucosal cells of 1-day-old chickens was faster than the replacement time in 3-week-old and 6-month-old chickens. In 1-day-old chickens, epithelial cell replacement was nearly complete in 5 days, whereas during the same period of time, epithelial replacement in 3-week-old and 6-month-old chickens was only 75 and 50% complete, respectively. Clarke (1967) noted that with age duodenal villi in chickens varied in size but not in number. In adults, villi were larger in size, but the total number of villi was approximately the same as during the late embryonic stage. Duodenal crypts, on the other hand, increased greatly in number and depth with age.

In contrast to chickens, small intestinal epithelial migration in older rats was faster than in young rats. Within 48 hours, the enterocytes of 210 day-old-rats had migrated to the top of the villus, whereas the 7 day-old rats' enterocytes had reached only about 25% of the length of the villus (Koldovsky *et al*, 1966). Similar to rats, small intestinal mucosal cell replacement in young pigs is slower than in older animals. In day-old pigs, the epithelium is replaced in 7-10 days, while in 3-week-old pigs it takes only about 2-4 days (Moon, 1971).

The site of enterocyte proliferation is different during the various stages of development of the animal and in various portions of the intestinal tract. DNA synthesis and cell proliferation occur in epithelial cells along the

entire length of the villus of fetal rats. However, in 1-day-old suckling rats replication is confined to the extreme base of the villus (Hermos *et al*, 1971). Kenworthy (1976) observed that the mitotic indices of 2-week-old weaned and unweaned pigs were higher in the jejunum and ileum than in the duodenum. In addition, the overall mitotic indices in the small intestine of weaned pigs were higher than in unweaned pigs. Using tritiated labeled thymidine, Imondi and Bird (1966) reported that the mucosa of 2-day-old chick's jejunum was replaced approximately in 48 hours, whereas the replacement time for the epithelial cells in the ileum and duodenum was longer than 48 hours.

Studies from various other animals have also revealed that many intestinal mucosal functions are affected by age. Chen *et al* (1990) showed that there was a greater rate of transepithelial transport of D-glucose and 3-O-methyl-D-glucose in young growing mice compared to older animals. These changes were accompanied by decreases in the number and height of villi with age, which resulted in a decrease in the mucosal surface area. The ability of intestinal mucosal epithelial cells to ingest macromolecular complexes is also associated with age (William and Beck, 1969). Studies in rats, rabbits and guinea-pigs, utilizing trypan blue as a marker for pinocytic activity, indicated that epithelial maturation was complete about 3-4 weeks after birth. Perozzi *et al* (1993) described the temporal expression of intestinal functions during fetal and

postnatal development in the pig. Results of studies using Northern hybridization indicated the presence of the messenger ribonucleic acids (mRNAs) for cellular retinol binding protein II (CRBP II), for the digestive enzyme aminopeptidase N, and for the microvillar proteins, villin and ezrin, in the small intestine of both weaned and 40-day fetal pigs. The mRNAs for two types of fatty acid-binding proteins (FABPs), I-FABP and L-FABP, which are involved in the metabolism of long chain fatty acids, were detected only in weaned animals, whereas the mRNA for CRBP I was detected only in the fetal intestine.

Nutrition

Starvation in rats leads to marked loss of small intestinal weight, and RNA and protein content (Steiner *et al*, 1968). The presence of food in the intestine may exert either direct or indirect effects on the growth of the small intestinal mucosa. The direct effect of the presence of food in the intestinal lumen on the mucosal growth may be a result of epithelial desquamation, or food may provide local nutrition and direct stimulation by particular dietary constituents may act as growth factors. The indirect effect of the presence of food in the intestinal lumen may be a result of nerve stimulation and the release of gastrointestinal peptides (Henning *et al*, 1994).

Dietary nucleotides, adenosine monophosphate, guanosine monophosphate, cytidine monophosphate, uridine monophosphate

and inosine monophosphate, appear to be modulators of intestinal development following chronic diarrhea (Nuñez *et al*, 1990). Administration of vitamin D to vitamin D deficient chicks for more than 110 hours partially restored the size of villi and improved epithelial cell migration (Spielvogel *et al*, 1972).

Intestinal flora

The intestinal flora affect intestinal epithelial transit time. Bacterial colonization in the small intestine causes 'physiologic inflammation', a condition that leads to higher rates of intestinal epithelial proliferation and migration (Abrams *et al*, 1963). In addition, the transit time of cells moving from the crypts to the villous tips is faster in conventional mice than in germ-free animals (Lesher *et al*, 1964).

Feedback control mechanism

Epithelial cell renewal is also believed to be regulated by a feedback control mechanism of the functional villous cell. Irradiation is known to kill actively dividing cells. Following cell depletion in the crypt after irradiation, initial development of crypt epithelium was similar in germ-free and conventional rats. However, subsequent proliferation and maturation of the cells were slower in germ-free rats. This probably was associated with the cellular density of the maturing non-dividing cells, determined by the level of esterase activity which is higher

in germ-free rats (Galjaard *et al*, 1972). This phenomenon was also observed by Sherman and Quastler (1960), who concluded that the continuous migration of intestinal epithelium is not dependent upon a push from dividing crypt cells but is a response to the loss of villous epithelial cells. Villous epithelial loss caused by intestinal parasites enhances epithelial regeneration. Intestinal epithelial turnover time in chickens infected with *Eimeria acervulina* was faster than the epithelial turnover time of normal chickens (Fernando and McCraw, 1973).

Epithelial cell number in the crypts and villi of rats that have had their intestines partially resected is higher than normal. The increase in epithelial cell number begins as early as 2-4 days postoperation, reaching its maximum in 12 days, and is proportionate to the amount of tissue removed (Hanson *et al*, 1977a,b).

Chalones, soluble substances that act as endogenous inhibitors of mitotic activity, have long been implicated as effectors of a negative feedback mechanism on intestinal crypts (Brugal, 1976; Houck, 1973). Recent *in vitro* and *in vivo* studies indicate that transforming growth factor beta (TGF β) acts as a chalone. Exogenous addition of TGF β has been shown to inhibit the proliferation of a non-transformed rat jejunal cell line (Lamprecht *et al*, 1989) and of a primary culture of rat small intestinal epithelium (Booth *et al*, 1995). Studies in mice shortly after whole body

irradiation have shown that TGF β-1 was an effective inhibitor of the small intestinal crypt proliferation of unirradiated and early regenerating animals (Potten *et al*, 1995). Using Northern blot analysis and immunohistochemistry in murine small intestines, Barnard *et al* (1993) showed that TGF β-1, TGF β-2 and TGF β-3 were expressed in the small intestinal epithelium, most prominently in the enterocytes located on the villous tip. No TGF β expression was detected in crypts.

Cell proliferation assessment in the small intestine

Intestinal mucosal regeneration has been studied extensively using morphological, kinetic or biochemical assessments. Most morphological studies were either based on epithelial cell counting (Hampson, 1986), linear measurements of mucosal thickness, villous height and crypt depth (Hart and Kidder, 1978) or mucosal surface complexity assessments (Bertram *et al*, 1991; Hart and Kidder, 1978; Rose *et al*, 1987; Dunnill and Whitehead, 1972). Mucosal surface complexity was assessed by determining the surface and volume ratio based on the method developed by Chalkley (Chalkley *et al*, 1949) with the use of a Weibel graticule (Weibel, 1963). Kinetic assessments were done with autoradiography, using tritiated thymidine to measure the rate of epithelial cell migration and replacement (Abrams *et al*, 1963). Biochemical analyses have been used to assess

intestinal DNA, RNA, mucosal protein and disaccharidase activities (Nuñez *et al*, 1990). In addition, a D-xylose absorption test has been used to assess intestinal malabsorption of carbohydrates (Bolton *et al*, 1976).

VITAMIN A

Vitamin A was first discovered by McCollum and Davis in 1913 in animal fat and fish oil as a factor that had growth promoting activity. The substance was called vitamin A by Drummond in 1920. In 1930, Moore showed that β-carotene extracted from plants had provitamin A activity.

Vitamin A or its precursors may be present in various forms, such as β-carotene, retinaldehyde, retinol, retinyl acetate and retinoic acid. The major sources of vitamin A or its precursors in the diet are carotenoid pigments, such as β-carotene from plants, and preformed vitamin A, mostly in the form of retinyl esters from animals. The various forms of vitamin A are involved in vision, reproduction and the maintenance and growth of epithelial cells. Only the role and the mechanism of retinaldehyde on the visual cycle have been clearly explained (Morton, 1944; Wald, 1953), whereas the action of the other forms of vitamin A are still under investigation.

Interconversion between vitamin A forms is known to exist but the functional activity of certain forms of

vitamin A cannot be replaced by other forms. For example, retinol and retinaldehyde can be utilized by all target tissues, whereas administration of retinoic acid to vitamin A deficient rats successfully maintained the growth of the rats but failed to maintain visual and reproductive functions (Dowling and Wald, 1960).

Either a deficiency of vitamin A or excessive dietary vitamin A can cause various clinical signs and lesions. Vitamin A deficiency causes night blindness which then proceeds to xerophthalmia (Fridericia and Holm, 1925). Also, vitamin A deficiency increases the incidence of diarrhea and respiratory disease (Sommer *et al*, 1984; Bloem *et al*, 1990), as well as the mortality rate among preschool children in developing countries (Sommer *et al*, 1983). Furthermore, vitamin A supplementation in children decreases the incidence of keratomalacia (Vijayaraghavan *et al*, 1984), diarrhea, respiratory diseases (Bloem *et al*, 1990) and childhood mortality (Sommer *et al*, 1986).

Vitamin A also causes abnormalities if administered in excess. Clinical cases and experimental studies of vitamin A intoxication in human beings and animals have been documented and reviewed by many authors (Clarke, 1971; Bendich and Langseth, 1989). Excess vitamin A causes teratogenic effects (Rosa *et al*, 1986), bone deformities (Irving, 1948, Tang *et al*, 1985), intracranial hypertension (Bhettay and Bakst, 1988) and hepatic fibrosis (Bioulac-Sage *et al*, 1988). Most of the previously listed conditions were

caused by the consumption of animal liver rich in vitamin A or by the excessive ingestion of a vitamin A preparation. Most conditions were reversible with a cessation of consumption.

Knowing the deleterious effects of either vitamin A deficiency or toxicity, many authors have reviewed the recommended daily intake for human beings (Bendich and Langseth, 1989; Sklan, 1987) and for animals (Baumann, 1953). Herdt and Stowe (1991) reported the range of serum vitamin A concentrations in dairy cattle. They also indicated that various factors, such as physiologic state, diet and environmental conditions should be considered prior to vitamin A supplementation in dairy cows. Similar studies have not been done with pigs.

Studies on the metabolism and mode of action of vitamin A have led to the discovery of various vitamin A carrier proteins, including retinol binding protein (RBP) (Kanai *et al*, 1968), transthyretin (TTR), CRBP (Bashor *et al*, 1973; Hollander *et al*, 1978b), CRBP II (Ong *et al*, 1987), CRBP III (Nishiwaki *et al*, 1990), cytoplasmic retinoic acid binding protein (CRABP) (Chytil and Ong, 1984) and the nuclear receptor for retinoic acid (Giguere *et al*, 1987; Petkovich *et al*, 1987).

Metabolism

The metabolism of vitamin A (Figure 1) has been extensively reviewed by many authors (Blomhoff *et al*, 1990;

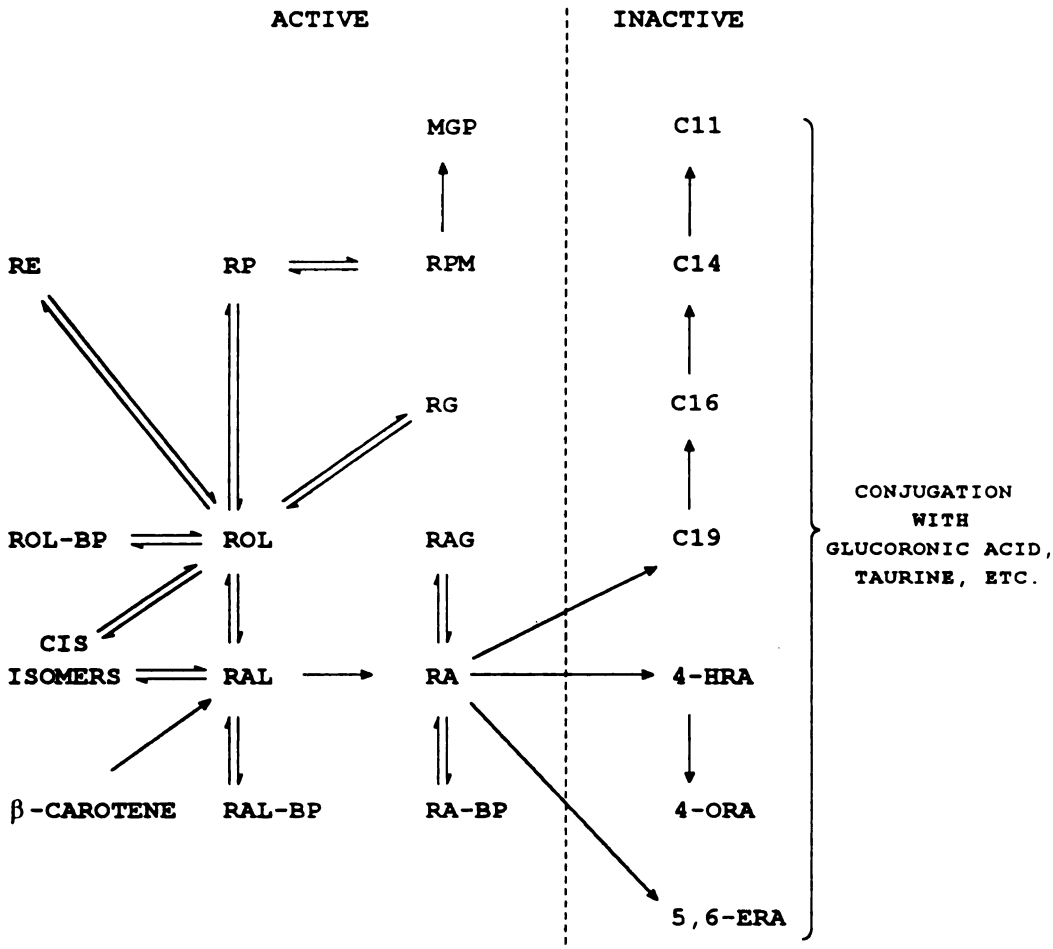

Figure 1. Major metabolic transformations of vitamin A. Metabolites with high biological activity are on the left side of dotted line. ROL, retinol; RAL, retinaldehyde; RA, retinoic acid; RE, retinyl esters; RP, retinyl phosphate; RG, Retinyl β-glucoronide; RAG, retinoyl β-glucoronide; RPM, retinyl phosphomannose; ROL-BP, cellular retinol-binding protein; RAL-BP, cellular retinaldehyde-binding protein; RA-BP, cellular retinoic acid-binding protein; MGP, mannosylated glycoprotein; 4-HRA, 4-hydroxyretinoic acid; 4-ORA, 4-oxoretinoic acid; 5,6-ERA, 5,6-epoxyretinoic acid; C19, C16, etc., oxidized metabolites of retinoic acid containing 19, 16, etc., carbon atoms (Olson, 1986).

Frolik, 1984; Goodman, 1969; Olson, 1969; Sklan, 1987; Herdt and Stowe, 1991). The absorption of β-carotene by the intestine requires the contribution of fat and bile salts to form mixed-micellar solutions (El-Gorab and Underwood, 1973). In all species, β-carotene appears to reach the intestinal mucosa in the same manner, that is through a passive diffusion phenomenon which is directly proportional to the concentration of β-carotene in the intestinal lumen (Hollander and Ruble, 1978).

There are two pathways for β-carotene absorption from the intestine before it reaches the circulation. The β-carotene is either directly transported unchanged to the lymph and blood or it may be cleaved prior to absorption. Direct transport of β-carotene from the small intestinal mucosa to circulation occurs in human beings in which significant amounts of carotene are present in the lymph and blood (Goodman *et al*, 1966). However, in rats virtually no intact β-carotene is absorbed beyond the intestinal mucosa (Huang and Goodman, 1965).

Within the intestinal mucosa, β-carotene is cleaved into 2 molecules of retinaldehyde by a soluble enzyme, β-carotene 15-15'-oxygenase. Immediately after it is formed, retinaldehyde is reversibly converted to retinol by a retinaldehyde reductase enzyme. Also, some retinaldehyde is

irreversibly oxidized to retinoic acid. Evidence suggests that the intestinal mucosa also serves as a target organ for retinoic acid. As an intermediate active metabolite in the intestinal mucosa, retinoic acid is converted to its isomer and glucoronyl derivatives (Cullum and Zile 1985), as well as to 5,6-epoxyretinoic acid (Napoli *et al*, 1978).

Dietary preformed vitamin A, primarily in the form of retinyl esters, is hydrolyzed to retinol by digestive esterase enzymes originating from the pancreas (Hollander and Muralidhara, 1977) and brush border intestinal membranes (Rigtrup and Ong, 1992). Both sets of enzymes are stimulated by the presence of bile salts. In pharmacological concentrations, absorption occurs through passive diffusion. However, under physiological concentrations, absorption occurs through a carrier-mediated-process using an intraluminal retinol carrier (Hollander and Muralidhara, 1977).

Inside intestinal epithelial cells, retinol formed from β-carotene or dietary retinyl esters is carried by CRBP to the endoplasmic reticulum (Hollander and Ruble, 1978). Within the endoplasmic reticulum, retinol is esterified mainly to form retinyl-palmitate (Ong, 1985), irrespective of other types of esters ingested (Mahadevan *et al*, 1963).

Almost all retinyl esters, derived from β-carotene or dietary retinyl esters, are incorporated with acylglyceride-rich chylomicrons prior to entering the lacteals and

reaching the circulation through the thoracic duct (Goodman, *et al* 1966). After extrahepatic hydrolysis of many of the chylomicron acylglycerides, the chylomicron remnants containing retinyl ester are then taken up by hepatocytes (Goodman *et al*, 1965) via receptor-mediated endocytosis (Windler *et al*, 1980). The retinyl esters are subsequently transferred to perisinusoidal stellate cells for storage (Blomhoff *et al*, 1982; 1985). Prior to the transfer, retinyl esters are hydrolyzed to retinol in the hepatocytes and then transferred to the stellate cells (Norum *et al*, 1986). Transfer from the hepatocytes to stellate cells is influenced by the vitamin A status of the animal (Blomhoff *et al*, 1982).

From the liver, retinol is mobilized and delivered to target organs by a specific carrier, RBP (Kanai *et al*, 1968). One molecule of RBP carries one molecule of retinol. The RBP-retinol is found in the circulation as a ternary complex with one molecule of thyroxin-binding prealbumin (transthyretin, TTR). The RBP-retinol-TTR complex is a stable complex and it is not susceptible to glomerular filtration (Wolf, 1984). Besides retinol, RBP can also bind retinaldehyde, retinoic acid and retinyl acetate. Cytoplasmic binding proteins for retinol and retinoic acids are present in a wide variety of tissues (Ong *et al*, 1982). Cytoplasmic retinol binding protein is highly specific, binding only to retinol but not to retinaldehyde, retinoic acid nor retinyl esters. However, CRABP can bind to 13-*cis*-

retinoic acid, as well as to all-*trans*-retinoic acid (Wolf, 1984).

Vitamin A homeostasis

The concentration of vitamin A in plasma is highly regulated. Plasma retinol concentration is fairly constant, independent of diet and liver stores. With very high consumption of vitamin A, plasma concentrations of vitamin A increase simultaneously with the appearance of retinyl esters in the circulation. Decreases of serum vitamin A concentration are observed when liver stores are completely exhausted (Sklan, 1987). The presence of retinyl esters in the circulation in human beings and rats indicates hypervitaminosis A (Sklan, 1987). However, even under normal physiological conditions, vitamin A in the circulation of dogs is in the form of retinyl esters (stearate and palmitate) bound to lipoprotein (Schweigert *et al*, 1990).

Under normal physiological conditions, retinol circulates in the blood as 1:1:1 trimolecular complex with RBP and TTR (Goodman, 1984). Four processes inside hepatocytes regulate the release of stored retinol to the circulation. The processes are 1) hydrolysis of stored retinol to free retinol, 2) synthesis of RBP, 3) attachment of retinol to RBP and 4) release of retinol-RBP-TTR complex.

A review by Wolf (1984) postulates that the normal homeostatic set point for vitamin A may be regulated by an

extrahepatic signal, since the plasma retinol concentration is independent of the hepatic concentration of vitamin A. Retinol metabolites such as retinoic acid may also serve as a regulatory signal for homeostasis. Administration of retinoic acid lowered the plasma homeostatic set point, presumably due to a reduction of the tissue utilization and mobilization of retinol.

Gerlach and Zile (1990) suggested that the kidney may have a direct or indirect role on vitamin A homeostasis. In a study using nephrectomized rats or rats with signs mimicking acute renal failure, they found that an increase in circulatory retinol concentrations was not associated with increased enzyme activity necessary for the deposition and/or release of vitamin A. They suggested that the kidney may serve as a producer of a specific regulatory substance. A decrease in the substance may act as a stimulus for the release of liver retinol or possibly the kidney fails to remove a peripheral signal that increases the homeostatic set point.

Transthyretin plays an important role in retinol transport and homeostasis. Disruption of the transthyretin (*ttr*) gene in mice results in a depression of plasma retinol and RBP to 3% and 6% of normal concentrations, respectively (Episkopou, 1993).

Effect of vitamin A status in the intestine

Using tritiated thymidine in rats, Zile *et al* (1981) indicated that at a very early stage of vitamin A deficiency, the DNA labeling index in jejunal crypt cells was not altered. However, a similar study has revealed that in more advanced stages of vitamin A deficiency, the jejunal crypt cell cycle is slowed by 14% due to a lengthening of DNA synthesis rate (Zile *et al*, 1977). Vitamin A deficiency decreased the number of goblet cells in the duodenal mucosal epithelium and reduced the synthesis of protein by membrane bound polyribosomes in the rat intestinal mucosa (De Luca *et al*, 1969). Readministration of vitamin A to vitamin A deficient rats increased nuclear RNA synthesis in the intestinal epithelial mucosa (Johnson *et al*, 1969; Zachman, 1967) and increased the number of goblet cells (De Luca *et al*, 1970). However, Rojanapo *et al* (1980) showed that reduced numbers of goblet cells in the duodenal mucosal cells of rats was only apparent 4 days after the withdrawal of retinoic acid from the diet. Subsequently, goblet cell numbers returned to normal. Furthermore, they suggested the presence of two different populations of goblet cells in the intestine, one relatively insensitive and one sensitive to vitamin A deficiency.

Molecular biology of vitamin A

De Luca (1977) reviewed the molecular role of retinol and retinoic acid. He suggested that retinol is involved in

the direct transfer of mannosyl residues to membrane glycoproteins through phosphorylation and glycosylation pathways. Retinoic acid might also have a similar involvement in mannosyl transfer. However, it is not known whether the receptor molecule(s) is a constituent of the cell membrane or a secretory product. Increased mannose incorporation is also associated with an increase in adhesive properties of spontaneously transformed mouse fibroblasts (De Luca *et al*, 1979).

Substantial evidence has been reported for the nuclear activity of retinoids. *In vivo* and *in vitro* studies indicate that retinol increases RNA synthesis and uridine incorporation into RNA in the mucosa of the small intestine and colon of vitamin A deficient rats (Zachman, 1967). Similarly, potassium retinoate increases uridine incorporation into the intestinal mucosa and liver "rapidly labeled" nuclear RNA (Johnson *et al*, 1969).

Furthermore, the finding of a nuclear receptor for retinoic acid in the human chromosome, hRAR (Petkovich *et al*, 1987) or hRR (Giguere *et al*, 1987), has partially elucidated the molecular mechanism of retinoids in cellular proliferation and differentiation. The retinoid receptors are structurally similar to steroid and thyroid hormone receptors which may act as either a transcriptional mediator or as a transcriptional regulator where their interaction with retinoic acid induces a cascade of regulatory events controlling morphogenesis and homeostasis or acts as a

negative regulator of oncogenesis. A review by Desbois (1993) classified these receptors as the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) which are apparently universal among vertebrates. The proposed physiological ligand for RARs is all-*trans*-retinoic acid and the ligand for RXRs is the stereoisomer 9-*cis*-retinoic acid. All-*trans*-RA binds only to RAR, whereas 9-*cis*-RA binds both RARs and RXRs. However, both RARs and RXRs can be activated by either all-*trans*-RA or 9-*cis*-RA. It is often observed that the induction of cell differentiation is associated with a decrease in cell proliferation after the addition of retinoids, and it has been suggested that retinoids may disturb both processes independently. Additional data indicated that both RARs and RXRs positively control genes involved in promoting cell differentiation through their binding to retinoid-response elements. However, RARs also indirectly repress expression of AP-1-regulated genes by inactivating AP-1 transcription factor (Figure 2). Genes involved in the control of cell division are included in the genes regulated by AP-1 transcription factor.

POLYAMINES

The polyamines, spermidine and spermine and diamine putrescine, are found in all prokaryotic and eukaryotic cells. They are polycationic, aliphatic, nitrogenous compounds with a low molecular weight. Polyamines play an important role in cellular growth and differentiation.

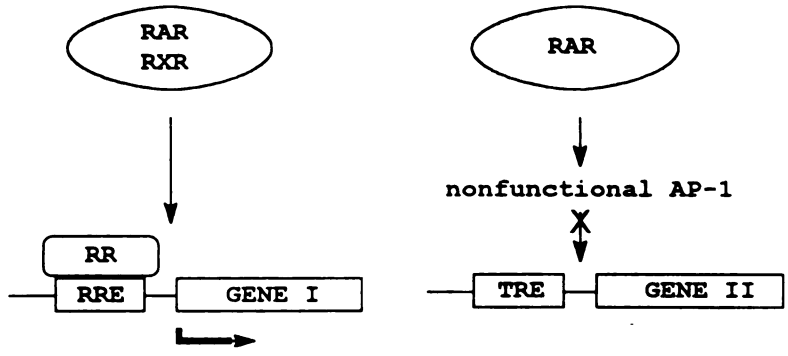

Figure 2. Mode of action of retinoid receptors. RAR, RXR, RR, RRE and TRE are the respective abbreviations of retinoic acid receptors, retinoid X receptors, retinoid receptors, retinoid-response element and tissue plasminogen activator (TPA)-receptor element. Gene I refers to genes, whose expressions are regulated by retinoid receptors, involved in promoting cell differentiation. Gene II refers to genes, whose expressions are regulated by AP-1, involved in the control of cell division (modified from Desbois, 1993).

Although polyamine cellular concentrations are highly regulated, their exact role in cellular growth and differentiation is unknown. Generally, prokaryotes have a higher concentration of putrescine than spermidine, and lack spermine. Eukaryotes, on the other hand, have little putrescine but contain high concentrations of spermidine and spermine (Heby, 1981). The cellular concentration of polyamines can be artificially depleted by either selection of mutant bacteria or by the use of various enzyme inhibitors for polyamine biosynthesis in mammalian cells.

Metabolism

The pathway for polyamine biosynthesis and the enzymes involved in their synthesis are shown in Figure 3, and the structure of polyamines and one of their biosynthetic inhibitors, DL-α-difluoromethylornithine (DFMO), are shown in Figure 4 (Pegg and McCann, 1982; Heby, 1981; McCann *et al*, 1987). Putrescine is a precursor of polyamines. In mammalian cells, putrescine is formed from ornithine by the action of ornithine decarboxylase (ODC). Ornithine is available directly from plasma or it may be formed from arginine by the action of arginase. In many microorganisms, putrescine can be formed from agmatine by the action of agmatinase. Agmatine is formed from arginine by the action of arginine decarboxylase, an enzyme that mammalian cells lack. Putrescine is then converted to spermidine with the

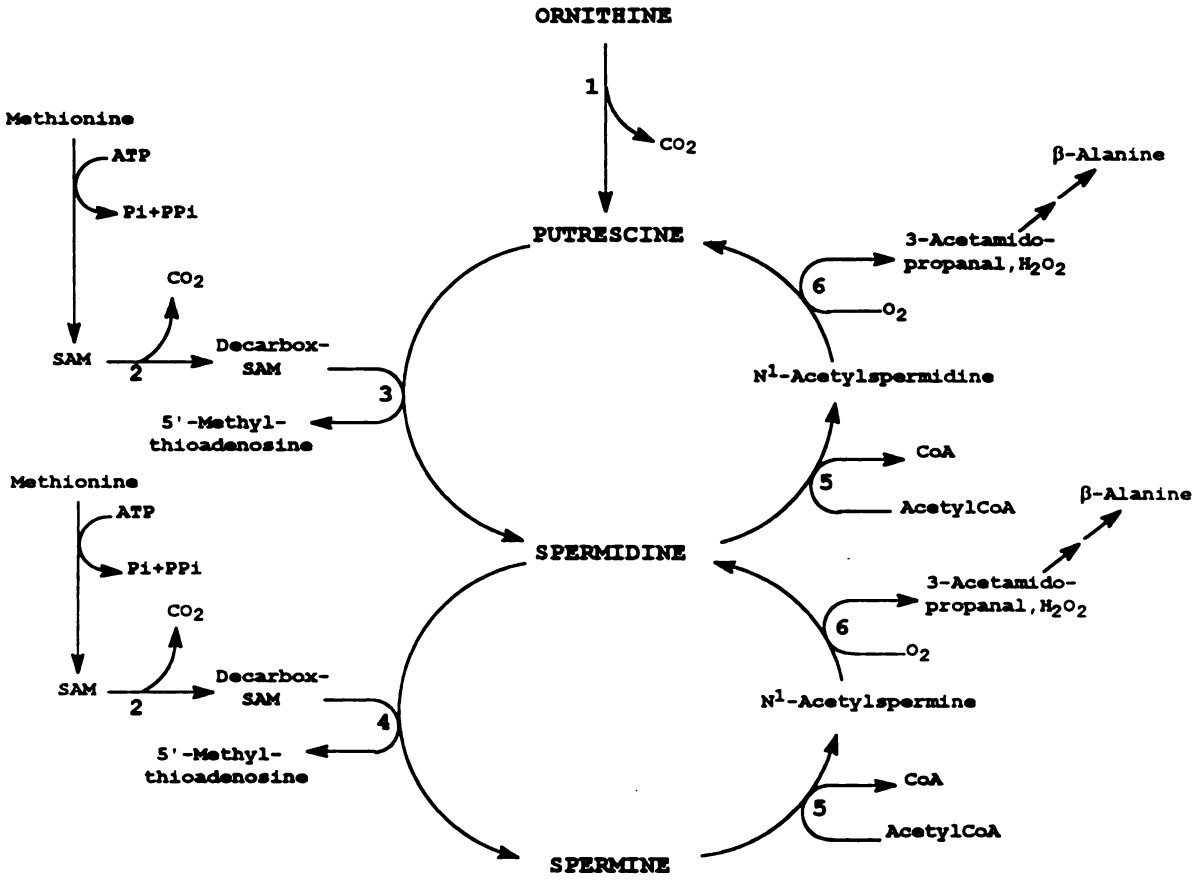

Figure 3. Metabolic pathway of polyamines. Enzymes involved in the polyamine metabolism are ornithine decarboxylase (1), S-adenosyl-L-methionine decarboxylase (2), spermidine synthase (3), spermine synthase (4), acetylCoA:spermidine/ spermine N1-acetyltransferase (5) and polyamine oxidase (6) (modified from Seiler, 1989).

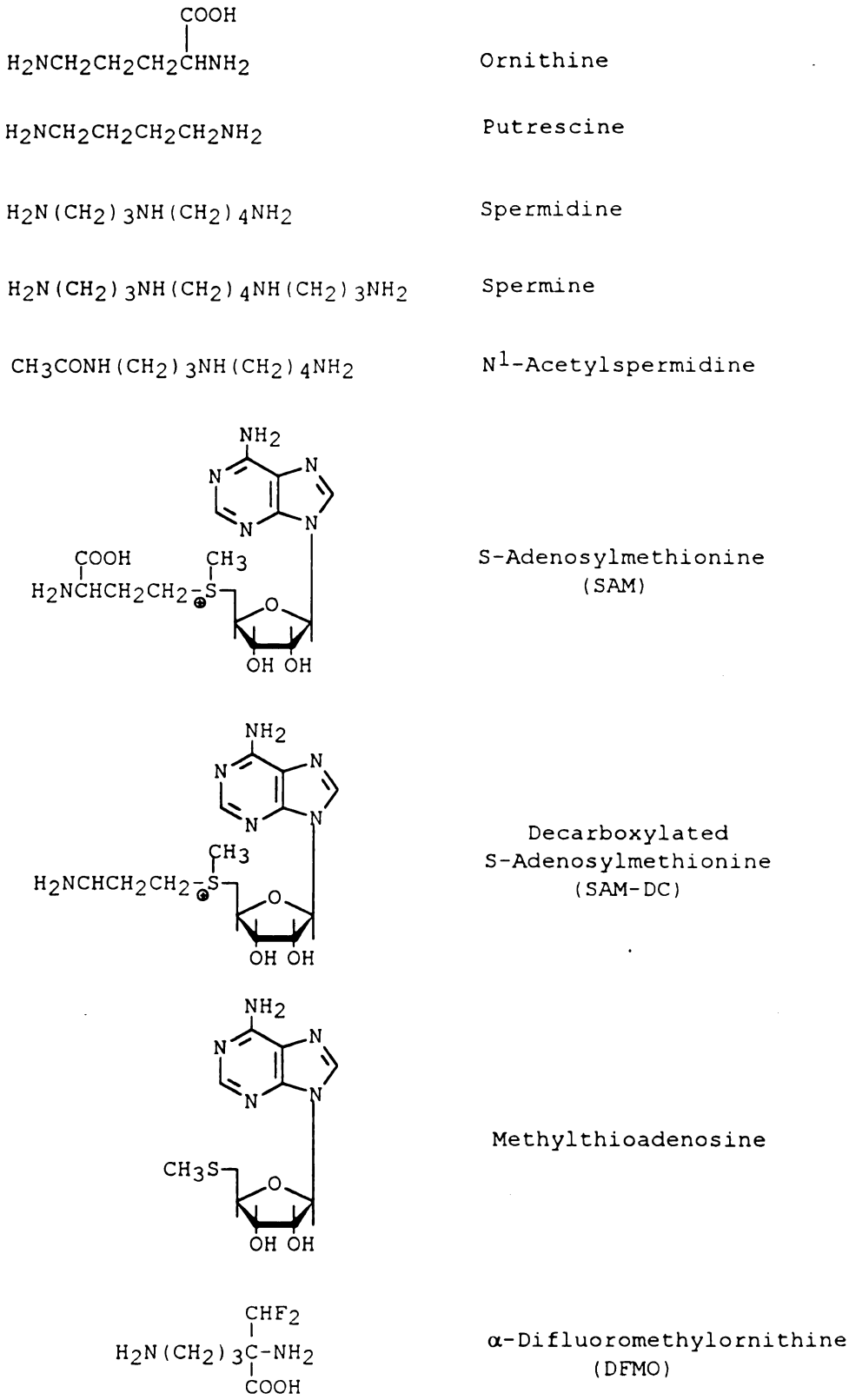

Figure 4. Structure of polyamines and related compounds (Bitonti and McCann, 1987; Mondovi *et al*, 1989).

addition of an aminopropyl compound from the decarboxylated S-adenosylmethionine.

The conversion of putrescine to spermidine is catalyzed by spermidine synthase. The formation of decarboxylated S-adenosyl methionine is catalyzed by S-adenosylmethionine decarboxylase (SAM-DC). The action of S-adenosylmethionine is activated by putrescine and repressed by spermidine.

Spermidine is converted to spermine by the action of spermine synthase. The conversion of spermidine to spermine also needs an aminopropyl moiety from the decarboxylated S-adenosylmethionine.

Spermidine and spermine synthase reactions are irreversible; interconversion between polyamine products can occur *in vivo*. The interconversion needs acetyl CoA and the action of spermidine N'-acetyltransferase and polyamine oxidase enzymes.

Enzymes for polyamine biosynthesis

Inhibitors for ODC and SAM-DC enzymes have been widely used to study the effects of both *in vivo* and *in vitro* polyamine depletion in various cells or tissues. Many studies have indicated that the activity of enzymes involved in polyamine biosynthesis and cellular concentrations of polyamines are associated with tissue growth. Luk and Baylin (1983) found increased activities of ODC and SAM-DC, as well as their synthetic products (putrescine and spermidine), in rat ileal mucosa after intestinal resection.

These increases were closely associated with crypt cell production rate, the rate of villous and crypt lengthening, mucosal thickening and mucosal DNA content and synthesis. Correlation coefficients between the ODC activity and crypt cell production rate, villous length and crypt lengthening rate were 0.97, 0.98 and 0.94, respectively.

Except in the prostate, the ODC activity is extremely low in "nondividing" cells. In early phases of cellular response to a variety of hormones and other substances that stimulate growth and division, the ODC activity increases significantly up to a thousandfold (Heby, 1981).

Studies using ODC inhibitors revealed that polyamines are important for cell proliferation and differentiation. Mamont *et al* (1976) suggested that continual synthesis of polyamines was necessary to maintain cell division processes. Addition of the ODC inhibitor DL-α-methyl ornithine to rat hepatoma tissue culture (HTC) cells reduced the concentration of putrescine and spermidine, parallel to the reduction of cell proliferation and thymidine incorporation into DNA. Addition of exogenous putrescine, spermidine or spermine restores the proliferation of the inhibited HTC. Although the concentration of spermine was not decreased by the presence of DL-α-methyl ornithine, addition of spermine restored cell proliferation, probably due to its conversion to spermidine.

Intestinal adaptation, a proliferative response of the small intestine to a variety of stimuli, such as intestinal resection, cold exposure and lactation, has been shown to be affected by ODC activity and the concentration of intestinal mucosal polyamines. Yang *et al* (1984) showed that an increase in the ODC activity is not the only phenomenon observed during intestinal adaptation in rats but that it plays an important role in mucosal hyperplasia during lactation. During the adaptation, increases in mucosal weight and thickness were accompanied by an increase in ODC and SAM-DC activities and polyamine content. Addition of DFMO, resulted in the suppression of both ODC activity and the adaptive response of the intestine during lactation.

Using DFMO on the jejunectomized rat, the suppression of polyamine synthesis was associated with the suppression of DNA synthesis and resulted in no increases in intestinal weight, mucosal thickness or crypt cell production (Luk and Baylin, 1984). Accumulation of putrescine rather than spermidine is needed for DNA synthesis after partial jejunectomy in the rat (Pösö and Pegg, 1982).

An increase in total RNA, DNA and protein concentrations was also observed in the enterocytes proximal to the ligated intestine. Increases in total RNA, DNA and protein concentrations were lower in the DFMO treated rats (Seidel *et al*, 1984). Also, DFMO prevented intestinal repair and the recovery of DNA, RNA and protein concentrations in the duodenal mucosa of the rat following

stress (Wang and Johnson, 1991), and resulted in delayed intestinal mucosal maturation and recovery subsequent to injury (Luk *et al* 1980). A similar phenomenon was also observed in rats infected with the nematode *Trichinella spiralis* (Wang *et al*, 1991).

As an ODC inhibitor, DFMO, has an effect on intestinal mucosal cell proliferation in young animals. Addition of DFMO to the diet of weaning rats causes villous atrophy and significant decreases in small intestinal mucosal weight, total protein, DNA and various intestinal enzymes (Alarcon *et al* 1987). Holland *et al* (1992) have also observed that the addition of DFMO to *Cryptosporidium parvum* infected calves resulted in more severe villous atrophy.

Addition of exogenous polyamines bypasses some of the effects of the inhibited ODC enzyme in various organs. Luk (1986) showed that the addition of putrescine to DFMO + hepatectomy treated rats reversed the inhibitory effect of DFMO on ODC activity. Protein synthesis, DNA synthesis and liver weights in hepatectomized rats receiving DFMO and putrescine reached similar concentrations of those in hepatectomized rats without DFMO. However, the addition of putrescine had no effect on hepatectomized rats which did not receive DFMO. In addition, putrescine or diethylamine stimulate enterocyte proliferation and partially prevent the reduction of small intestinal absorption in calves caused by a soybean protein diet (Grant *et al*, 1989).

Role of polyamines in cell proliferation

Spermidine plays an essential role in DNA synthesis by stabilizing DNA folds (Flink and Pettijohn, 1975), activating DNA-dependent DNA polymerase (Chiu and Sung, 1972), and stimulating the replication of single strand phage DNA (Wickner *et al*, 1973). Mannen and Russell (1977a,b) indicated that ODC increases the rate of initiation of the RNA polymerase I reaction. Putrescine, on the other hand, has no effect on the RNA polymerase I activity.

Young and Srinivasan (1972) showed that the addition of putrescine into a polyamine deficient *E. coli* mutant resulted in the resumption of protein synthesis before the synthesis of DNA and RNA. Furthermore, Geiger and Morris (1980) showed that polyamines affect the DNA replication fork movement rather than the initiation of DNA synthesis.

Shinki *et al* (1991) suggested a possible association between putrescine and vitamin D_3 action. Administration of vitamin D to vitamin D deficient chicks significantly increased intestinal calcium transport activity. Administration of polyamine synthesis inhibitors, DFMO or N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine, to the chicks decreased the calcium transport activity induced by vitamin D_3. The decrease in the calcium transport can be completely restored by supplementation with putrescine.

ASSOCIATION OF VITAMIN A AND POLYAMINE BIOSYNTHESIS

Several studies on the interaction of vitamin A and polyamine biosynthesis during cell proliferation and differentiation have been done. Studies where various retinoids have been given in association with other substances have focused on the effect of the combined substances on the ODC activity and the synthesis of polyamines, RNA and DNA.

A study on ODC activity using Chinese hamster ovary (CHO) cells revealed that retinoids inhibit ODC induction during G1 progression (Russell and Haddox, 1981). In the G1 phase, ODC synthesis is transcriptionally regulated by the activation of a cAMP-dependent protein kinase. Retinoid did not affect the production of the cAMP-dependent protein kinase nor the production of other proteins required for ODC synthesis. However, in the retinol treated cells, there was no increase in RNA synthesis during G1 phase. Furthermore, they suggested that the specific effect of retinol was on the inhibition of messenger RNA synthesis for ODC production during the G1 phase. The ODC activity appeared to increase again when the cells progressed to the S phase.

Using an initiation-promotion model system, a study involving the effect of retinoids on skin carcinogenesis in mice (Boutwell and Verma, 1981) revealed that retinoic acid increased ODC activity and tumor formation when applied with dimethylbenzanthracene (DMBA), a known tumor initiator.

However, when retinoic acid was applied in conjunction with phorbol ester, a cancer promoter agent, it significantly reduced ODC activity and tumor formation. The DNA synthesis, measured by thymidine incorporation, was significantly reduced initially but increased above normal 42 hours after phorbol ester treatment. In addition, since the phorbol ester acts through stimulation of the AP-1 complex, inactivation of AP-1 by RARs (retinoic acid receptors) might provide an explanation for the reduction of tumor formation (Desbois, 1993).

Daliam *et al* (1988) showed that vitamin A deficiency increased the basal activity of ODC in the lung, esophagus and liver of rats. Addition of 100 μg of retinoic acid significantly reduced or normalized the ODC basal activity in the lung and esophagus, but increased the ODC activity in the liver.

ROTAVIRUS

Rotaviruses are major viral agents causing acute gastroenteritis and diarrhea in young children (Banatvala *et al*, 1978) and a wide variety of young animals including calves (Mebus *et al*, 1969; Hall *et al*, 1993), piglets (Bohl, 1979), foals (Traub-Dargatz, 1988), suckling mice (Ijaz *et al*, 1989), and birds (McNulty *et al*, 1978). Rotavirus may also cause severe diarrhea in adult human beings (Bridger, 1987).

Rotaviruses, member of the genus Reoviridae, are nonenveloped double-shelled infectious particles that possess 11 segments of double-stranded RNA genome. The complete virion has an average diameter of 60-70 nm (Estes *et al* 1983). The name rotavirus was suggested due to the morphological resemblance of the virus to small wheels (Flewett and Woode, 1978). Three types of particles, including double-shelled, single-shelled and core particles, can be seen ultrastructurally. Only the double-shelled particles are infectious (Estes *et al* 1989).

Rotaviruses are extremely infectious. As low as 90 OSU strain porcine rotavirus particles may induce infection in piglets (Payment and Morin, 1990). Graham *et al* (1987) indicated that the lowest dose for the same strain of rotavirus was 1 PFU.

Classification and serotyping

Initial serological studies on rotaviruses isolated from cases of gastroenteritis in children, calves, piglets, mice and foals have indicated that the viruses share common group antigens located within the inner capsid of the virus, irrespective of their species of origin. The common group specific antigens were demonstrable with a variety of techniques including complement fixation (CF), immunofluorescence (FA), gel-diffusion (GD) and immunoelectron microscopic (IEM) tests (Woode *et al*, 1976; Flewett and Woode, 1978).

Further findings of viruses that are morphologically identical to but antigenically distinct from conventional rotaviruses have led investigators to classify the viruses into 6 groups designated as group A, B, C, D, E and F, based on the presence of group specific antigens (Bridger, 1987; Pedley *et al,* 1983, 1986). Reference virus isolates for grouping were the Ohio State University (OSU) strain for group A, NIRD-1 for group B, Cowden and S strain for group C, strain D/132 for group D and strain E/DC-9 for group E. Bohl *et al* (1984) have further classified porcine rotaviruses using a plaque reduction neutralization (PRN) test on MA-104 cells, cross protection studies in gnotobiotic piglets and electropherotype migration of the RNA genome. Porcine rotaviruses can be divided into 2 subgroups on the basis of the ability to produce plaques on initial culture isolation. Subgroup or biogroup 1, which produces plaques, contains the OSU, EE and A-580 strains. Subgroup or biogroup 2, which is unable to produce plaques, consists of Gottfried (G), SB-1b, SB-3 and SB-5 strains.

Although serotyping was done based on the antigenic differences and similarities found in the PRN test and cross protection studies, classification of the rotaviruses based on serotyping has been somewhat variable. For example, Bohl *et al* (1984) classify the OSU strain as serotype 1 and the G strain as serotype 2, whereas Hoshino *et al* (1984) classify these strains as serotype 5 and 4, respectively. In addition to the neutralization test, Sato *et al* (1982) used

an immunofluorescence technique for serotyping rotavirus isolates.

Genome and viral proteins

Electropherotype studies indicate that rotavirus contains an 11-segmented genome. Each segment encodes either a structural or nonstructural viral protein. Nine structural proteins (VP1-VP9) and 4 nonstructural viral proteins have been identified in rotavirus SA11. Inner capsid structural proteins, VP1, VP2 and VP6 are coded by gene segments 1, 2 and 6, respectively. Outer capsid protein VP3 is coded by gene segment 4. Precursors for outer capsid viral proteins, VP7 and VP9, are encoded by gene segments 9 and 11, respectively. Gene segments 5, 7, 8 and 10 code for non-structural viral proteins (Estes *et al*, 1983).

Biological properties of both structural and nonstructural glycoproteins of some rotaviruses have been studied. The outer capsid protein VP4 has been implicated in several important functions such as cell penetration, hemagglutination activity, generation of neutralizing antibody, and virulence (Burns *et al*, 1988). Prasad *et al* (1990) showed that the surface spikes on rotavirus particles were made up of VP4. Ball *et al* (1996) showed that the rotavirus nonstructural glycoprotein NSP4 acts as a viral enterotoxin by potentiating chloride secretion through a calcium-dependent signaling pathway.

Serotype specificity has been attributed to the outer capsid protein VP7 (Kalica *et al*, 1981). Studies on bovine rotavirus (neonatal calf diarrhea virus, NCDV) and simian rotavirus (SA11) have revealed that two genes encoding viral proteins VP3 and VP7 - gene segments 4 and 9, respectively - cosegregate with neutralization specificity (Offit and Blavat, 1986). Reassortant (recombinant) rotaviruses containing VP3 and VP7 from different parent rotaviruses induce a protective immune response against both parental serotypes (Offit *et al*, 1986a,b).

There is also evidence that interaction among viral proteins and calcium availability is required for the virus to function. In calcium-deprived cultures, the concentration of the outer capsid protein (VP7) decreases due to degradation (Shahrabadi *et al*, 1987). One study has determined that neutralization of VP7 requires interaction of VP7 with other rotaviral proteins (Dormitzer *et al*, 1992).

Viral entry and growth

Results of most rotaviral studies indicate that oral transmission is the common method of infection. Experimentally, the infection can also be transmitted via aerosol droplets in suckling rats (Prince *et al*, 1986). In a study using MA104 cells, Suzuki *et al* (1985) indicated that the KUN strain of human rotaviruses entered the cells by direct penetration of the nucleoid through the cell

membrane, and not by endocytosis as previously believed (Petrie *et al*, 1982). Furthermore, Kaljot *et al* (1988) found that addition of endocytosis inhibitors, such as sodium azide and dinitrophenol, had only a limited effect on viral entry and that neutralizing antibodies can inhibit direct membrane penetration. Pretreatment with trypsin resulted in cleavage of VP3 and dissolution of the virus capsid within the cell membrane, followed by direct penetration of the viral nucleoid into the cytoplasm, probably through a protease-dependent mechanism. The non-trypsin treated virus (with intact VP3) enters the cell through the relatively slower endocytic pathway and is ultimately degraded in lysosomes (Kaljot *et al*, 1988).

Virus replication has been studied with bovine and simian rotaviruses. Maximal protein synthesis of simian rotavirus SA11 was noted within 3-5 hours postinfection (Ericson *et al*, 1982). A review by Estes *et al* (1983) indicated that animal rotaviruses have fairly rapid generation cycles. A complete replication cycle was achieved within 12 hours, with a 2- to 3-hour viral eclipse phase. In experimentally infected suckling rats, rotavirus antigen was excreted in a biphasic pattern. Following oral inoculation, the antigen was detected at 1, 4 and 5 days postinfection in 100% of the experimental animals, whereas, at 2 and 3 DPI, antigen excretion was only detected in 70 and 20% of the experimental animals, respectively (Vonderfect *et al*., 1988).

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In addition to enterocytes, viral antigen can also be found in other tissues. Following infection, rotavirus has been found in the lungs of rats (Prince *et al*, 1986) and in the brain and spleen of pigs (Shaw *et al*, 1989b).

Factors affecting infection

Rotaviruses cause severe disease in young animals and human beings. Age-related resistance has been reported in mice (Patterson, 1987; Ball *et al*, 1996), but not in pigs (Gelberg, 1992), human beings and cattle (McNulty, 1978).

Breast-feeding or continual administration of colostrum is an important factor in protection against enteric pathogens. Total protection against rotavirus can not be achieved with breast-feeding, but breast-feeding lessens the severity of diarrhea and vomiting associated with a rotavirus infection. Immune colostrum protects or lessens the severity of rotavirus infection in young animals and human beings. Using cow milk collected at various times, ranging from 8 hours to 10 days postparturition, colostral antibody titers against rotavirus ranged from a high 8 hours postpartum to no titer on day 10 postpartum (Acres and Babiuk, 1978). Administration of colostral supplements have been shown to have a protective effect or lessen the severity of rotavirus infection in calves (Saif *et al*, 1983; Snodgrass and Wells, 1978).

Antibody directed against rotavirus present in chicken eggs is capable of inhibiting the growth of some rotavirus

strains in tissue culture (Yolken *et al*, 1988). Antibody levels can be increased by immunizing hens with rotavirus. The antibody can then be used to prevent the development of rotaviral enteritis in experimentally infected animals (Yolken *et al*, 1988). In addition, Archambault *et al* (1988) have shown that colostral antibodies and colostral lymphocytes from immune cows have a protective effect on calves infected with rotavirus.

The continual presence of antibody in the small intestinal lumen appears to be the prime mediator of protection against rotavirus infection (Woode *et al*, 1975; McNulty *et al*, 1976). In addition, virus-neutralizing antibody present in the intestinal lumen is predominantly in the form of IgA (Chanock, 1978; Burns, 1996). Recently, Burns *et al* (1996) indicated that rotavirus VP6-specific IgA has the capability to inactivate rotavirus intracellularly.

Besides antibodies and lymphocytes present in the milk, another substance has been shown to have a protective effect against rotavirus infection. Mucin, a macromolecule found in human milk, directly binds to rotavirus and inhibits viral replication both in vivo and in vitro (Yolken *et al*, 1992). Polyions having negative charges, such as mucin, α-1-acid glycoprotein heparin, heparan sulphate and dextran sulphate, inhibit rotaviral infectivity and replication, whereas polymers having positive charges, such as protamine, protamine sulphate, DEAE-dextran, histone and poly-L-lysine

hydrobromide, enhance viral infectivity (Superti *et al* 1993). A trypsin inhibitor present in human milk has also been shown to have a protective effect against rotavirus infection in infants (McLean and Holmes, 1978).

The presence of rotavirus severely enhanced the lesions caused by enterotoxigenic *E. coli* in neonatal gnotobiotic calves (Torres-Medina, 1984). Similarly, Hall and Parsons (1989) showed that the combined effect of weaning and rotavirus infection in gnotobiotic piglets led to more severe damage in the distal part of the small intestine, than rotavirus infection alone.

Vitamin A deficiency caused dramatic lesions in rotavirus-infected mice (Ahmed *et al*, 1990). In the small intestine of vitamin A deficient mice, rotavirus caused almost complete destruction of the tips of the villi compared to the control animals. They concluded that although rotavirus infection and vitamin A deficiency caused few changes alone, their combined effect caused significant destruction of the mucosal barrier of the small intestine.

Clinical signs

Many authors have described the clinical signs associated with rotavirus infection, both experimentally and under natural conditions, in infants and animals. Among the clinical signs, diarrhea is the most prevalent. Vomiting is inconsistently seen in piglets. In infants, fever and vomiting also quite often accompany the diarrhea.

Pigs develop clinical signs, including depression, anorexia, vomiting and diarrhea, starting from 12-18 hours postinfection (Theil *et al*, 1978). In a study by Svensmark *et al* (1989a,b), profuse watery diarrhea, weakness and dehydration were observed 1-2 days after inoculation and the surviving pigs recovered in 1-2 weeks. Later, McAdaragh *et al* (1980) reported that diarrhea in gnotobiotic piglets caused by rotavirus was usually observed within 12 to 96 hours postinfection. The fecal consistency varied from a mixture of solids and liquid to a liquid with no detectable solids. The fecal consistency returned to normal 96 hours later.

Diarrhea, indicated by increased quantities of bright yellow feces, and anorexia were noted in calves experimentally infected with a virulent strain of bovine rotavirus (Hall *et al*, 1993). Woode and Crouch (1978) reported that calves either infected with human or bovine rotaviruses showed clinical signs of depression, weight loss and diarrhea. The diarrhea consisted of watery light green feces with mucus and fresh blood or yellow-white feces. Some of the experimental calves took 28 days to regain their birth body weight.

Lesions

The virus causes lesions restricted to the small intestine. The lesion usually is comprised of detached absorptive epithelial cells leading to villous atrophy. In

piglets, the earliest evidence of epithelial detachment and villous atrophy was noted 12 hours postinfection in the proximal jejunum and by 24 hours, the lesion extended to the distal jejunum and proximal ileum (McAdaragh *et al*, 1980).

Villous atrophy in the caudal two-thirds of the small intestine is the consistent lesion caused by the OSU strain of porcine rotavirus (Theil *et al* 1978a). Villi were shortened, blunted and covered with cuboidal epithelial cells. McAdaragh *et al* (1980) on the other hand, found that the lesion was present throughout, but variably distributed in, the small intestine and frequently, both atrophic and normal villi were present within the same intestinal section.

The presence of detectable rotavirus antigen in the small intestinal mucosal cells always precedes the onset of diarrhea and declines after the onset of diarrhea. With an immunofluorescence technique, Theil *et al* (1978a) reported that the OSU strain of porcine rotavirus antigen was demonstrable throughout the small intestine but was detected most consistently in the jejunum and ileum as early as 4 hours postinfection. Within 16-24 hours postinfection almost all columnar epithelial cells covering the apical half of the jejunal and ileal villi were infected. After 24 hours, the number of infected cells became fewer, but the cells still remained positive up until 96 hours postinfection. Rotavirus antigen was not detected after 168 hours. These results differ from the finding of McAdaragh

et al (1980). They found that the rotavirus antigen was first detected in the duodenum and progressed to the distal part of the small intestine.

In a study in calves experimentally infected with either one of two bovine rotaviruses of different virulence, Hall *et al* (1993) reported that even though both strains produced small intestinal lesions, villous atrophy and fusion were more consistently observed in calves infected with the virulent strain. Immunohistochemical staining revealed that the virulent strain was primarily found in the mid and distal small intestine whereas the low-virulent strain was mostly found in the proximal and mid small intestine.

Diagnosis

A definitive diagnosis of rotavirus infection is based on the finding of rotaviral antigen in the diarrheic specimen and its correlation with histological lesions. The presence of rotaviral antigen can be demonstrated with a variety of methods including electron microscopy (Flewett, 1978), immunoelectron microscopy, complement fixation, counter-immunoelectroosmophoresis, immunofluorescence, enzyme-linked immunosorbent assay, radioimmunoassay, hemagglutination assay, cytopathic effect and peroxidase-antiperoxidase technique (Estes *et al*, 1983). A solid-phase immune electron microscopy double-antibody colloidal-gold technique has been recently developed. The technique is 800

times more sensitive than direct EM technique (Wu *et al*, 1990).

CHAPTER 1

EFFECT OF DAILY DIETARY ADMINISTRATION OF RETINYL PALMITATE AND/OR PUTRESCINE ON THE CLINICAL SIGNS, LESIONS AND ROTAVIRAL ANTIGENIC EXPRESSION IN ROTAVIRUS-INFECTED PIGLETS

ABSTRACT

Experiments were conducted to determine the effects of daily, dietary administration of vitamin A and/or putrescine on the clinical signs, lesions and rotaviral antigenic expression in rotavirus-infected piglets. One hundred and twenty-eight piglets were removed from the sows at two days of age. Starting on day 3, piglets were randomly divided into 4 groups of 32 piglets. Three of the groups received milk containing either retinyl palmitate, putrescine or a combination of retinyl palmitate and putrescine. One group received milk only. On day 5, half of each group was infected with porcine rotavirus. Piglets were euthanatized on days 6, 7, 8 and 13 (1, 2, 3 and 8 days postinoculation). Results of this experiment indicate that treatment failed to produce any significant effect on the course of rotaviral infection in piglets, including clinical signs, gross and histologic lesions, and the expression of rotavirus antigen in the small intestinal mucosa.

INTRODUCTION

Rotavirus is the most frequent cause of viral gastroenteritis in young children (Estes, 1983) and animals (Holland, 1990). Rotaviral infections may be asymptomatic (Banatvala and Chrystie, 1978), or cause mild to severe disease which may result in death (Rocchi *et al*, 1981; Carlson *et al*, 1978). Common clinical signs of rotaviral infection in young animals are diarrhea, vomiting and dehydration (Mebus *et al*, 1969; Collins *et al*, 1989; Theil *et al*, 1978).

Lesions induced by rotavirus are limited to the small intestine. Rotavirus infects mature enterocytes, resulting in intestinal epithelial cell desquamation followed by villous atrophy (McAdaragh *et al*, 1980; Theil *et al*, 1978; Woode and Crouch, 1978). The desquamated epithelial cells are replaced by immature squamous to cuboidal cells which lack both digestive and absorptive capacities. The loss of digestive and absorptive capacities leads to an osmotic diarrhea due to nutrient (primarily carbohydrate) malabsorption (Graham *et al*, 1984).

Severity of rotaviral infections, as indicated by the number of deaths, slowed weight gain and the severity of diarrhea, may be influenced by pre- and postnatal dietary effects. Low caloric and/or low protein diets increased the severity of rotaviral infections in suckling mice (Noble *et al*, 1983). Also, malnourishment increased the

susceptibility and the severity of rotaviral infection (Offor *et al*, 1985), as well as rotaviral antigenic expression in small intestinal enterocytes (Riepenhoff-Talty *et al*, 1985, 1989) when compared to well-nourished suckling mice. Additionally, vitamin A deficiency caused more severe clinical signs in rotavirus-infected mice than in mice with adequate vitamin A status (Ahmed *et al*, 1990).

Vitamin A and putrescine have received much attention because of their potential beneficial effects in mucosal cell regeneration. Vitamin A affects the growth and differentiation of various epithelial tissues, including small intestinal epithelial cells (Wolf, 1984). Supplementation of vitamin A to children living in vitamin A deficient populations in many developing countries has been reported to reduce the morbidity and mortality rate associated with diarrheal and respiratory diseases (Sommer *et al*, 1984, 1986; Bloom *et al*, 1990). Ahmed *et al* (1990) indicated that the degree of destruction of small intestinal epithelium in rotavirus-infected mice was negatively associated with the concentration of vitamin A. Rotaviral infection in vitamin A deficient mice caused severe epithelial damage when compared to the infection in normal mice.

Putrescine has been reported to improve small intestinal functions and maintain normal morphology in calves fed soy protein. Villous atrophy occurs in calves fed diets containing soy protein (Kilshaw and Slade, 1982).

Supplementation with putrescine in calves' diets containing soy protein prevented a reduction in small intestinal absorption and enhanced enterocyte proliferation caused by the soy protein diet (Grant *et al*, 1989).

The purpose of this study was to evaluate the usefulness of vitamin A and/or putrescine in preventing or reducing rotaviral infectivity and the clinical signs and intestinal mucosal damage caused by a rotaviral infection in piglets. The objectives of this study were 1) to determine if the clinical signs and lesions produced by a rotaviral infection in piglets could be altered by the daily dietary administration of vitamin A and/or putrescine and 2) to determine if rotaviral antigenic expression in the small intestinal mucosa of piglets could be altered by the daily dietary administration of vitamin A and/or putrescine.

MATERIALS AND METHODS

Experimental animals

One hundred and twenty eight, five-day-old piglets from 16 litters were obtained from the Michigan State University swine farm. All piglets received colostrum from the sows for two days. Experimental design is shown in Figure 1.1. Piglets within the same litter were allocated for a variety of treatments. Piglets were reared in individual cages in a temperature controlled room (30-32^{o}C). Each piglet was

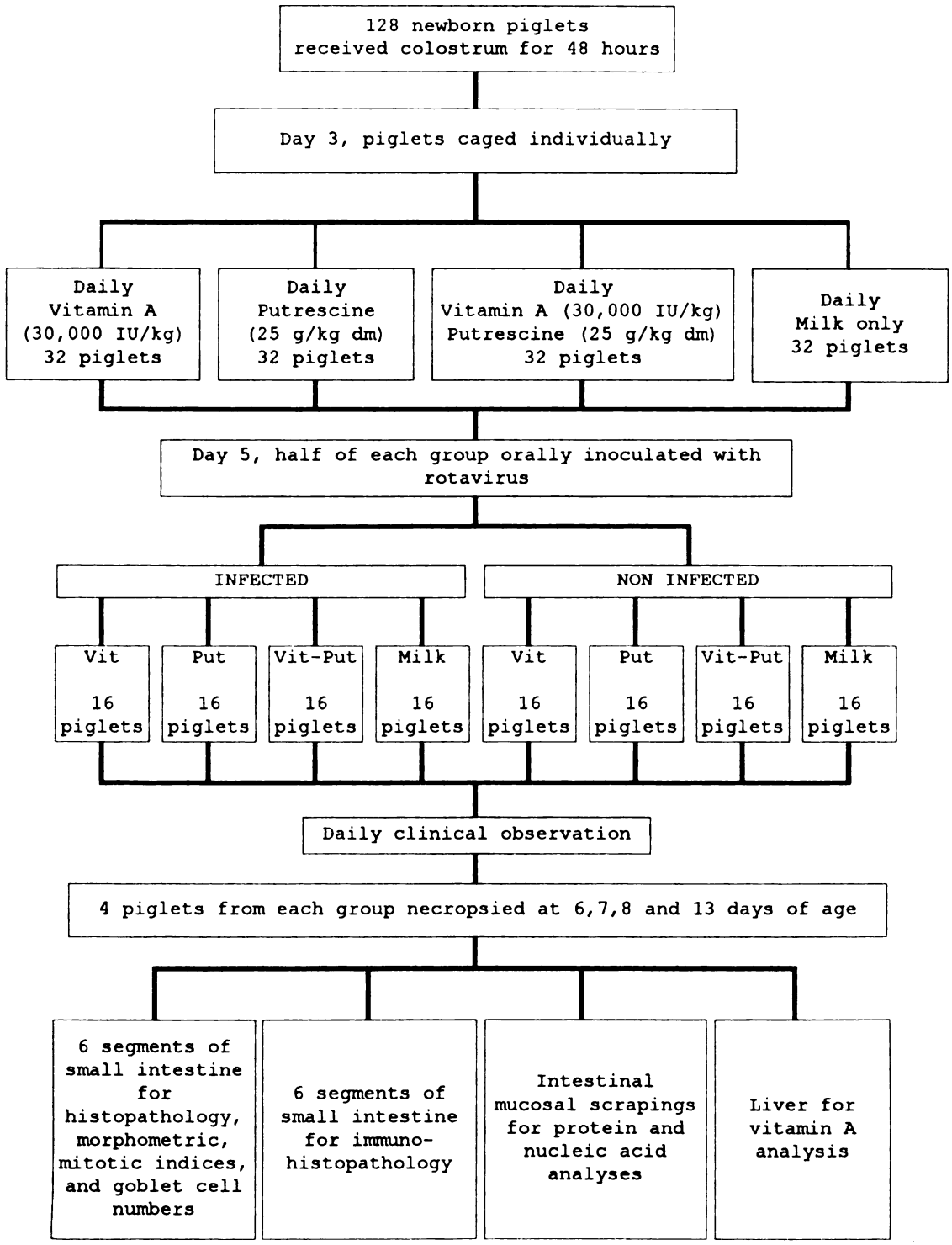

Figure 1.1. Experimental design.

given a total of 300 ml evaporated milk[1] (14% dry matter) daily. The nutritional composition of the milk is shown in Table 1.1. Milk was offered three times a day at 8:00 a.m., 12:00 p.m. and 5:00 p.m. Starting from day 3, subgroup 1 (RP) was given milk containing vitamin A in the form of retinyl palmitate[2] (30,000 IU/kg of milk) daily. Subgroup 2 (P) received putrescine[3] (25 g/kg dry matter of milk) in the milk. Subgroup 3 (RPP) was fed milk containing retinyl palmitate (30,000 IU/kg of milk) and putrescine (25 g/kg dry matter of milk). Subgroup 4 (MO) received milk only. Addition of vitamin A and putrescine in the milk was done just prior to feeding by diluting each of the substances in approximately two ml of distilled water. On day 5, the rotavirus-infected piglet group was orally inoculated with 1 ml 10^{-10} dilution of the stock intestinal homogenate. Following inoculation, all animals were observed three times a day during the feeding for clinical signs of diarrhea. Body weight, as well as combined fecal and urinary output weight, were determined[4]. Weighing was performed daily prior to the morning feeding. Four piglets from each group were euthanatized on days 6, 7, 8 and 12. The time for necropsy was scheduled so that only five to six animals were euthanatized in a day.

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[1] Carnation Evaporated Milk, Nestle Food Co., Glendale, CA.
[2] Sigma Chem. Co., St. Louis, MO.
[3] Sigma Chem. Co., St. Louis, MO.
[4] Mettler, Type BD6000, Mettler Instrument Corp., Highstown, NJ.

Table 1.1. Nutritional composition of milk used in the experiments

Nutrients	100 grams of milk
Calories	134
Protein, g	6.81
Carbohydrate, g	10.00
Fat, g	7.56
Ash, g	1.55
Crude fiber, g	0
Vitamin A, IU	159

Necropsy and specimen collection

Pigs were euthanatized by an overdose of sodium pentobarbital[5] administered intravenously. Three-cm segments from six sites of the small intestine were removed immediately and placed in buffered neutral formalin for histopathologic and immunohistochemical examinations. The first segment was removed from the duodenum approximately 5-10 cm distal to the pyloric junction and the last segment was obtained from the lower part of the ileum, approximately 5 cm proximal to the ileocecal junction. The remaining segments were obtained at approximately a sixth, third, half and two-thirds of the distance from the pylorus to the ileocecal junction. These segments were designated as duodenum (DU), upper jejunum (UJ), lower jejunum (LJ), mid-intestine (MI), upper ileum (UI) and lower ileum (LI). The lumen of the segments were rinsed with 10% neutral buffered formalin and the segments were then immersed in the fixative.

Histopathology

Formalin-fixed, small intestinal samples were routinely processed for histopathologic examination. The tissues were oriented in cross sections (ring shaped) and embedded in paraffin. Tissues were cut by a microtome into 6-μm thick sections. One slide contained DU, UJ, LJ segments and the

[5] The Butler Co., Columbus, OH.

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b

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p

u

p

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second slide contained MI, UI and LI segments. The sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy for lesions.

Qualitative ELISA for rotavirus antigen in feces

Pre-infected and post-infected feces of experimental pigs were assayed for the presence of rotavirus antigen using a commercially available qualitative ELISA kit[6]. To a polystyrene tube coated with rabbit anti-rotavirus IgG, 100 µl horseradish peroxidase (HRP)-conjugated monoclonal antibody and 300 µl of 10% raw stool was added. Three hundred µl samples of an inactivated, simian rotavirus (SA-11) suspension and the sample diluent were used as positive and negative controls. After gentle mixing, the suspension was incubated at room temperature for 60 minutes. The suspension was then discarded and the tube was washed 5-7 times with 2-4 ml distilled water per wash. To each tube a 0.5 ml chromogen substrate solution of tetramethylbenzidine was added. The tube was then covered and incubated for 15 minutes at room temperature to allow for color development. A blue color indicated the presence of rotavirus antigen.

Immunohistochemical assay

Unstained 4 µm sections from six parts of the small intestine, designated as DU, UJ, LJ, MI, UI and LI, were used in an avidin-biotin-based immunohistochemical assay for

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[6] Pathfinder™®, Kallestad Diagnostics, Austin, TX.

the presence of rotavirus antigen in enterocytes. Two intestinal slides from known rotavirus-infected piglets and two intestinal slides from uninfected piglets were used as controls. The immunohistochemical assay kit and its protocol were obtained from SIGMA[7]. The protocol for the standard procedure outlined in the kit was followed with some modification (Parsons *et al*, 1984). The unstained sections were deparaffinized in two consecutive xylene baths of 10 minutes each. The sections were rehydrated by immersing the slides for 2-3 dips in consecutive baths of 100, 100, 100, 95, 95, 80, 70, 50 percent ethanol and distilled water. Then the slides were washed in PBS for 5 minutes. The slides were then placed for 10 minutes in 3% hydrogen peroxide, 10 minutes in tap water and 5 minutes in a phosphate-buffered saline (PBS) wash. The slides were then incubated with diluted goat serum (blocking reagent) for 10 minutes at room temperature. After wiping the slides and removing the excess reagent, the slides were incubated with the primary antibody (1:100 rabbit antihuman rotavirus antibody[8] in PBS), for 24 hours at 4°C in a humid chamber. In this step, normal rabbit antiserum containing the same amount of protein as the primary antibody was added to one positive control and one negative control slide (DeLellis *et al*, 1979). After a 5 minute PBS wash, the slides were incubated with the secondary biotinylated antibody

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[7] Sigma Chemical Co., St. Louis, MO.
[8] DAKO Corp., Santa Barbara, CA.

(biotinylated goat-antirabbit IgG). After another 5 minute PBS wash, the slides were incubated with peroxidase reagent (ExtrAvidin®-conjugated peroxidase in buffered saline) at room temperature for 20 minutes. After a 5 minute wash in PBS, the slides were incubated with the substrate reagent (0.1% 3,3'-diaminobenzidine in PBS with an addition of a drop of 3% hydrogen peroxide per 10 ml of substrate solution) at room temperature for 10 minutes. The slides were then washed in deionized water for 5 minutes, counterstained with Mayer's hematoxylin and mounted with a coverslip. The cytoplasm of the infected epithelial cells developed a golden-brown color. The severity of infection in each segment of the intestine was scored as 0 if no rotavirus antigen was found in any enterocyte, 1+ if less than 5 percent of the villi contained positive enterocytes, 2+ if between 5 to 25 percent of the villi contained positive enterocytes, 3+ if between 25 to 50 percent of the villi contained positive enterocytes and 4+ if more than 50 percent of the villi contained positive enterocytes.

Statistical analysis

Data were grouped based on the infection status, treatment and time. Each group consisted of four infected and four control animals as in the experimental design. Statistical analyses were performed using three-factor analysis of variance (three-way ANOVA) with main effects of treatment, infection and time (Devore, 1991). The numbers

of levels of the three factors were denoted as 2, 4 and 4 for the infection status, treatment and time factors, respectively. Differences between group means within each factor were analyzed by one-way ANOVA followed by Tukey's procedure (Fowler and Cohen, 1990). Differences between groups were considered significant at the level of $P \leq 0.05$. Statistical analyses were done using SPSS Computer Software[9].

RESULTS

Clinical signs

Before inoculation with the SDSU strain of porcine rotavirus, piglets were active, alert and healthy. Their appetites were usually very good, indicated by the disappearance of milk from the pan. Their feces were yellowish to tan with a firm to pasty texture.

Clinical signs of diarrhea were observed from day 1 to day 8 postinoculation (Table 1.2). The highest incidence of diarrhea occurred 2, 3 and 4 days postinoculation (DPI). Clinical signs in infected piglets were similar and were not altered by treatment. Diarrhea was determined by the presence of yellowish watery feces on the floor pan and hindquarters of the piglets. Occasionally, the diarrheic feces contained curds of undigested milk. Also, vomitus containing curds of undigested milk was observed with some

[9] SPSS Inc., Chicago, IL.

Table 1.2. Number of diarrheic piglets 1 - 8 days postinoculation with the SDSU strain of porcine rotavirus

Treatment group[1]	Days postinoculation							
	1	2	3	4	5	6	7	8
RP	5/16[2]	8/12	2/8	2/4	1/4	1/4	0/4	0/4
P	5/16	6/12	2/8	1/4	0/4	0/4	0/4	0/4
RPP	7/16	6/12	5/8	3/4	2/4	2/4	1/4	1/4
MO	2/16	6/12	3/8	2/4	2/4	3/4	2/4	1/4

[1] Group RP: 9,000 IU retinyl palmitate/day.
Group P: 1,050 mg putrescine/day.
Group RPP: 9,000 IU retinyl palmitate and 1,050 mg putrescine/day.
Group MO: milk only.

[2] Number of diarrheic piglets/observed piglets. On days 1, 2 and 3 postinoculation, four piglets were euthanatized, thus the decline in animal numbers.

infected piglets. Vomiting was observed in two piglets from the P group, one piglet from the RPP group and two piglets from the MO group. Most piglets, especially those which had diarrhea, became anorectic, indicated by their reluctance to drink milk at the time of feeding or the presence of milk remaining in the pan at the time of the next feeding. Also, diarrheic animals were mildly dehydrated. At 5 DPI, signs of diarrhea began to subside and animals regained their appetites. Most piglets recovered by 7 DPI. All piglets survived until the time of necropsy. Combined fecal and urinary weights between treatment groups of animals were not significantly different (Table 1.3).

Piglets that were not inoculated with the virus remained active and alert throughout the experiment. They rapidly consumed their milk and had firm to pasty, golden yellow feces.

Body weight

On 1, 2 and 3 DPI, infected piglets in the MO group had average body weight and daily weight gains similar to the treated groups (Tables 1.4 and 1.5). Body weights and weight gains were not significantly different. All piglets, irrespective of the treatment group, had highly variable body weights and daily body weight gains, as reflected by high standard deviations (data not shown). For example, on 1 DPI, one piglet in the infected, RP group had a weight loss of 10%, whereas, another piglet within the same group

Table 1.3. Average of combined fecal and urinary weights (grams) for control piglets *vs.* piglets infected with the SDSU strain of porcine rotavirus

	Infected				Infected DPI mean	Control				Control DPI mean	All DPI mean ± SE[d]
	RP [a]	P	RPP	MO		RP	P	RPP	MO		
0 DPI [b]	93 [c]	100	141	82	104	101	115	98	98	103	104 ±6
1 DPI	103	94	114	97	102	93	90	97	108	97	100 ±5
2 DPI	134	101	99	81	104	121	116	112	126	119	111 ±6
3 DPI	101	129	98	90	105	132	109	150	140	133	119 ±7
8 DPI	170	169	155	145	160	102	141	145	140	132	146 ±10
Treatment mean ± SE	111 ±9	108 ±5	119 ±10	92 ±7	107 ±4	108 ±8	109 ±6	112 ±8	116 ±9	111 ±4	109 ± 3

[a] RP: 9,000 IU retinyl palmitate/day, P: 1,050 mg putrescine/day, RPP: 9,000 IU retinyl palmitate and 1,050 mg putrescine/day, MO: milk only.

[b] DPI: day postinoculation.

[c] The number of piglets on 0, 1, 2, 3 and 8 DPI were 16, 16, 12, 8 and 4, respectively.

[d] Fecal and urinary weights 8 DPI > 0 and 1 DPI ($P<0.01$).

Table 1.4. Average of daily body weights (grams) for control piglets *vs*. piglets infected with the SDSU strain of porcine rotavirus

	Infected				Infected DPI mean	Control				Control DPI mean	All DPI mean ± SE
	RP [a]	P	RPP	MO		RP	P	RPP	MO		
0 DPI [b]	1741 [c]	1638	1581	1650	1653	1618	1501	1670	1653	1610	1632 ±28
1 DPI	1799	1701	1611	1653	1705	1683	1556	1714	1705	1665	1685 ±31
2 DPI	1925	1810	1780	1637	1828	1874	1633	1855	1601	1741	1784 ±35
3 DPI	2067	1910	1927	2071	1960	1993	1768	1921	1738	1855	1908 ±42
8 DPI	2623	2525	2511	1457	2511	2377	2112	2155	2180	2206	2358 ±54
Treatment mean ± SE	2031 ±54	1917 ±60	1882 ±55	1895 ±58	1810 ±28	1909 ±47	1714 ±37	1863 ±44	1775 ±47	1731 ±22	1771 ± 18 [d]

[a] RP: 9,000 IU retinyl palmitate/day, P: 1,050 mg putrescine/day, RPP: 9,000 IU retinyl palmitate and 1,050 mg putrescine/day, MO: milk only.

[b] DPI: day postinoculation.

[c] The number of piglets on 0, 1, 2, 3 and 8 DPI were 16, 16, 12, 8 and 4, respectively.

[d] Statistical analyses:
Body weights 8 DPI > 2 and 3 DPI > 0 and 1 DPI ($P<0.01$).
Body weights infected piglets > control piglets ($P<0.01$).
Body weights RP groups > P groups ($P<0.05$).

Table 1.5. Average daily body weight gains (grams) for control piglets *vs*. piglets infected with the SDSU strain of porcine rotavirus

	Infected				Infected DPI mean	Control				Control DPI mean	All DPI mean ± SE
	RP [a]	P	RPP	MO		RP	P	RPP	MO		
0 DPI [b]	74 [c]	47	36	64	55	12	-23	-3	-10	-6	25 ±8
1 DPI	66	55	33	60	54	39	16	20	21	24	39 ±6
2 DPI	75	59	51	80	66	60	45	52	25	45	56 ±5
3 DPI	85	68	62	95	78	77	63	60	51	63	70 ±5
8 DPI	109	96	86	83	93	97	90	78	76	85	89 ±4
Treatment mean ± SE	76 ±8	58 ±8	46 ±10	72 ±8	63 ±4	45 ±9	23 ±10	30 ±9	21 ±10	30 ±5	47 ± 3 [d]

[a] RP: 9,000 IU retinyl palmitate/day, P: 1,050 mg putrescine/day, RPP: 9,000 IU retinyl palmitate and 1,050 mg putrescine/day, MO: milk only.

[b] DPI: day postinoculation.

[c] The number of piglets on 0, 1, 2, 3 and 8 DPI were 16, 16, 12, 8 and 4, respectively.

[d] Statistical analyses:
Daily body weight gains (DBWG) 0 DPI < 2, 3 and 8 DPI ($P<0.01$).
DBWG 1 DPI < 3 and 8 DPI ($P<0.01$).
DBWG of infected 0 and 1 DPI piglets > control 0 and 1 DPI piglets ($P<0.01$).

had a weight gain of 10%. Similarly, on 2 DPI, one piglet in the RPP group had a weight loss of 1% whereas another piglet in the same group had a weight gain of 18%. Although differences were not statistically significant, all infected piglets in the supplemented groups 8 DPI had higher body weights and body weight gains than the MO group of infected piglets 8 DPI.

Gross lesions

The external appearance of some of the diarrheic piglets necropsied on 1, 2 and 3 DPI was: thin, dehydrated, as judged by a decrease in skin pliability and the prominence of bony processes, rough hair coat and fecal staining on the hindquarters. Some diarrheic piglets, especially those euthanatized 8 DPI, appeared in good condition, except for the presence of fecal staining on the hindquarters.

Internal lesions in the infected piglets, regardless of treatment, were very similar. Most diarrheic piglets that were euthanatized 1, 2 and 3 DPI had minimal to no chyle in lymphatics. Piglets euthanatized 8 DPI, regardless of the state of diarrhea, had normal amounts of chyle in the lymphatics. Stomachs of all piglets contained various amounts of curdled milk. Small intestines of diarrheic piglets euthanatized 1, 2 and 3 DPI were pale, flaccid, thin-walled and contained large amounts of watery, yellow fluid. The ceca and colons of the diarrheic piglets

euthanatized 1, 2 and 3 DPI were distended with a large amount of watery yellow fluid which occasionally contained a small amount of curdled, undigested milk. Other organs were grossly normal.

Histologic lesions

Enterocyte vacuolation was seen in all of the experimental piglets (Figures 1.2 and Table 1.6), but the number of vacuolated enterocytes was higher in the infected group than in the control piglets ($P<0.01$). Vacuolated enterocytes contained either a single large vacuole or multiple small vacuoles. Piglets 1 DPI had less vacuolated enterocytes than piglets 8 DPI ($P<0.01$). The number of vacuolated enterocytes did not differ between treatment groups.

Vacuolar degeneration of enterocytes (VDE) in the villi was present in almost all infected groups (Figure 1.3 and Table 1.7). The VDE was indicated by separation and clumping of epithelial cells, lack of columnar form, irregular appearance of the cell walls and pleomorphism. Cells had distended cytoplasms and the nuclei were displaced centrally. The numbers of degenerated enterocytes were highest in animals 1 to 3 DPI, and lowest in animals 8 DPI ($P<0.01$). No VDE was observed in the control group. No differences existed in the number of degenerated enterocytes between treatment groups.

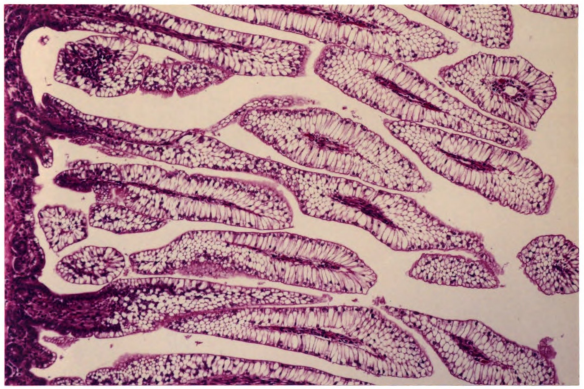

Figure 1.2. Photomicrograph of a section of ileum from a rotavirus-infected piglet in the retinyl palmitate and putrescine group, 2 DPI. Normal enterocyte vacuolation. Each epithelial cell contains a single, large, clear, cytoplasmic vacuole (Hematoxylin and eosin, 120X).

Table 1.6. Enterocyte vacuolation[1] of control piglets *vs.* piglets infected with the SDSU strain of rotavirus

DPI[2]	Treatment group[3]		Intestinal sites[4]					
			DU	UJ	LJ	MI	UI	LI
1	Infected	RP	1.25	1.00	1.25	2.75	2.75	1.75
		P	2.75	3.25	3.25	3.00	2.50	2.25
		RPP	3.25	2.50	2.50	2.50	1.00	1.00
		MO	1.50	1.75	1.25	3.25	4.00	0.75
	Control	RP	3.25	3.00	3.25	3.00	2.25	1.00
		P	0.75	1.25	1.25	1.25	1.25	1.00
		RPP	1.50	2.00	1.00	2.00	1.50	1.00
		MO	-	1.00	2.75	4.00	4.00	4.00
2	Infected	RP	0.75	1.50	2.50	1.75	2.00	1.25
		P	0.25	0.75	1.75	3.25	3.25	1.50
		RPP	2.50	2.75	3.25	4.00	3.25	1.00
		MO	2.00	1.50	2.50	3.00	3.00	1.00
	Control	RP	0.25	-	1.00	1.00	1.00	1.00
		P	1.25	1.00	2.00	1.00	1.00	1.00
		RPP	-	-	0.75	1.75	1.50	-
		MO	1.00	2.25	1.75	2.00	1.00	1.50
3	Infected	RP	0.25	1.00	1.00	2.00	2.00	1.00
		P	0.50	1.00	0.50	3.25	2.25	1.25
		RPP	0.50	0.50	0.50	2.00	2.00	2.00
		MO	0.75	1.50	2.25	3.50	3.50	1.00
	Control	RP	1.00	-	-	1.50	2.00	1.00
		P	0.75	-	-	-	-	0.75
		RPP	-	1.00	0.50	-	1.00	2.00
		MO	-	-	-	2.00	2.00	3.00
8	Infected	A	-	-	-	-	0.75	0.50
		B	0.25	0.50	1.50	1.75	2.25	2.00
		C	-	-	1.75	0.50	2.00	-
		D	-	-	-	-	-	-
	Control	A	-	-	-	-	1.00	-
		B	0.50	-	0.50	-	-	-
		C	-	-	-	-	2.00	1.00
		D	0.25	-	-	-	1.00	0.50

[1] Data are presented as average values (n=4). Values expressed as -: no enterocyte vacuolation, 1: < 25% villi contain vacuolated enterocytes, 2: between 26-50% villi contain vacuolated enterocytes, 3: between 51-75% villi contain vacuolated enterocytes, 4: > 75% villi contain vacuolated enterocytes.

[2] DPI: days postinoculation.

[3] RP: 9,000 IU retinyl palmitate/day, P: 1,050 mg putrescine/day, RPP: 9,000 IU retinyl palmitate and 1,050 mg putrescine/day, MO: milk only.

[4] DU: duodenum, UJ: upper jejunum, LJ: lower jejunum, MI: mid intestine, UI: upper ileum, LI: lower ileum.

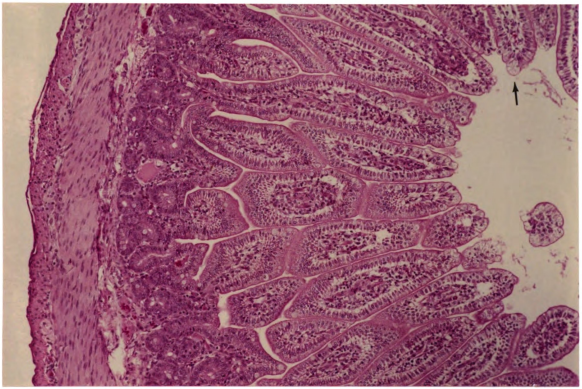

Figure 1.3. Photomicrograph of a section of jejunum from a rotavirus-infected piglet in the putrescine group, 2 DPI. Notice enterocyte vacuolar degeneration (arrow), especially in the tips of the villi (Hematoxylin and eosin, 120X).

Table 1.7. Vacuolar degeneration of enterocytes[1] in piglets infected with the SDSU strain of rotavirus

DPI[2]	Group[3]	Intestinal sites[4]					
		DU	UJ	LJ	MI	UI	LI
1	RP	0.5	0.25	1	1.5	1	1
	P	2.5	3	2.25	2	1.5	0.75
	RPP	1.5	1	0.75	1.5	-	-
	MO	1.25	0.5	-	2	1.5	-
2	RP	0.75	1.5	2	1	0.5	-
	P	0.25	0.75	0.75	1.25	1.25	-
	RPP	2	2.5	2.5	1	1.5	0.75
	MO	1.25	1.5	2.25	1	1	-
3	RP	0.25	1	1	2	1	-
	P	0.5	1	0.5	2.25	2.25	-
	RPP	0.5	0.5	-	1	1	1
	MO	0.75	1.5	2.25	2.5	2.5	-
8	RP	-	-	-	-	0.75	0.5
	P	0.25	0.25	0.25	0.5	0.25	-
	RPP	-	-	0.25	-	-	-
	MO	-	-	-	-	-	-

[1] Data are presented as average values (n=4). Values expressed as -: no vacuolar degeneration of enterocyte (VDE), 1: < 25% villi contain VDE, 2: between 26-50% villi contain VDE, 3: between 51-75% villi contain VDE, 4: > 75% villi contain VDE.

[2] DPI: days postinoculation.

[3] RP: 9,000 IU retinyl palmitate/day. P: 1,050 mg putrescine/day. RPP: 9,000 IU retinyl palmitate and 1,050 mg putrescine/day. MO: milk only.

[4] DU: duodenum, UJ: upper jejunum, LJ: lower jejunum, MI: mid-intestine, UI: upper ileum, LI: lower ileum.

Immature enterocytes (Figure 1.4), indicated by the presence of cuboidal and squamous epithelial cells, were seen in the infected group 1, 2, 3 and 8 DPI (Table 1.8). The infected group had significantly higher numbers of immature enterocytes than the control group ($P<0.01$). There were no statistical differences between treatment groups and the time after inoculation.

Dilatation of villous lymphatic vessels was occasionally observed in both infected and control piglets. Desquamated cells, either from degenerated or infected enterocytes, were not detected in the intestinal lumen.

Immunohistochemistry

Immunohistochemical staining was used to indicate the presence of rotaviral antigen in small intestinal enterocytes. Villous enterocytes containing a fine to globular intracytoplasmic, golden-brown precipitate were considered positive cells. Enterocytes containing a golden-brown precipitate were seen lining the sides and the tips of small intestinal villi of some, but not all, rotavirus-infected piglets euthanatized on 1, 2, 3 and 8 DPI (Figure 1.5, Table 1.9). When present, the predominant site of rotaviral antigenic expression was either the middle or posterior part of the small intestine (Table 1.10). No rotavirus antigen was detected in the intestinal lumen. The statistical differences between treatment groups were not significant.

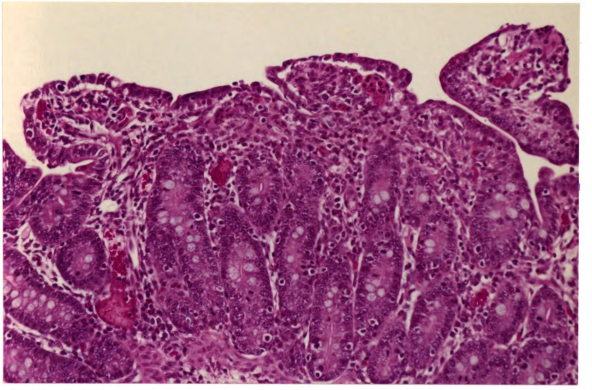

Figure 1.4. Photomicrograph of a section of jejunum from a rotavirus-infected piglet in the milk-only group, 8 DPI. Notice the atrophic villi covered by squamous to cuboidal enterocytes (Hematoxylin and eosin, 220X).

Table 1.8. Immature enterocytes[1] in piglets infected with the SDSU strain of rotavirus

DPI[2]	Group[3]	Intestinal sites[4]					
		DU	UJ	LJ	MI	UI	LI
1	RP	-	0.75	-	0.25	-	-
	P	1.25	1.5	1	1.75	1	1
	RPP	-	2	1.5	0.5	0.75	-
	MO	0.5	-	-	-	-	-
2	RP	0.5	-	-	1	1	1.25
	P	2	2	2	0.75	0.75	0.25
	RPP	0.75	-	0.25	-	-	0.5
	MO	1	1	-	-	-	-
3	RP	2	1	0.5	-	-	-
	P	1.5	0.5	-	-	0.5	1
	RPP	1	1	1	1	1	-
	MO	-	-	-	-	-	-
8	RP	1	-	0.5	1	1	1
	P	-	-	-	-	-	-
	RPP	-	-	-	-	-	-
	MO	1	1	1	1	1	1

[1] Data are presented as average values (n=4). Values expressed as -: no immature enterocytes, 1: < 25% villi contain immature enterocytes, 2: between 26-50% villi contain immature enterocytes, 3: between 51-75% villi contain immature enterocytes, 4: > 75% villi contain immature enterocytes.

[2] DPI: days postinoculation.

[3] RP: 9,000 IU retinyl palmitate/day. P: 1,050 mg putrescine/day. RPP: 9,000 IU retinyl palmitate and 1,050 mg putrescine/day. MO: milk only.

[4] DU: duodenum, UJ: upper jejunum, LJ: lower jejunum, MI: mid-intestine, UI: upper ileum, LI: lower ileum.

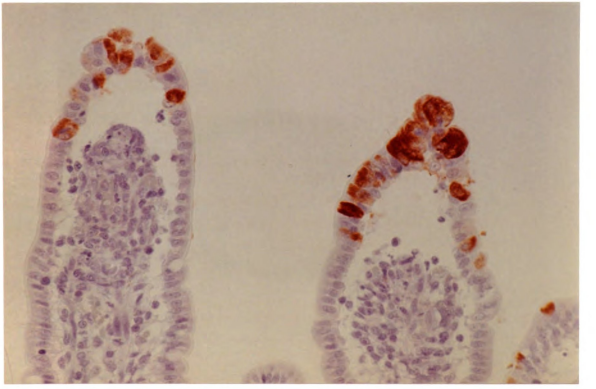

Figure 1.5. Photomicrograph of a section of duodenum showing the presence of rotaviral antigen. The section was taken from a rotavirus-infected piglet in the retinol palmitate group, 1 DPI. Notice the presence of rotaviral-antigen-positive enterocytes as indicated by the golden brown pigment. Immunohistochemical staining based on avidin-biotin binding with DAB as the chromogen, counterstained with Mayer's hematoxylin, 440X.

Table 1.9. Number of piglets with rotavirus-antigen-positive enterocytes, based on an immunohistochemistry

Treatment Groups[1]	Days Postinoculation			
	1	2	3	8
RP	2/4[2]	1/4	1/4	0/4
P	3/4	1/4	1/4	1/4
RPP	2/4	1/4	4/4	2/4
MO	1/4	0/4	3/4	2/4

[1] Group RP: 9,000 IU retinyl palmitate/day.
Group P: 1,050 mg putrescine/day.
Group RPP: 9,000 IU retinyl palmitate and 1,050 mg putrescine/day.
Group MO: milk only.

[2] Number of piglets with rotavirus-antigen-positive enterocytes/observed piglets.

Table 1.10. Expression of rotavirus antigen in enterocytes of piglets infected with the SDSU strain of porcine rotavirus[1]

DPI[2]	Group[3]	Intestinal sites						
		DU[4]	UJ	LJ	MI	UI	LI	Average
1	RP	2.00	0.25	1.00	1.00	1.00	0.25	0.92
	P	2.25	2.25	3.00	3.00	2.00	0.25	2.13
	RPP	-	1.25	0.25	0.25	0.25	-	0.33
	MO	-	-	0.25	0.25	0.25	-	0.13
2	RP	-	-	1.00	0.50	0.50	0.25	0.38
	P	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	RPP	-	0.25	-	-	-	-	0.04
	MO	-	-	-	-	-	-	-
3	RP	-	1.00	-	-	-	-	0.17
	P	1.00	-	-	1.00	1.00	-	0.50
	RPP	1.75	1.75	1.75	2.50	2.00	1.00	1.79
	MO	3.00	2.25	2.25	3.00	3.00	3.00	2.75
8	RP	-	-	-	-	-	-	-
	P	-	-	-	1.00	0.75	-	0.29
	RPP	-	1.00	1.00	2.00	1.00	0.25	0.88
	MO	-	-	-	-	1.00	2.00	0.50

[1] Values are presented as Mean + Standard Deviation. Values expressed as -: no rotavirus antigen was expressed in any enterocyte, 1: <5 percent villi contain positive enterocytes, 2: 5-25% villi contain positive enterocytes, 3: 25-50% villi contain positive enterocytes, 4: >50% villi contain positive enterocytes.

[2] DPI: day postinoculation.

[3] Each group consisted of 4 piglets. RP: 9,000 IU retinyl palmitate/day, P: 1,050 mg putrescine/day, RPP: 9,000 IU retinyl palmitate and 1,050 mg putrescine/day, MO: milk only.

[4] DU: duodenum, UJ: upper jejunum, LJ: lower jejunum, MI: mid intestine, UI: upper ileum, LI: lower ileum, Average: average from all intestinal sites.

DISCUSSION

Findings in this study indicated that supplementation with vitamin A and/or putrescine did not cause differences in clinical signs, histopathologic lesions and rotavirus antigenic expression in the small intestinal enterocytes of piglets infected with the SDSU strain of porcine rotavirus. Most inoculated piglets became infected, as indicated by clinical signs of diarrhea or the demonstration of rotaviral antigen in enterocytes with immunohistochemistry. Although most of the piglets in the study were euthanatized in the acute stage of disease (1, 2 and 3 DPI), those euthanatized 8 DPI were in good condition or had recovered, indicating that the rotavirus infection was self-limiting. Generally, clinical signs observed in this study agree with those previously reported (McNulty *et al*, 1976; Theil *et al*, 1978).

All piglets survived until the time of necropsy. The lack of mortality caused by the SDSU strain of porcine rotavirus infection agrees with the findings of other investigators (Theil *et al*, 1978; McAdaragh *et al*, 1980). Mortality associated with rotavirus infection has been reported in gnotobiotic piglets (Tzipori and Williams, 1978), in colostrum-deprived piglets (McNulty *et al*, 1976) and in conventional piglets (Bohl *et al*, 1978).

Supplementation with retinyl palmitate and/or putrescine did not cause any significant differences in the

body weights and body weight gains of both rotavirus-infected and control piglets. Body weights between piglets varied greatly, as can be seen from the high standard deviations. Piglet-to-piglet variation in body weight and body weight gain associated with rotavirus infection was also reported by Woode *et al* (1979). The reason for piglet-to-piglet variation is not known, but may be associated with host physiological factors. However, even though not significantly different, at 8 DPI, body weights and body weight gains of infected piglets given RP and/or P were higher than infected piglets given MO. Similarly, body weights and body weight gains of the control piglets given RP were higher than control piglets given MO. This result may indicate the potential of long term beneficial effects of vitamin A and/or putrescine supplementation. Enterocyte replacement time in young pigs is about 7-10 days (Moon, 1971), therefore, the effects of treatment might have been significant if the time for terminating the experiment was extended.

Clinical signs of diarrhea observed in this study corresponded with findings by other investigators (McNulty *et al*, 1976; Theil *et al*, 1978; McAdaragh *et al*, 1980). In the present study, diarrhea was based on clinical signs only and could not be judged based on the fecal output weight due to an inability to separate the fecal and urinary output. No differences were observed between treatment groups in the

severity of the diarrhea and the number of animals with diarrhea.

In the present study, vacuolated enterocytes were observed in both infected and control piglets. Vacuolation is normally present in the jejunal and ileal epithelial cells of young, especially gnotobiotic, piglets. Most vacuolated enterocytes contain a single large vacuole which distends the cytoplasm and displaces the nucleus towards the base or apex of the cell (Moon, 1972). Vacuolated ileal epithelium is present up to the third week of age and vacuolated jejunal epithelium is present up to eleven days of age (Moon, 1972; Moon *et al*, 1973). In the first few days of age, the vacuole does not contain lysosomal enzymes (Kraehenbuhl and Campiche, 1969) which enable the cells to actively transport intact antibody (Staley *et al*, 1968). No reports regarding the enzyme content in the ileal vacuoles after cessation of intact antibody transport were found in the literature. Moon *et al* (1973) speculated that ileal vacuoles in pigs after antibody transport cessation might be lysosomes which transport or process antigen for active immunization.

Infected piglets had higher numbers of vacuolated enterocytes than control piglets. This finding was in contrast to the findings of Theil *et al* (1978), McAdaragh *et al* (1980) and Collins *et al* (1989) who found that the vacuolation in the small intestine of rotavirus-infected piglets was less conspicuous than in control piglets.

However, some investigators reported the presence of numerous vacuoles (Pearson and McNulty, 1977) containing neutral fat (Hall *et al*, 1976) in rotavirus-infected enterocytes. The increased number of vacuolated enterocytes in the present study was probably a result of a mixture of both normal enterocyte vacuolation present in young pigs and pathologic vacuolation occurring in association with rotaviral infection.

Vacuolar degeneration of villous enterocytes was observed only in infected piglets. Increased numbers of degenerated enterocytes in infected piglets 1 to 3 DPI corresponded with the onset of diarrhea, indicating that degenerated enterocytes may have lost their absorptive function. No differences were observed between treatment groups based on the number of degenerated enterocytes in rotavirus-infected piglets.

The preference for rotavirus replication in the mature epithelial cells on the tips of the villi has been demonstrated using a variety of methods, including immunofluorescence and immunohistochemistry (McAdaragh *et al*, 1980; Gelberg, 1992). As a result of desquamation and lysis of infected epithelial cells, free or cell-associated rotaviral antigen can also be demonstrated in the intestinal lumen (McAdaragh *et al*, 1980; Theil *et al*, 1978). In the present study, rotaviral antigenic expression was only observed in enterocytes that were still attached to intestinal villi. No rotaviral antigen was detected in the

intestinal lumen. The inability to detect antigenic expression in the intestinal lumen was probably due to the flushing of fixative during tissue preparation.

Although not statistically significant, the number of piglets with rotaviral antigen positive enterocytes at 1 and 3 DPI tended to be higher in piglets fed diets containing putrescine than in piglets fed diets not containing putrescine. Polyamines, because of their multiple amino groups, are known to have a strong positive charge at cellular pH, which makes them highly reactive with a variety of cellular molecules, including DNA and RNA (Wolfe, 1993). Polymers having positive charges, such as protamine, protamine sulphate, DEAE-dextran, histone and poly-L-lysine hydrobromide, enhance rotaviral infectivity, whereas polymers having negative charges such as mucin, α-1-acid glycoprotein heparin, heparan sulphate and dextran sulphate inhibit rotaviral infectivity and replication (Superti *et al*, 1993). Since polyamines are known to have a strong positive charge, polyamines may also enhance rotaviral infectivity. In the present study, at 1 DPI, the P group had the highest number of piglets showing rotaviral antigenic expression in enterocytes and at 3 DPI, the RPP group had the highest number of positive animals.

Based on the results of this study the daily dietary administration of vitamin A and/or putrescine was ineffective in the prevention or alleviation of rotaviral infectivity, and the clinical signs and intestinal mucosal

damage caused by rotaviral infection in piglets. Putrescine appeared to increase rotaviral infectivity as indicated by the increased number of infected piglets and a greater distribution of enterocytes showing rotaviral antigenic expression.

CHAPTER 2

EFFECT OF DAILY DIETARY ADMINISTRATION OF RETINYL PALMITATE AND/OR PUTRESCINE ON THE SMALL INTESTINAL MUCOSAL MORPHOMETRY OF ROTAVIRUS-INFECTED PIGLETS

ABSTRACT

Experiments were conducted to determine the effects of the daily dietary administration of retinyl palmitate and/or putrescine on the small intestinal morphometry of rotavirus-infected piglets. One hundred and twenty-eight piglets were removed from sows at two days of age. On day 3, piglets were divided into four groups of 32 piglets. Each group received either milk only, or retinyl palmitate and/or putrescine. On day 5, half of each group was infected with porcine rotavirus. Piglets were euthanatized on days 6, 7, 8 and 13 (1, 2, 3 and 8 days postinoculation, DPI). Complexity indices did not differ between the treatment groups. Infected animals had greater complexity indices than control animals ($P<0.05$). Piglets 1, 2 and 3 DPI had greater complexity indices than piglets 8 DPI ($P<0.01$). Relative mucosal volume of the small intestine was less in infected than control piglets ($P<0.01$). Crypt depth of piglets 8 DPI was greater than the crypt depth of piglets 1, 2 and 3 DPI ($P<0.05$). Average mitotic indices of supplemented piglets were higher than piglets given milk

only. Supplementation significantly increased the average mitotic indices of the infected piglets 3 DPI and control piglets 1 DPI ($P<0.01$). Rotaviral infection in piglets reduced the relative small intestinal mucosal volume. Supplementation with retinyl palmitate and/or putrescine increased mitotic indices in the small intestinal crypts of both rotavirus-infected and control piglets.

INTRODUCTION

Small intestinal mucosal regeneration is essential for animals and human beings, especially for recovery from intestinal injury. Intestinal injury with mucosal damage can be caused by viral pathogens, including rotavirus. Rotavirus is one of the most important viral agents causing destruction of absorptive enterocytes in young animals (Holland, 1990) and children (Estes, 1983). The virus replicates within mature enterocytes located at the tips of villi, causing cell detachment and villous atrophy (McAdaragh *et al*, 1980).

Vitamin A and putrescine have received much attention because of their potential beneficial effects in mucosal cell regeneration. Vitamin A deficiency affects the growth and differentiation of various epithelial tissues, including small intestinal epithelial cells (Wolf, 1984). Also, vitamin A can protect rats from intestinal damage caused by

the antitumor drug, methotrexate (Tsurui *et al*, 1990). Methotrexate, as an antitumor drug, inhibits mitosis of small intestinal epithelial cells in the crypts, disrupts the steady state system of the epithelium and progressively reduces the size of crypts and villi (Jeynes and Altmann, 1978).

Putrescine affects small intestinal functions and morphology in calves fed soy protein. Villous atrophy may occur in calves (Kilshaw and Slade, 1982) and in early-weaned pigs (Dunsford *et al*, 1989) fed diets containing soy protein. Addition of putrescine to the soy protein containing diet of calves enhanced enterocyte proliferation (Grant *et al*, 1989).

The purpose of this study was to evaluate the usefulness of vitamin A and/or putrescine on intestinal mucosal regeneration in piglets during and after the onset of rotaviral infection. The objectives of this study were to determine the effect of daily dietary administration of vitamin A and/or putrescine to rotavirus-infected piglets on the following: 1) small intestinal mucosal villous height and crypt depth, 2) small intestinal mucosal complexity indices, and 3) small intestinal mitotic indices and goblet cell numbers.

MATERIALS AND METHODS

Experimental animals

One hundred and twenty eight, five-day-old piglets from 16 litters were obtained from the Michigan State University swine farm. All piglets received colostrum from the sows for two days. Experimental design is shown in Figure 1.1. Piglets within the same litter were allocated for a variety of treatments. Piglets were reared in individual cages in a temperature controlled room (30-32^{o}C). Each piglet was given a total of 300 ml evaporated milk[10] (14% dry matter) daily. The nutritional composition of the milk is shown in Table 1.1. Milk was offered three times a day at 8:00 a.m., 12:00 p.m. and 5:00 p.m. Starting from day 3, subgroup 1 (RP) was given milk containing vitamin A in the form of retinyl palmitate[11] (30,000 IU/kg of milk) daily. Subgroup 2 (P) received putrescine[12] (25 g/kg dry matter of milk) in the milk. Subgroup 3 (RPP) was fed milk containing retinyl palmitate (30,000 IU/kg of milk) and putrescine (25 g/kg dry matter of milk). Subgroup 4 (MO) received milk only. Addition of vitamin A and putrescine was done just prior to feeding by diluting each of the substances in approximately two ml of distilled water. On day 5, the rotavirus-infected piglet group was orally inoculated with 1 ml 10^{-10} dilution of the stock intestinal homogenate. Four piglets from each

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[10] Carnation Evaporated Milk, Nestle Food Co., Glendale, CA.
[11] Sigma Chem. Co., St. Louis, MO.
[12] Sigma Chem. Co., St. Louis, MO.

group were euthanatized on days 6, 7, 8 and 12. The time for necropsy was scheduled so that only five to six animals were euthanatized in a day.

Necropsy and specimen collection

Pigs were euthanatized with an overdose of intravenous injection of sodium pentobarbital[13]. Three-cm segments from six sites of the small intestine were removed immediately and placed in buffered neutral formalin for histopathologic and immunohistochemical examinations. The first segment was removed from the duodenum approximately 5-10 cm distal to the pyloric junction and the last segment was obtained from the lower part of the ileum, approximately 5 cm proximal to the ileocecal junction. The remaining segments were obtained at approximately a sixth, third, half and two-thirds of the distance from the pylorus to the ileocecal junction. These segments were designated as duodenum (DU), upper jejunum (UJ), lower jejunum (LJ), mid-intestine (MI), upper ileum (UI) and lower ileum (LI). The lumen of the segments were rinsed with 10% neutral buffered formalin and the segments were then immersed in the fixative.

Morphometric measurements

Formalin-fixed small intestines were routinely processed for histopathologic examination. The tissues were oriented in cross sections (ring shaped), embedded in

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[13] BUTLER®, The Butler Co., Columbus, OH.

paraffin and marked accordingly. Tissues were cut by a microtome into 6-μm thick sections. One slide contained DU, UJ, LJ segments and the second slide contained MI, UI and LI segments. Sections were stained with hematoxylin and eosin (H&E) and subsequently, morphometric assessments were made, including mucosal complexity index, villous height, crypt depth, mitotic index and goblet cell number.

Mucosal complexity index was determined using six segments of intestine. Complexity of the intestinal mucosal surface (intestinal mucosal surface to volume ratio) was measured using a Weibel graticule mounted in the microscope eyepiece (Weibel, 1963; Dunnill and Whitehead, 1972). A Weibel graticule, consisting of 15 equal length lines ('l'), was superimposed on the intestinal section so that the most lateral graticule line fell on the border of the lamina propria and the muscularis mucosa (Figure 2.1). The magnification (10 X objective and 10 X ocular lenses) was kept constant throughout the experimental studies. With this magnification, the length of 'l' was 1.8 X 10^{-2} cm. Lines cutting the mucosal surface were counted as 'cuts' ('c') and the end points of the lines falling in the tissue between the mucosal cells and the muscularis mucosae were counted as 'hits' ('h'). The complexity index of the villous pattern was obtained by using c:lh ratio. The number of 'h' per field also represented the relative mucosal volume.

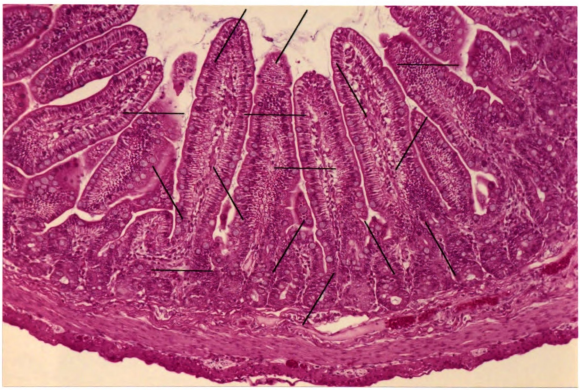

Figure 2.1. Photomicrograph of a section of ileum with a Weible graticule superimposed on the image to illustrate the use of a Weible graticule. The lines cutting the mucosal surface are counted as 'cuts' ('c') and the end points of the lines falling in the tissue between the mucosal cells and the muscularis mucosae are counted as 'hits' ('h'). The length of the line ('l') is measured with a micrometer. The complexity index is obtained by calculating the c:lh ratio. In this example, the number of cuts is 23 and the number of hits is 26.

The villous height and crypt depth of 5 well-oriented villi and crypts were measured using a computer-assisted video image analyzer. All segments of intestinal sections were viewed with an MTI series 68 video camera mounted on a Nikon Microphot-FX microscope[14]. Villous length and crypt depth were measured with the aid of JAVA Image Analysis Software[15].

Counting epithelial cells from approximately 30 crypts, the mitotic index and goblet cell number was determined using a light microscope. The mitotic index was calculated as the total number of mitotic figures in any stage of mitosis divided by the number of crypts.

Statistical analysis

Morphological measurement data were grouped based on the infection status, treatment and time. Each group consisted of 4 animals, as in the experimental design. Statistical analyses were performed using three-factor analysis of variance (three-way ANOVA) with main effects of treatment, infection and time (Devore, 1991). The numbers of levels of the three factors were denoted as 2, 4 and 4 for the infection status, treatment and time factors, respectively. Differences between group means within each factor were analyzed by one-way ANOVA followed by Tukey's procedure (Fowler and Cohen, 1990). Differences between groups were considered significant at the level of $P \leq 0.05$.

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[14] Nikon Inc., Instrument Group, Garden City, NY.
[15] Jandel Scientific, Corte Madera, CA.

Statistical analyses were done using SPSS Computer Software[16].

RESULTS

Mucosal complexity

The average values of mucosal complexity indices as measured by using a Weibel graticule (Table 2.1 and Figure 2.2), ranged from 45.48 (± 4.66) to 68.58 (± 8.82). Three-way ANOVA results indicated that the complexity indices were influenced by infection status and time after infection, but not by treatment. There were no interaction existed between the main effects. The infected group had higher average complexity indices than the control group ($P<0.05$). When each intestinal segment was examined, the DU, UI and LI of the infected group had higher complexity indices than the control group. Regardless of the infection status, piglets 1, 2 and 3 DPI had higher complexity indices than piglets 8 DPI.

Three-way ANOVA results indicated that the relative volume of the small intestinal mucosa (average number of `hits' per field) of the control group was higher than the infected group. Piglets 8 DPI had higher average relative mucosal volumes than piglets 1, 2 and 3 DPI, but the average relative volumes were not significantly different. However, the relative volumes of the duodenum and upper jejunum of

[16] SPSS Inc., Chicago, IL.

Table 2.1. Complexity indices in the small intestines of control piglets *vs.* piglets infected with the SDSU strain of porcine rotavirus

	Infected				Infected DPI mean	Control				Control DPI mean	All DPI mean ± SE[d]
	RP [a]	P	RPP	MO		RP	P	RPP	MO		
1 DPI [b]	69 [c]	63	65	61	64	65	59	59	63	62	63 ±1
2 DPI	67	62	62	60	63	57	53	61	64	59	61 ±1
3 DPI	61	62	59	60	60	56	57	59	61	58	59 ±2
8 DPI	54	55	53	45	52	54	50	49	45	50	51 ±1
Treatment mean ± SE[e]	63 ±2	60 ±2	60 ±2	57 ±2	60 ±1	58 ±2	55 ±2	57 ±2	58 ±2	57 ±1	58 ± 1

[a] RP: 9,000 IU retinyl palmitate/day, P: 1,050 mg putrescine/day, RPP: 9,000 IU retinyl palmitate and 1,050 mg putrescine/day, MO: milk only.

[b] DPI: day postinoculation.

[c] Average from 4 piglets. Six segments of small intestinal section per piglet.

[d] Complexity indices (CI) of 1, 2 and 3 DPI piglets > CI of 8 DPI

[e] CI of infected piglets > CI of control piglets ($P<0.05$).

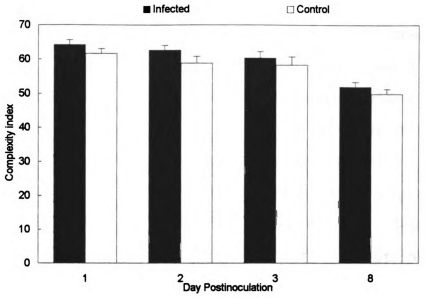

Figure 2.2. Pooled small intestinal mucosal complexity indices (SIMCI) for control piglets *vs.* piglets infected with the SDSU strain of porcine rotavirus (mean ± standard error). SIMCI of infected piglets > SIMCI control piglets ($P<0.05$). SIMCI of 1, 2 and 3 DPI piglets > SIMCI of 8 DPI piglets ($P<0.01$).

piglets 8 DPI were significantly higher than those of piglets 1, 2 and 3 DPI ($P<0.05$).

Villous height and crypt depth

Results from a three-way ANOVA indicated that villous height (Table 2.2 and Figure 2.3) was not influenced by time, treatment nor infection. Also, crypt depth (Table 2.3 and Figure 2.4) was not influenced by treatment nor infection. However, crypt depth was influenced by time. No interaction was found between the main effects on both villous height and crypt depth. The average crypt depth 8 DPI, was significantly higher than the average crypt depth 1, 2 and 3 DPI ($P<0.05$). The average crypt depth 3 DPI was significantly higher than the average crypt depth 1 and 2 DPI, but significantly lower than the crypt depth 8 DPI ($P<0.05$). When each intestinal segment was examined, the crypt depth values of the DU on 3 and 8 DPI were significantly higher than the values on 1 and 2 DPI ($P<0.05$). The crypt depth values of the UJ, LJ, MI, UI and LI 8 DPI were significantly higher than the values 1, 2 and 3 DPI ($P<0.05$). In addition, the crypt depth value of the LJ 3 DPI was higher than the value 1 DPI.

Ratios of villous height to crypt depth were influenced by time, but were not influenced by treatment nor infection status. The average villous height to crypt depth ratio from all parts of the small intestine was higher on 1 DPI than on 8 DPI ($P<0.05$). When each individual segment

Table 2.2. Villous lengths (μm) in the small intestines of control piglets *vs.* piglets infected with the SDSU strain of porcine rotavirus

	Infected				Infected DPI mean	Control				Control DPI mean	All DPI mean ± SE
	RP [a]	P	RPP	MO		RP	P	RPP	MO		
1 Dpi [b]	682 [c]	633	602	615	633	666	645	598	972	720	677 ±47
2 Dpi	736	568	691	644	660	686	726	560	582	638	649 ±39
3 Dpi	722	623	536	597	619	603	618	584	645	612	616 ±25
8 Dpi	631	661	644	526	615	625	662	685	530	625	620 ±20
Treatment mean ± SE	693 ±47	621 ±50	618 ±58	595 ±51	632 ±26	645 ±46	663 ±36	607 ±38	682 ±56	649 ±22	640 ± 17 [d]

[a] RP: 9,000 IU retinyl palmitate/day, P: 1,050 mg putrescine/day, RPP: 9,000 IU retinyl palmitate and 1,050 mg putrescine/day, MO: milk only.

[b] DPI: day postinoculation.

[c] Average from 4 piglets. Six segments of small intestinal section per piglet.

[d] No effect of DPI, treatment nor infection.

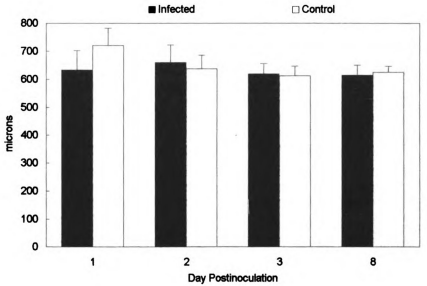

Figure 2.3. Pooled small intestinal mucosal villous length (SIMVL) for control piglets *vs.* piglets infected with the SDSU strain of porcine rotavirus (mean $\pm$ standard error). No significant differences were found between groups.

Table 2.3. Crypt depths (μm) in the small intestines of control piglets *vs.* piglets infected with the SDSU strain of porcine rotavirus

	Infected				Infected DPI mean	Control				Control DPI mean	All DPI mean ± SE[d]
	RP [a]	P	RPP	MO		RP	P	RPP	MO		
1 DPI [b]	161 [c]	161	148	144	153	172	176	191	155	173	163 ±5
2 DPI	168	159	193	168	172	183	202	195	158	184	178 ±6
3 DPI	197	185	184	144	178	211	213	214	182	205	191 ±7
8 DPI	240	194	237	243	229	193	232	233	242	225	227 ±7
Treatment mean ± SE	192 ±11	175 ±7	190 ±11	175 ±12	183 ±5	190 ±9	206 ±11	208 ±11	184 ±12	197 ±5	190 ± 4

[a] RP: 9,000 IU retinyl palmitate/day, P: 1,050 mg putrescine/day, RPP: 9,000 IU retinyl palmitate and 1,050 mg putrescine/day, MO: milk only.

[b] DPI: day postinoculation.

[c] Average from 4 piglets. Six segments of small intestinal section per piglet.

[d] Crypt depth (CD) of 8 DPI piglets > CD of 1, 2 and 3 DPI piglets ($P<0.05$).

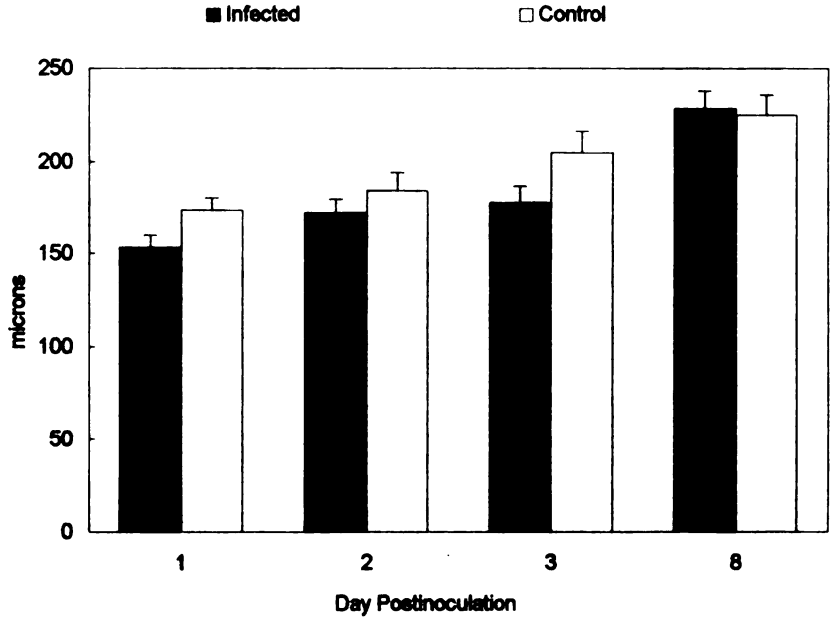

Figure 2.4. Pooled crypt depths for control piglets *vs.* piglets infected with the SDSU strain of porcine rotavirus (mean ± standard error). Crypt depth of 8 DPI piglets > crypt depth of 3 DPI piglets > crypt depth of 1 and 2 DPI piglets ($P<0.05$).

was examined, the villous height to crypt depth ratios of DU, LJ, MI and UI were higher on 1 DPI than on 8 DPI ($P<.05$). The villous height to crypt depth ratio of the LJ 1 DPI was also higher than the villous height to crypt depth ratio 3 DPI.

Mitotic index and goblet cell number

Results from a three-way ANOVA indicated that the mitotic indices in the small intestinal mucosa (Figures 2.5, 2.6, Table 2.4 and Figure 2.7) were significantly influenced by treatment and in some portions were also significantly influenced by time. However, mitotic indices were not influenced by infection.

At 2, 3 and 8 DPI, the average value of mitotic indices of the small intestine of infected piglets given RP and/or P were higher than the mitotic indices of the infected piglets given MO. However, results from a one-way ANOVA showed that only those in the 3 DPI groups were significantly different ($P<0.05$). The average value of mitotic indices of the control piglets given RP and/or P were also higher than the mitotic indices of the control piglets given MO. Statistical differences were noted in piglets 1 and 3 DPI. Results from a one-way ANOVA showed that at 1 DPI, all the control piglets given RP and/or P had significantly higher mitotic indices than the control piglets given MO ($P<0.05$). However, at 3 DPI, only the control piglets given P had a

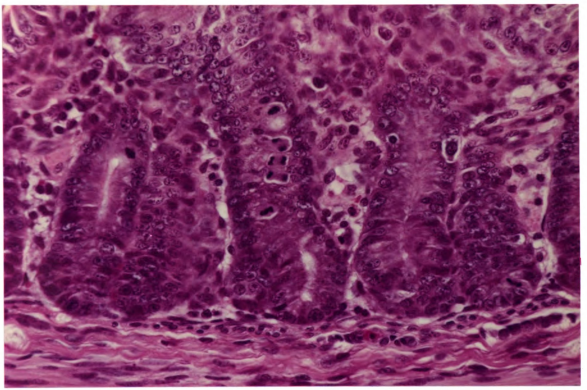

Figure 2.5. Photomicrograph of a section of jejunum with a high number of mitotic figures. The section was taken from a rotavirus-infected piglet, 3 days postinoculation in the putrescine group (Hematoxylin and eosin, 440X).

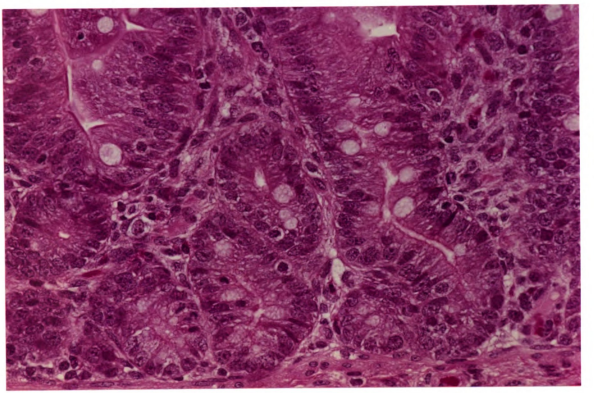

Figure 2.6. Photomicrograph of a section of jejunum with a low number of mitotic figures. The section was taken from a rotavirus-infected piglet, 3 days postinoculation in the milk only group (Hematoxylin and eosin, 440X).

Table 2.4. Mitotic indices in the small intestines of control piglets *vs.* piglets infected with the SDSU strain of porcine rotavirus

	Infected				Infected DPI mean	Control				Control DPI mean	All DPI mean ± SE
	RP [a]	P	RPP	MO		RP	P	RPP	MO		
1 DPI [b]	0.72[c]	0.69	0.77	0.78	0.74	0.75	0.96	0.86	0.32	0.72	0.73 ±0.05
2 DPI	1.01	0.99	0.89	0.56	0.86	0.81	0.94	0.94	0.68	0.84	0.85 ±0.05
3 DPI [d]	0.98	1.10	1.05	0.49	0.91	0.82	1.22	0.86	0.55	0.86	0.88 ±0.05
8 DPI	1.16	0.85	1.12	0.62	0.93	0.75	0.88	0.79	0.61	0.76	0.85 ±0.06
Treatment mean ± SE	0.97 ±0.06	0.91 ±0.08	0.95 ±0.09	0.61 ±0.04	0.86 ±0.04	0.78 ±0.04	1.00 ±0.07	0.86 ±0.05	0.54 ±0.05	0.80 ±0.03	0.83 ± 0.03

[a] RP: 9,000 IU retinyl palmitate/day, P: 1,050 mg putrescine/day, RPP: 9,000 IU retinyl palmitate and 1,050 mg putrescine/day, MO: milk only.

[b] DPI: day postinoculation.

[c] Average from 4 piglets. Six segments of small intestinal section per piglet.

[d] Mitotic indices (MI) of RP,P and RPP 3 DPI piglets > MI of MO 3 DPI piglets ($P<0.05$).

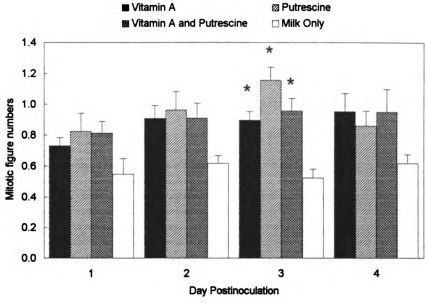

Figure 2.7. Pooled mitotic indices of control and infected piglets (mean ± standard error).

* Significantly different from milk only groups ($P<0.01$).

significantly higher mitotic indices than the control piglets given MO ($P<0.05$).

When the data were analyzed from individual segments of the small intestine, results of the effect of treatment were similar to the results from the average values. However, results on the effect of time after infection were not consistent. Time had no effect on mitotic indices in the upper part of small intestine, but it had a significant effect in the lower portions of the intestine. Mitotic indices in the UI and LI of piglets 3 and 8 DPI were higher than those of piglets 1 DPI ($P<0.05$).

No statistical differences existed in average goblet cell numbers among the RP, P, RPP and MO groups and also, no differences existed between the infected and control groups (Table 2.5). However, the goblet cell number at 8 DPI was higher than the number of goblet cells at 1,2 and 3 DPI ($P<0.05$).

DISCUSSION

Results of morphometric studies using a Weibel graticule indicated that control piglets had lower complexity indices than the rotavirus-infected piglets (57.19 vs. 59.78), but no differences existed between the treatment groups. However, linear measurement results indicated that treatment, infection status and time postinoculation did not have an effect on villous height.

Table 2.5. Goblet cell numbers per crypt in the small intestines of control piglets *vs.* piglets infected with the SDSU strain of porcine rotavirus

	Infected				Infected DPI mean	Control				Control DPI mean	All DPI mean ± SE[d]
	RP [a]	P	RPP	MO		RP	P	RPP	MO		
1 DPI [b]	2.66[c]	2.49	3.38	2.18	2.68	2.07	3.07	2.57	2.09	2.45	2.56 ±0.13
2 DPI	2.58	2.80	2.59	2.50	2.61	2.53	2.14	2.98	2.90	2.64	2.62 ±0.11
3 DPI	2.97	3.07	2.52	2.32	2.72	2.45	2.55	2.62	2.24	2.46	2.59 ±0.13
8 DPI	3.66	3.44	3.92	3.02	3.51	3.04	3.21	3.16	3.09	3.12	3.32 ±0.13
Treatment mean ± SE	2.97 ±0.20	2.95 ±0.18	3.10 ±0.25	2.50 ±0.17	2.88 ±0.10	2.52 ±0.18	2.74 ±0.18	2.83 ±0.20	2.58 ±0.15	2.67 ±0.09	2.77 ± 0.07

[a] RP: 9,000 IU retinyl palmitate/day, P: 1,050 mg putrescine/day, RPP: 9,000 IU retinyl palmitate and 1,050 mg putrescine/day, MO: milk only.

[b] DPI: day postinoculation.

[c] Average from 4 piglets. Six segments of small intestinal section per piglet.

[d] Goblet cell number (GCN) of 8 DPI piglets > GCN of 1, 2 and 3 DPI piglets ($P<0.01$).

In the following discussion, unless specifically mentioned, there were no interaction existed between the main effects. Normal porcine complexity index values for comparison were not available. However, reduced complexity index values indicate the presence of villous atrophy (Dunnill and Whitehead, 1972; Wright and Tomkins, 1978). Thus, results from the present experiment indicate that the control group had more mucosal atrophy than the infected group. However, this is probably not the case, since histopathologic results indicated that the infected animals had more villous atrophy than the control animals. Since the complexity indices were calculated from the formula 'c:lh', any increase of 'c' and/or decrease of 'h' would result in an increased complexity indices. The average value of 'c' per field reflects the surface area and the value of 'h' per field reflects the relative volume of the small intestinal mucosa (Dunnill and Whitehead, 1972; Wright and Tomkins, 1978; Hart and Kidder, 1978). For example, the complexity index value in normal, human jejunum is 46.0 $\pm$ 12.0 (Dunnill and Whitehead, 1972) and ranged between 40 to 60 (Wright and Tomkins, 1978), whereas in healthy dogs, which have a thicker mucosa than human beings, complexity indices ranged between 22.1 to 31.3 for young dogs and 22.8 to 28.1 for adult dogs (Hart and Kidder, 1978). In the present experiment, no differences were found in the 'c' values. When the value of 'h' in the present experiment was calculated, the infected group had a smaller 'h' value than

the control group, indicating that the infected group had thinner intestinal walls than the control group. The thinning of the intestinal wall in the rotavirus-infected group was consistent with the thin-walled gross appearance of the intestine.

The thinning of the intestinal wall may have been caused by reduced crypt depth or villous atrophy. In this experiment, the histological appearance of the crypts was uniform and no differences were noted based on the linear measurement. Thus, the volume or thickness of the crypts was not affected by the rotaviral infection. However, the histological appearance of the villi was not uniform. This agrees with a previous investigator who observed the presence of atrophic and normal villi within the same intestinal section (McAdaragh, 1980). Linear measurements of the villous lengths in the present study were obtained from the longest remaining villi, resulting in an inability to demonstrate villous atrophy. Although not statistically significant, the average villous length of the infected piglets was lower than the control piglets (632 $\pm$ 208 μm for infected piglets versus 649 $\pm$ 178 μm for control piglets). By evaluating both the results from the Weible graticule and the linear measurement of the crypt depth, it was concluded that the reduction in mucosal volume was caused by a reduction of villous volume, which was to be expected in a rotaviral infection.

Complexity indices of piglets 8 DPI were less than in piglets 1, 2 and 3 DPI. The 'h' value and the linear measurement of the crypt depth from piglets 8 DPI were greater than piglets 1, 2 and 3 DPI ($P<0.05$). This may be associated with the increased thickness of the mucosa as the piglets grew (Moon, 1971).

The number of mitotic figures in the crypts appeared to be influenced by vitamin A and/or putrescine supplementation. In response to villous atrophy caused by a rotaviral infection, crypt hyperplasia is usually present (McAdaragh *et al*, 1980; Theil *et al*, 1978; Woode and Crouch, 1978). However, results of the present experiment indicate that the number of mitotic figures per crypt in infected and control groups was not significantly different. Treatment with either vitamin A and/or putrescine significantly increased the number of mitotic figures both in the infected and control piglets. Piglets fed milk supplemented with either RP, P or RPP had greater mitotic indices than those fed with milk only. Retinyl palmitate may exert its effect in increasing mitotic figures by its association with TGF β. Transforming growth factor β stimulates proliferation of mesenchymal cells (Lamprecht *et al*, 1989; Blachowski *et al*, 1994), but in contrast, inhibits the growth of a large variety of epithelial cells, including actively dividing enterocytes (Lampert *et al*, 1989; Potten *et al*, 1995; Booth *et al*, 1995). Expression of TGF β-1 is largely governed by

AP-1 promoter (Salbert *et al*, 1993). The activity of AP-1 is inhibited by RAR and RXR (Salbert *et al*, 1993; Chen *et al*, 1995; Desbois, 1993; Fanjul *et al*, 1994). Thus, inhibition of AP-1 activity by administration of vitamin A would result in the suppression of TGF β, which would consequently increase cell proliferation. In the present experiment, the increase in cell proliferation may have been reflected by an increased number of mitotic figures.

An increase in mitotic figures was also observed in piglets treated with putrescine. Putrescine, as a polyamine, is required for cell growth and differentiation (Heby, 1981). The finding in the present study supports the suggestion that exogenous polyamines are needed for maximal cell growth (Pegg and Cann, 1982).

Data from the present study indicate that the number of goblet cells per crypt was not influenced by infection and treatment. A reduced number of goblet cells in the small intestine of rats has been associated with a vitamin A deficiency (De Luca *et al*, 1969; Rojanapo *et al*, 1980). Thus the result of the present study may indicate that all piglets, regardless of the infection status and treatment, still had adequate concentrations of vitamin A. However, goblet cell numbers were influenced by intestinal site and time. Goblet cell numbers in the LI of piglets 1, 2, 3 and 8 DPI were significantly greater than those of other segments of intestine ($P<0.01$). Also, the average goblet

cell numbers in piglets 8 DPI was higher than in piglets 1, 2 and 3 DPI. The finding of a greater number goblet cells in the LI was consistent with the normally high population of goblet cells in the posterior part of the small intestine (van-Kruinigen, 1988). The greater numbers of goblet cells in piglets 8 DPI were probably related to the increased size of the crypts.

In summary, this study indicated that rotaviral infection in piglets reduced small intestinal mucosal volume. The volume reduction occurred in the villous portion of the intestine which is consistent with the pathogenesis of rotaviral infection. Daily dietary administration of retinyl palmitate or putrescine alone or in combination increased the mitotic index in small intestinal crypts, suggesting a possible beneficial effect of retinyl palmitate or putrescine on the regeneration of the intestinal mucosa.

CHAPTER 3

EFFECT OF DAILY DIETARY ADMINISTRATION OF RETINYL PALMITATE AND/OR PUTRESCINE ON INTESTINAL MUCOSAL NUCLEIC ACID AND PROTEIN, AND ON HEPATIC VITAMIN A CONCENTRATIONS OF ROTAVIRUS-INFECTED PIGLETS

ABSTRACT

Experiments were conducted to determine the effects of the daily dietary administration of retinyl palmitate and/or putrescine on the concentrations of small intestinal nucleic acids and protein, and the concentration of hepatic vitamin A in rotavirus-infected piglets. One hundred and twenty-eight piglets were removed from the sows at two days of age. On day 3, piglets were divided into four groups of 32 piglets. Three of the groups received milk containing either retinyl palmitate, putrescine, or retinyl palmitate and putrescine combined. One group received milk only. On day 5, half of each group was infected with porcine rotavirus. Piglets were euthanatized on days 6, 7, 8 and 13 (1, 2, 3 and 8 days postinoculation, DPI). Results of this experiment indicated that mucosal protein and RNA concentrations were higher in piglets 8 DPI than in piglets 1 and 2 DPI. Mucosal protein and RNA concentrations were not affected by infection status and treatments. Mucosal DNA concentrations were higher in piglets 8 DPI than in piglets 1 DPI. Mucosal DNA concentrations were decreased in

not affected by treatment. The hepatic vitamin A concentration was influenced by treatment and time, but not by infection status. Daily dietary vitamin A administration increased hepatic concentrations of vitamin A. Putrescine tended to decrease the concentration of hepatic vitamin A in the infected animals.

INTRODUCTION

Vitamin A is necessary for the maintenance, growth and differentiation of various epithelial cells (Wolf, 1984; Zile and Cullum, 1983). Many factors, such as dietary intake, absorption and tissue utilization of vitamin A, affect the availability of this vitamin in the liver (Herdt and Stowe, 1991). Malabsorption of vitamin A may occur in association with diarrhea, intestinal infections and intestinal parasitic infestations (Holland, 1992; Rosenberg *et al*, 1977; Mansour *et al*, 1979). Increased utilization of vitamin A may occur in animals with increased demand for cell renewal (Herdt and Stowe, 1991).

Vitamin A and putrescine have received much attention because of their potential beneficial effects in mucosal cell regeneration. Intestinal mucosal growth is associated with increased intestinal mucosal synthesis of DNA, RNA and protein (Dembinski *et al*, 1984). Several reports indicate that in the small intestinal mucosa of rats, vitamin A deficiency reduces protein synthesis (Rojanapo *et al*, 1980; De Luca *et al* 1969; Olson, *et al*, 1981)), lengthens the DNA

synthesis phase (Zile *et al*, 1977) and reduces RNA synthesis (Johnson *et al*, 1969). Administration of vitamin A to vitamin A deficient animals reverses the effects.

Several reports also indicate that putrescine affects the growth of intestinal mucosal cells. When putrescine was infused into the rat small intestine, small intestinal mucosal growth was stimulated (Seidel *et al*, 1985). Also, 30 hours after the oral administration of spermine in rats, increased concentrations of DNA in the small intestinal mucosa were noted (Wery and Dandrifosse, 1993).

The objectives of this study were the following: 1) to determine the effect of the daily dietary administration of vitamin A and/or putrescine on small intestinal nucleic acid and protein concentrations of rotavirus-infected piglets and 2) to determine the effect of the daily dietary administration of vitamin A and/or putrescine on the hepatic vitamin A concentration of rotavirus-infected piglets.

MATERIALS AND METHODS

Experimental animals

One hundred and twenty eight, 5-day-old piglets which had received colostrum from the sow for two days were used for the study. The experimental design is illustrated in Figure 1.1. Piglets were reared in individual cages in a temperature controlled room (30-32^{o}C). Each piglet was

given a total of 300 ml evaporated milk[17] (14% dry matter) daily. The nutritional composition of the milk is shown in Table 1.1. Milk was served three times a day at 8:00 a.m., 12:00 p.m. and 5:00 p.m. Starting from day 3, subgroup 1 (RP) was given milk containing vitamin A in the form of retinyl palmitate[18] (30,000 IU/kg). Subgroup 2 (P) received putrescine[19] (25 g/kg dry matter) in the milk. Subgroup 3 (RPP) was fed milk containing retinyl palmitate (30,000 IU/kg) and putrescine (25 g/kg dry matter). Subgroup 4 (MO) received milk-only. Addition of vitamin A and putrescine was done just prior to feeding by diluting each of the substances in approximately two ml of distilled water. On day 5, the rotavirus-infected group was orally inoculated with 1 ml 10^{10} dilution of the stock intestinal homogenate. Four piglets from each group were euthanatized on days 6, 7, 8 and 12. Necropsies were scheduled so that only five to six animals were euthanatized in a day.

Necropsy and specimen collection

Pigs were euthanatized with an overdose of intravenous injection of sodium pentobarbital[20]. Three-cm segments from six small intestinal sites were removed immediately and placed in buffered neutral formalin for histopathologic and immunohistochemical examinations. Intestinal mucosal

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[17] Carnation Evaporated Milk, Nestle Food Co., Glendale, CA.
[18] Sigma Chem. Co., St. Louis, MO.
[19] Sigma Chem. Co., St. Louis, MO.
[20] BUTLER®, The Butler Co., Columbus, OH.

scrapings were obtained from the remaining small intestine. The intestinal lumen was rinsed with cold saline and then the intestine was incised longitudinally along the mesenteric attachment. The intestine was opened, and placed on a cold glass surface, blotted with a paper towel and then scraped with a blunt glass slide. Mucosal scrapings were stored at -20°C in plastic microtubes prior to further testing to determine mucosal nucleic acid and protein concentrations. Approximately five grams of liver were collected from each pig, placed in a plastic container, and stored at -80°C for vitamin A analysis.

Nucleic acid analyses

A modified Fleck and Munro procedure for nucleic acid estimation (Munro and Fleck, 1966) was performed to determine the amount of DNA and RNA present in the intestinal mucosal preparations (Figure 3.1). Approximately 200 mg (± 20 mg) of thawed mucosa was homogenized in 5 ml distilled water with a motor-driven Teflon homogenizer[21] at 4°C. One ml of the homogenate was used for protein analysis and 4 ml was used for RNA and DNA analyses. The homogenate for protein analysis was then diluted with 4 ml of distilled water. One-hundred μl of the solution (in duplicate) were transferred to test tubes and stored at -20°C for subsequent protein analysis.

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[21] THOMAS® Teflon pestle Tissue Homogenizer, Thomas Scientific™ USA, Philadelphia, PA.

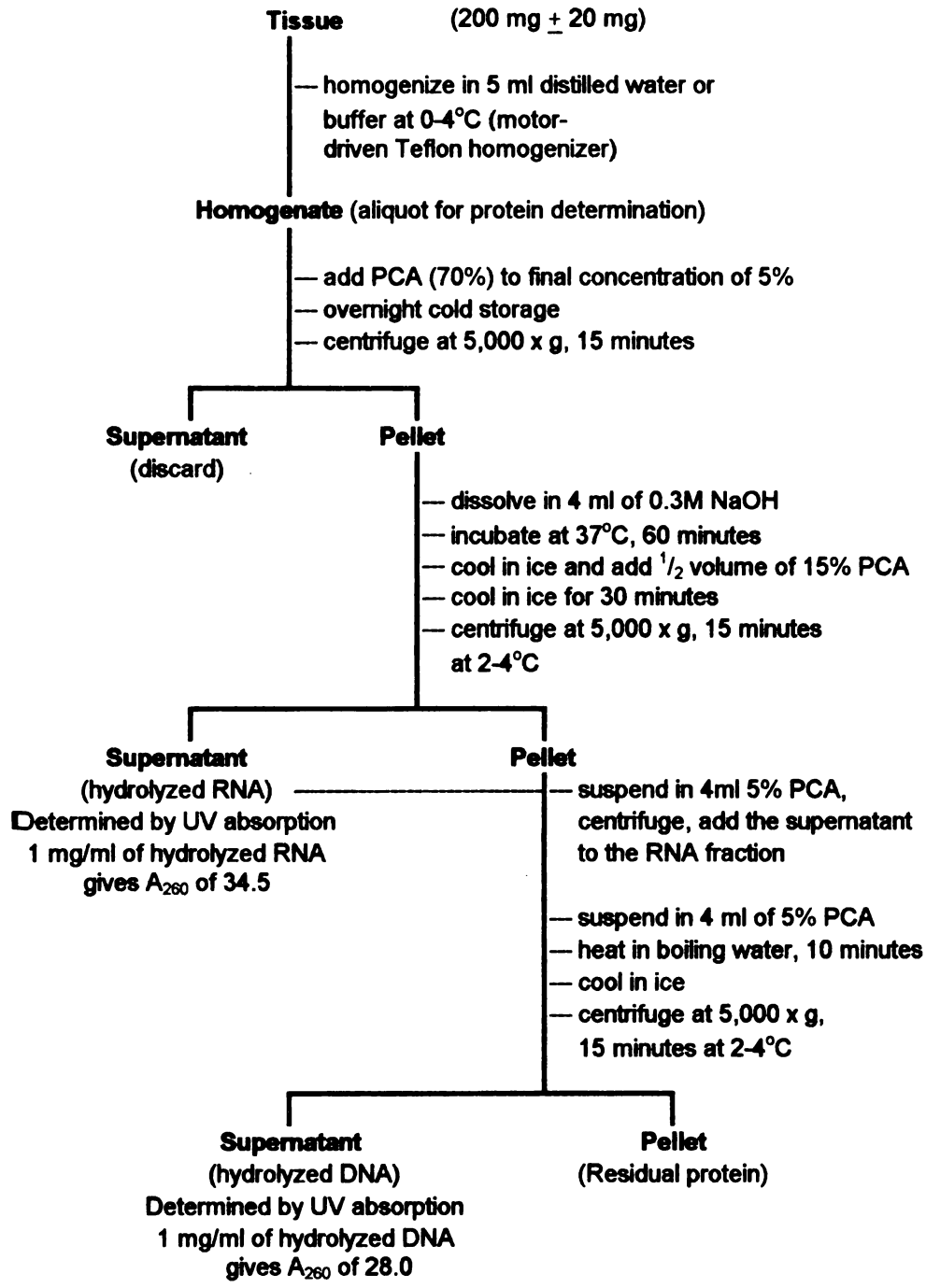

Figure 3.1. Outline of nucleic acid and protein analyses (Munro and Fleck, 1962, with some modification).

A 70% solution of perchloric acid (PCA)[22] was added to 4 ml of homogenate for RNA and DNA analyses to make a final 5% PCA concentration. The solution was vortexed for 5 seconds. After keeping the solution overnight at 4°C, the solution was centrifuged[23] at 5,000 g for 15 minutes at 4°C and the supernatant was discarded. The pellet was dissolved in 4 ml 0.3M NaOH and incubated at 37°C for 60 minutes with multiple agitations during the incubation. Two ml of 15% PCA were added to the sample which was then cooled on ice for 30 minutes. Next, the sample was centrifuged at 5,000 g for 15 minutes at 4°C and the supernatant was saved for RNA analysis. The pellet was washed with 4 ml of 5% cold PCA, centrifuged at 5,000 g for 15 minutes at 4°C, and the supernatant added to the initial RNA fraction. The remaining pellet was used for DNA analysis. One ml of the collected supernatant was diluted with 5% PCA to a final volume of 5 ml and the optical density was measured in a spectrophotometer[24] set at 260 nm. One mg/ml of hydrolyzed RNA gives an optical density of 34.5.

DNA was analyzed by resuspending the pellet in 4 ml of 5% PCA. The suspension was heated in boiling water for 10 minutes. After cooling on ice for 10 minutes, the sample was centrifuged at 5,000 g for 15 minutes at 4°C and the

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[22] Baker Analyzed®, Phillipsburg, NJ.
[23] Refrigerated Centrifuge, Model PR-7000, International Equipment Co., Needham, MA.
[24] Shimadzu. UV-Visible Recording Spectrophotometer, Model UV-260, Shimadzu Co., Kyoto, Japan.

optical density of the supernatant was measured in a spectrophotometer set at 260 nm. One mg/ml of hydrolyzed DNA gives an optical density of 28.0.

Protein analysis

Protein concentrations were determined by using the Bradford method (Bradford, 1976). The dye reagent was prepared by diluting 1 part dye reagent concentrate[25] with 4 parts distilled water. The solution was then filtered using a Whatman #1 filter[26]. Protein standards were prepared by diluting bovine serum albumin (BSA)[27] to the following concentrations: 0.194, 0.388, 0.583, 0.777 and 0.971 mg/ml. Standards and samples, 100 μl of each in duplicate, were pipetted into test tubes, and 5 ml of diluted dye reagent was added to each tube. The mixtures were vortexed for 5 seconds. After a 5 minute incubation at room temperature, the absorbance was read in a spectrophotometer at 595 nm.

Vitamin A analysis

The determination of vitamin A in the liver was performed in the Nutrition Laboratory of the Animal Health Diagnostic Laboratory, Michigan State University. Vitamin A was quantitated by the method established by Stowe (1982) using a modification of the high performance liquid chromatography procedure described by Dennison and Kirk

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[25] Bio-Rad Protein Assay, BIO-RAD Lab., Inc., Richmond, CA.
[26] Whatman® Int., Ltd., Maidstone, England.
[27] Bio-Rad Protein Assay, BIO-RAD Lab., Inc., Richmond, CA.

(1977). One gram of liver tissue was placed in a tube and up to 5 ml of distilled water was added. The mixture was then homogenized and from this homogenate, 0.5 ml was pipetted into a disposable test tube. An equal amount of absolute ethanol was added to the homogenate (0.5 ml) and the mixture was vortexed for 5 seconds. After the addition of 5 ml hexane (68^{o}-69^{o}C b.p.), the mixture was vortexed for 1 minute and centrifuged at 3000 rpm for 10 minutes. The hexane supernatant was removed and passed through a 0.45 Millipore filter in a Swinny-type filter holder. The aliquot (100μl) was then injected into a chromatograph. Separation was isocratic in a Microporasil column (3.9 mm ID x 30 cm long) with a 60:40 mixture of degassed and filtered hexane. The mixture was pumped through the HPLC unit at 2.5 ml/minute at 63.4 kg/cm^2. Forms of vitamin A were detected with a 35 μl flow cell in a spectrofluorometer[28] set at 330 and 470 nm for the excitation and emission wave lengths, respectively. Liver vitamin A content was calculated from the value derived from the chromatogram, the wet weight and the dry weight of the tissue prepared for the assay. The dry weight of the liver was determined by placing 2 grams of the tissue in an aluminum pan with subsequent drying in an oven at 56^{o}C for 24 hours.

[28] Waters Assoc., Inc., Milford, MA.

Statistical analysis

Analytical data were grouped based on the infection status, treatment and time. Each group consisted of 4 animals as in the experimental design. Statistical analyses were performed using three-factor analysis of variance (three-way ANOVA) with main effects of treatment, infection and time (Devore, 1991). For intestinal mucosal protein and nucleic acid concentrations, the numbers of levels of the three factors were denoted as 2, 4 and 4 for the infection status, treatment and time factors, respectively. For hepatic vitamin A concentration, the numbers of levels of the three factors were denoted as 2, 4 and 3 for the infection status, treatment and time factors, respectively. Differences between group means within each factor were analyzed by one-way ANOVA followed by Tukey's procedure (Fowler and Cohen, 1990). Differences between groups were considered significant at the level of $P \leq 0.05$. Statistical analyses were done using SPSS Computer Software[29].

RESULTS

Nucleic acid and protein concentrations

Intestinal mucosal protein and nucleic acid concentration results (dry matter weight) are presented in Tables 3.1, 3.2, 3.3, Figures 3.2, 3.3 and 3.4. Intestinal mucosal RNA concentrations of piglets 3 and 8 DPI were

[29] SPSS Inc., Chicago, IL.

Table 3.1. Small intestinal mucosal protein concentrations (mg/g mucosal dry matter) of control piglets *vs.* piglets infected with the SDSU strain of porcine rotavirus

	Infected				Infected DPI mean	Control				Control DPI mean	All DPI mean ± SE[d]
	RP [a]	P	RPP	MO		RP	P	RPP	MO		
1 DPI [b]	389 [c]	385	354	366	374	358	391	384	373	376	375 ±6
2 DPI	401	379	376	372	382	385	327	393	424	382	382 ±10
3 DPI	422	401	392	394	402	403	411	440	398	413	408 ±6
8 DPI	419	426	406	427	419	417	435	429	430	428	424 ±6
Treatment mean ± SE	408 ±8	398 ±9	382 ±10	390 ±11	394 ±5	391 ±10	391 ±18	412 ±8	406 ±11	400 ±6	397 ± 4

[a] RP: 9,000 IU retinyl palmitate/day, P: 1,050 mg putrescine/day, RPP: 9,000 IU retinyl palmitate and 1,050 mg putrescine/day, MO: milk only.

[b] DPI: day postinoculation.

[c] Average from 4 piglets.

[d] Small intestinal mucosal protein concentrations (SIMPC) of 3 and 8 DPI piglets > SIMPC of 1 DPI piglets $P<0.01$).

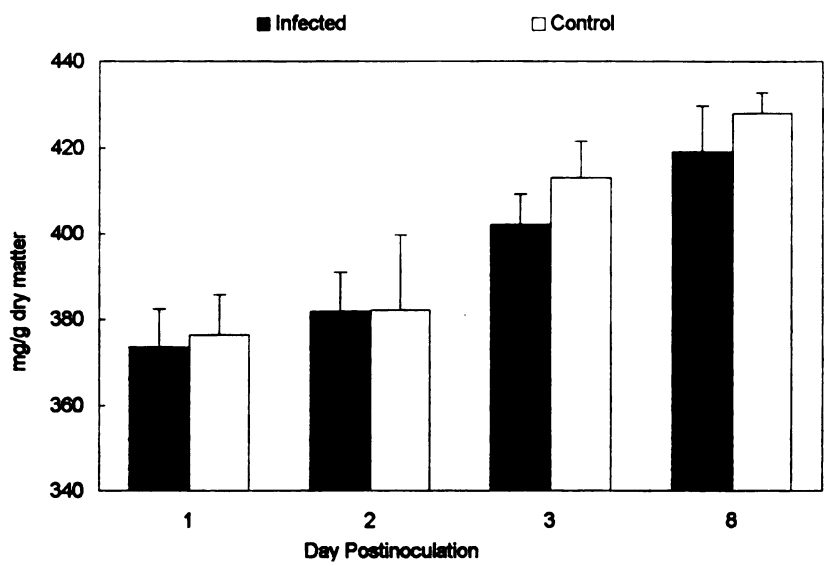

Figure 3.2. Pooled small intestinal mucosal protein concentration (SIMPC) for control piglets *vs.* piglets infected with the SDSU strain of porcine rotavirus (mean ± standard error). SIMPC of 3 and 8 DPI piglets > SIMPC of 1 and 2 DPI piglets ($P<0.01$).

Table 3.2. Small intestinal mucosal DNA concentrations (mg/g dry weight of mucosa) of control piglets *vs.* piglets infected with the SDSU strain of porcine rotavirus

	Infected				Infected DPI mean	Control				Control DPI mean	All DPI mean ± SE
	RP [a]	P	RPP	MO		RP	P	RPP	MO		
1 DPI [b]	13 [c]	12	12	13	12	13	13	14	14	14	13 ±0.27
2 DPI	14	13	14	13	14	14	12	14	13	13	13 ±0.34
3 DPI	15	14	14	14	14	15	14	15	14	15	15 ±0.22
8 DPI	13	14	14	14	14	14	15	15	16	15	14 ±0.23
Treatment mean ± SE	408 ±8	398 ±9	382 ±10	390 ±12	394 ±5	391 ±10	391 ±18	412 ±8	406 ±10	400 ±6	14 ± 0.14[d]

[a] RP: 9,000 IU retinyl palmitate/day, P: 1,050 mg putrescine/day, RPP: 9,000 IU retinyl palmitate and 1,050 mg putrescine/day, MO: milk only.

[b] DPI: day postinoculation.

[c] Average from 4 piglets.

[d] Statistical analyses:
Small intestinal mucosal DNA concentration (SIMDC) of 8 DPI piglets > SIMDC of 1 DPI piglets; SIMDC of 3 DPI piglets > SIMDC of 1 and 2 DPI piglets ($P<0.01$).
SIMDC of infected piglets < SIMDC of control piglets ($P<0.05$).

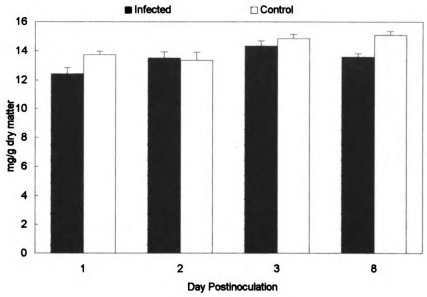

Figure 3.3. Pooled small intestinal mucosal DNA concentrations for control piglets *vs.* piglets infected with the SDSU strain of porcine rotavirus (mean $\pm$ standard error). DNA of 8 DPI piglets > DNA of 1 DPI piglets; DNA 3 DPI piglets > DNA 1 and 2 DPI piglets ($P<0.01$).

Table 3.3. Small intestinal mucosal RNA concentrations (mg/g dry weight) of control piglets vs. piglets infected with the SDSU strain of porcine rotavirus

	Infected				Infected DPI mean	Control				Control DPI mean	All DPI mean ± SE[d]
	RP [a]	P	RPP	MO		RP	P	RPP	MO		
1 DPI [b]	23 [c]	22	22	22	22	23	23	23	22	23	22 ±0.47
2 DPI	23	24	25	23	24	23	19	23	22	22	23 ±0.58
3 DPI	25	25	26	24	25	25	25	27	24	25	25 ±0.44
8 DPI	24	24	25	25	25	26	29	25	28	27	26 ±0.46
Treatment mean ± SE	24 ±0.63	24 ±0.86	24 ±0.64	23 ±0.54	24 ±0.33	24 ±0.60	24 ±1.37	25 ±0.66	24 ±0.76	24 ±0.44	24 ±0.28[d]

[a] RP: 9,000 IU retinyl palmitate/day, P: 1,050 mg putrescine/day, RPP: 9,000 IU retinyl palmitate and 1,050 mg putrescine/day, MO: milk only.

[b] DPI: day postinoculation.

[c] Average from 4 piglets.

[d] Statistical analyses:
Small intestinal mucosal RNA concentrations (SIMRC) of 3 and 8 DPI piglets > SIMRC of 1 and 2 DPI piglets ($P<0.01$).
Infection X DPI interaction ($P<0.05$).

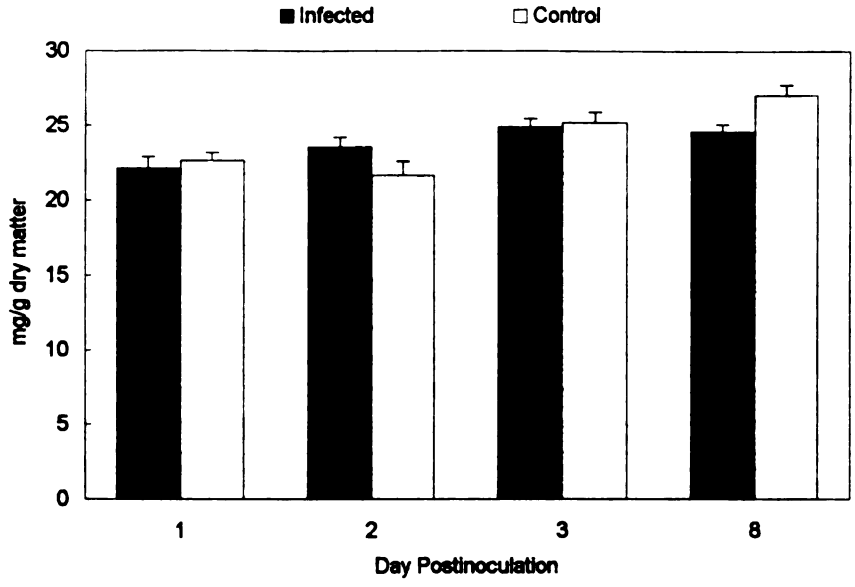

Figure 3.4. Pooled small intestinal mucosal RNA concentrations for control piglets *vs.* piglets infected with the SDSU strain of porcine rotavirus (mean ± standard error). RNA of 3 and 8 DPI piglets > RNA of 1 and 2 DPI piglets ($P<0.01$). Infection X DPI interaction existed ($P<0.05$).

higher than in piglets 1 and 2 DPI ($P<0.01$). A significant infection X time interaction existed in the mucosal RNA concentration. Intestinal mucosal DNA concentrations of infected piglets were lower than those in control piglets ($P<0.01$). Mucosal DNA concentrations of piglets 8 DPI were higher than in piglets 1 DPI. Mucosal DNA concentrations of piglets 3 DPI were higher than in piglets 1 and 2 DPI ($P<0.01$). Protein concentrations of piglets 3 and 8 DPI were higher than in piglets 1 DPI ($P<0.01$). The results of protein and nucleic acid ratios are presented in Table 3.4. There were no main effect interactions on intestinal mucosal protein and DNA concentrations. The ratio of protein:RNA between groups was not significantly different. The ratios of RNA:DNA and protein:DNA in the infected group were significantly higher than those in the control group ($P<0.01$).

Vitamin A concentration

Total hepatic vitamin A concentrations (Table 3.5, Figure 3.5) ranged from 108 µg/g dry weight in a control piglet 1 DPI treated with putrescine to 1433 µg/g dry weight in an infected piglet 8 DPI treated with retinyl palmitate. Total liver vitamin A, retinyl palmitate and retinol concentrations in RP and RPP groups were significantly higher than those in P and MO groups ($P<0.01$). Piglets 8 DPI had higher concentrations of liver retinol than piglets 1 DPI. Livers from piglets 3 and 8 DPI had higher

Table 3.4. Protein:DNA, RNA:DNA and protein:RNA ratios[1] from control piglets *vs.* piglets infected with the SDSU strain of porcine rotavirus

DPI[2]	Treatment group[3]		PROT:DNA	RNA:DNA	PROT:RNA
1	Infected	RP	29 ± 1.42	1.70 ± 0.09	17 ± 1.70
		P	32 ± 2.13	1.81 ± 0.09	18 ± 1.84
		RPP	31 ± 1.80	1.92 ± 0.07	16 ± 0.37
		MO	29 ± 2.21	1.72 ± 0.14	17 ± 1.12
	Control	RP	27 ± 1.65	1.69 ± 0.06	16 ± 1.35
		P	29 ± 1.34	1.70 ± 0.09	17 ± 1.43
		RPP	28 ± 1.13	1.66 ± 0.09	17 ± 1.18
		MO	26 ± 2.44	1.56 ± 0.06	17 ± 1.08
2	Infected	RP	30 ± 1.68	1.67 ± 0.09	18 ± 0.96
		P	29 ± 1.73	1.75 ± 0.09	16 ± 1.42
		RPP	27 ± 2.35	1.74 ± 0.11	15 ± 0.73
		MO	30 ± 3.86	1.87 ± 0.20	16 ± 0.74
	Control	RP	27 ± 1.18	1.58 ± 0.06	17 ± 0.39
		P	27 ± 0.84	1.56 ± 0.02	17 ± 0.46
		RPP	28 ± 1.26	1.66 ± 0.05	17 ± 0.72
		MO	32 ± 1.64	1.70 ± 0.09	19 ± 1.81
3	Infected	RP	29 ± 2.56	1.71 ± 0.05	17 ± 1.16
		P	29 ± 1.30	1.79 ± 0.07	16 ± 0.17
		RPP	27 ± 1.36	1.79 ± 0.06	15 ± 0.87
		MO	28 ± 2.15	1.69 ± 0.08	16 ± 0.80
	Control	RP	27 ± 1.25	1.63 ± 0.05	16 ± 0.52
		P	29 ± 1.30	1.75 ± 0.06	17 ± 0.96
		RPP	29 ± 0.69	1.78 ± 0.08	16 ± 0.68
		MO	28 ± 1.68	1.64 ± 0.14	17 ± 1.78
8	Infected	RP	32 ± 2.47	1.85 ± 0.13	17 ± 0.15
		P	32 ± 2.08	1.80 ± 0.05	17 ± 0.73
		RPP	29 ± 1.62	1.79 ± 0.07	16 ± 0.98
		MO	31 ± 1.28	1.84 ± 0.01	17 ± 0.71
	Control	RP	29 ± 0.85	1.81 ± 0.05	16 ± 0.83
		P	29 ± 0.41	1.96 ± 0.10	15 ± 0.67
		RPP	29 ± 1.82	1.67 ± 0.10	17 ± 0.65
		MO	27 ± 1.41	1.76 ± 0.10	15 ± 0.29

[1] Values are presented as Mean ± Standard Error.

[2] DPI: day postinoculation.

[3] Each group consisted of 4 piglets. Group RP: 9,000 IU retinol palmitate/day, Group P: 1,050 mg putrescine/day, Group RPP: 9,000 IU retinol palmitate and 1,050 mg putrescine/day, Group MO: milk only.

Table 3.5. Hepatic total vitamin A concentrations of control piglets *vs.* piglets infected with the SDSU strain of porcine rotavirus

	Infected				Infected DPI mean	Control				Control DPI mean	All DPI mean ± SE
	RP [a]	P	RPP	MO		RP	P	RPP	MO		
1 DPI [b]	352 [c]	133	323	193	250	362	158	372	150	260	255 ±22
3 DPI	725	188	595	177	421	597	258	603	277	434	427 ±44
8 DPI	1094	174	703	217	547	832	167	841	194	508	528 ±67
Treatment mean ± SE	723 ±89	165 ±10	540 ±57	196 ±13	406 ±39	597 ±56	194 ±15	605 ±67	207 ±27	401 ±34	403 ± 30[d]

[a] RP: 9,000 IU retinol palmitate/day, P: 1,050 mg putrescine/day, RPP: 9,000 IU retinol palmitate and 1,050 mg putrescine/day, MO: milk only.

[b] DPI: day postinoculation.

[c] Average from 4 piglets.

[d] Statistical analyses:
Hepatic total vitamin A concentrations (HTVAC) of 3 and 8 DPI piglets > HTVAC of 1 DPI piglets ($P<0.01$).
HTVAC of RP and RPP piglets > HTVAC of P and MO piglets ($P<0.01$).
Treatment X DPI interaction ($P<0.01$).

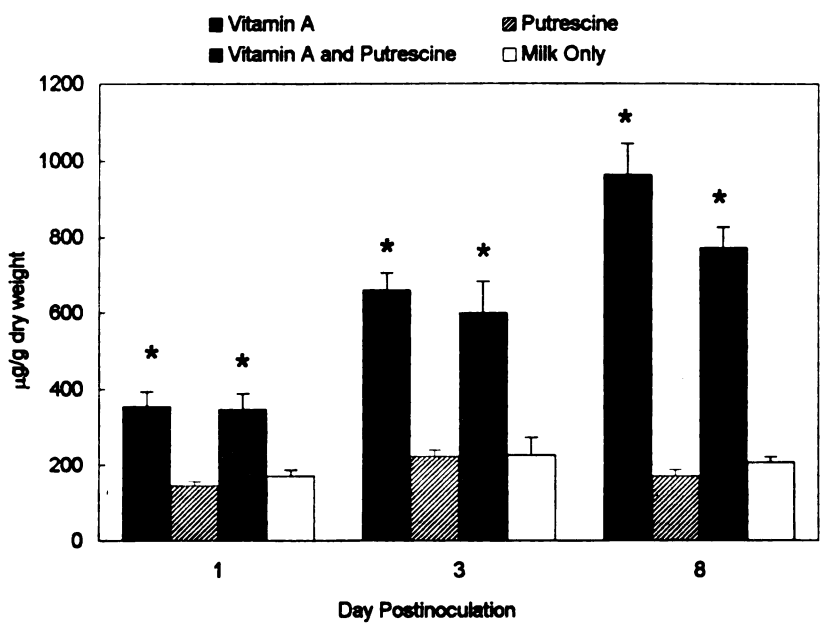

Figure 3.5. Pooled hepatic total vitamin A concentration of control and infected piglets (mean ± standard error).

* Significantly different from putrescine and milk only groups ($P<0.01$).

concentrations of total vitamin A and retinyl palmitate than the livers from piglets 1 DPI ($P<0.01$). A significant interaction of treatment X time effect existed ($P<0.01$). Livers from infected piglets 3 DPI which were treated with vitamin A only had higher concentrations of total vitamin A than those from piglets that were treated with vitamin A and putrescine combined ($P<0.01$).

DISCUSSION

The results of this experiment indicated that intestinal mucosal protein, DNA and RNA concentrations in dry matter weight were significantly influenced by time. Older piglets (13-day-old) had significantly higher RNA concentrations than younger piglets (six-day-old). These results are similar to the findings of Grant *et al* (1990). They found that the intestinal mucosa of 14-day-old piglets fed with milk only, contained higher protein, DNA and RNA than the intestinal mucosa of seven-day-old piglets. Higher concentrations of protein and nucleic acids were probably associated with the thickness of the intestinal mucosal crypts. Moon (1971) indicated that small intestinal mucosal cell replacement time in young piglets was slower than in older piglets. The shorter replacement time in older piglets was caused by a reduction in villous length and an increase in crypt depth (Moon, 1971). In the present study, crypt depths of older piglets were significantly higher than

those in younger piglets. As a proliferation compartment, crypts contain higher concentrations of protein, DNA and RNA. Thus the greater concentrations of protein, DNA and RNA in older piglets may have occured because they have larger crypts.

Results of the present study also indicated that protein and DNA concentrations of infected piglets were lower than control piglets. Except in piglets 2 DPI, RNA concentrations of the infected piglets were also lower than the control group. Several investigators have shown that the concentrations of protein and nucleic acids in the intestine are influenced by the level of nutrition and the degree of intestinal damage. Protein and nucleic acid concentrations in the intestine of sheep fed ad libitum were higher than in sheep with restricted feed intake (Burrin *et al*, 1991). Intestinal mucosal protein and nucleic acid concentrations of rats with intestinal mucosal atrophy were lower than those in normal rats (Wang and Johnson, 1991). In the present study, rotavirus infection reduced intestinal mucosal volume and caused a malabsorptive diarrhea. This might explain the decreased concentrations of protein and nucleic acids in the small intestinal mucosa of the infected piglets.

The ratios of RNA:DNA and protein:DNA in the intestinal mucosa were significantly influenced by infection status but not by treatment nor time. The ratios of RNA:DNA and protein:DNA in the infected piglets were significantly

higher than in the control piglets ($P<0.01$). Protein:DNA and RNA:DNA ratios are indicators of intestinal epithelial cell size (Allison *et al*, 1963, Robinson 1969, Winich and Noble 1965), therefore the results of this experiment indicate that the intestinal epithelial cell size was greater in the infected than in the control piglets. However, this is probably not the case, since histopathologic results indicated that the infected animals had higher numbers of squamous and cuboidal cells. Thus, the infected piglets had more attenuated or smaller cells than the control piglets. In addition, results also indicated that infected piglets had lower DNA concentrations without any increase in protein concentration. Thus, the increased ratios of protein:DNA and RNA:DNA in the infected animals may not indicate larger cell volume, but may be the result of a reduction in DNA concentration.

Daily dietary administration of retinyl palmitate and/or putrescine did not have any effect on the mucosal protein and nucleic acid concentrations in infected or control piglets. A deficiency of vitamin A reduced protein synthesis in the rat intestinal mucosa (De Luca, 1969). Administration of vitamin A to vitamin A deficient animals increased RNA synthesis in the small intestinal mucosa (Johnson *et al*, 1969; Zachman, 1967). The absence of vitamin A effects in the present study compared to the previous study was probably because the piglets were not in a deficient status.

Also, putrescine did not have any effect on the intestinal mucosal protein and nucleic acid concentrations. Reduced concentrations of intestinal mucosal protein and nucleic acids were seen in animals treated with DMFO, an inhibitor of the polyamine biosynthesis enzyme (Wang and Johnson, 1991; Seidel *et al*, 1984). In hepatectomized rats, exogenous putrescine reversed the reduction of protein and nucleic acid synthesis caused by DMFO. Exogenous putrescine did not have any effects on hepatectomized rats without DMFO (Luk, 1986). Since putrescine did not have any effect on the protein and nucleic acid concentrations in both infected and control piglets, rotaviral infections may not interfere with polyamine biosynthesis.

Increased DNA synthesis and content in intestinal mucosa were shown to be associated with increased cellular proliferation in the crypts (Luk and Baylin, 1984; Tsujikawa *et al*, 1990). Results of the present study indicate that administration of vitamin A and/or putrescine increased cellular proliferation in the crypts. However, the data regarding intestinal mucosal DNA concentrations were not significantly different. The inability to show a correlation between cellular proliferation and increased DNA concentrations in the present study may have been due to technical problems. The amount of DNA produced by the proliferative cells may have been too small to be detected adequately by the present method of analysis. In addition, the proliferating cells are located in the crypts, whereas

the specimens for the analyses were obtained from both crypt and villous portions of the intestinal mucosa, thus affecting the present results.

Hepatic vitamin A concentrations, including total vitamin A, retinyl palmitate and retinol, were significantly influenced by treatment and time, but not influenced by infection status. Reduced vitamin A absorption, as indicated by a reduced serum retinol and retinyl palmitate concentrations, was seen in calves infected with by *Cryptosporidium parvum* (Holland *et al*, 1990). In the present study, no analysis was done on serum vitamin A concentrations. The concentrations of hepatic vitamin A observed in this study appeared to be associated with the amount of vitamin A intake which corresponded with findings by other investigators (Hoppe *et al*, 1992; Wellenreiter *et al*, 1969; Pryor *et al*, 1969). Vitamin A concentrations of RP and RPP groups increased steadily, whereas the concentrations of vitamin A in P and MO groups remained at the base concentration. This pattern resulted in a significant interaction of treatment X time effect.

Reference values for normal concentrations of vitamin A in piglets were not available. Compilation of data and references generated by the Nutrition Laboratory in the Department of Animal Science, Michigan State University indicated that the normal concentration of liver total vitamin A in young piglets varied between 125 to 200 μg/g

dry weight, which agrees with a variety of sources (Steinhardt *et al*, 1985; Hennig *et al*, 1985). In the present study, all piglets (1, 3 and 8 DPI) given P and MO had normal concentrations of vitamin A.

Vitamin A concentrations tended to be lower in the liver of animals treated with putrescine. The infected piglets 1 and 8 DPI in the P group had lower total hepatic vitamin A concentrations than infected piglets in the MO group. Also, the infected piglets given RPP had lower hepatic total vitamin A concentrations than infected piglets given RP. The difference between infected piglets given RPP or RP was statistically significant 8 DPI. The reason for this trend is not known, and warrants further investigation. In the present study, vitamin A was given in the form of retinyl palmitate, stabilized in an acacia-starch matrix with antioxidants. The stability of vitamin A in dry feeds or premixes is reduced by hygroscopic agents such as salt and urea (Mitchell, 1967; Shields *et al*, 1982). Putrescine is a highly reactive substance, especially with a variety of cellular molecules (Wolfe, 1993). Also, putrescine is a strong hygroscopic substance, but this hygroscopicity was unlikely to affect the stability of vitamin A since in the present experiment both substances were given in liquid milk. Some concern has been expressed about the instability of vitamin A in the retinol form. Retinyl palmitate is converted to retinol prior to absorption by intestinal mucosal cells. Thus, retinol may be present in the

intestinal lumen or within the intestinal mucosal cells. Since putrescine is also present both in the intestinal lumen and within the intestinal mucosal cells, the degradation of retinol may occur intraluminally or intracellularly.

In conclusion, the results of this study indicate that 13-day-old piglets had higher protein and nucleic acid concentrations than six-day-old piglets. Rotavirus infection reduced intestinal mucosal protein and DNA concentrations in piglets. Supplementation with vitamin A and/or putrescine did not have any effect on the intestinal protein and nucleic acid concentrations. Rotavirus infection did not have any effect on the hepatic vitamin A concentrations. Daily administration of vitamin A increased hepatic vitamin A concentrations in piglets.

CONCLUSIONS

The results from the study presented in this dissertation indicate that:

1. Daily dietary administration of vitamin A (30,000 IU/kg of milk) and/or putrescine (25g/kg dry matter of milk) was ineffective in the prevention or attenuation of rotaviral infectivity, and the clinical signs and intestinal mucosal damage caused by rotaviral infection in piglets. Furthermore, putrescine appeared to increase rotaviral infectivity.
2. Rotaviral infection in piglets caused villous atrophy resulting in a reduction of the small intestinal mucosal volume. Daily dietary administration of retinyl palmitate or putrescine alone or in combination increased the mitotic index in small intestinal crypts, suggesting a possible beneficial effect of retinyl palmitate or putrescine on the regeneration of intestinal mucosa.
3. Rotavirus infection reduced intestinal mucosal protein and DNA concentrations in piglets. Supplementation with vitamin A and/or putrescine did not have any effect on the intestinal protein and nucleic acid concentrations.

Thirteen-day-old piglets had higher protein and nucleic acid concentrations than six-day-old piglets.

4. Rotavirus infection did not have any effect on the hepatic vitamin A concentrations. Hepatic vitamin A concentrations in piglets correlated with the amount of vitamin A consumed.

APPENDIX

Information regarding the dose of rotavirus inocula is generally unavailable and varies depending on the host and environmental factors. It was therefore necessary to perform a series of studies to propagate the rotavirus and determine the pig-infectious-dose fifty (PID_{50}, dilution of the virus pool that caused diarrhea in 50% of the piglets) and the dose of inocula allowing infected piglets to survive until eight days postinoculation (the end of the observation period).

MATERIALS AND METHODS

Two four-day-old, naturally born, colostrum-deprived piglets were used for virus propagation. Each piglet was orally inoculated with 1 ml of a filtered intestinal homogenate containing a South Dakota State University (SDSU) strain of porcine rotavirus, obtained from Dr. D. Benfield (South Dakota State University, Brookings, SD). Piglets were placed in individual cages in a temperature-controlled room (30-32^{o}C) and fed 100 ml of evaporated milk three times a day. Forty-eight hours after oral inoculation with rotavirus, piglets were euthanatized with sodium pentobarbital[30]. Immediately after opening the carcasses,

[30] The Butler Co., Columbus, OH.

the small intestine was placed on a cold glass surface. The intestine was opened longitudinally along the mesenteric attachment and the mucosa was scraped. Small intestinal mucosal scrapings and intestinal contents were collected and frozen at -20°C until further processing.

A 10% suspension of the intestinal mucosa and its contents in Hank's balanced salt solution (HBSS)[31] was prepared. The suspension was homogenized with a Teflon pestle tissue homogenizer[32] and then frozen and thawed twice to rupture the infected epithelial cells. The homogenate was centrifuged[33] 20 minutes at 5,000 x g at 4°C. The supernatant was collected and then filtered through a series of filters[34] with average pore diameter of 0.80 μm, 0.45 μm and 0.22 μm. The filtrate (10 ml) was then divided into 0.5 ml plastic tubes and frozen at -70°C for stock virus. Fecal samples from the two piglets were also collected and tested using an ELISA kit[35] for the presence of rotavirus antigen.

Virus infectivity titration was done on eight four-day-old, caesarean-derived, colostrum-deprived (CDCD) piglets. One tube containing 0.5 ml of virus suspension was thawed

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[31] Sigma Chemical Co., St. Louis, MO.

[32] Thomas® Teflon pestle tissue homogenizers, Thomas Scientific™ USA, Philadelphia, PA.

[33] Refrigerated Centrifuge, Model PR-7000, International Equipment Co., Needham, MA.

[34] Nalgene® Disposable Filterware, Nalge Comp., Rochester, NY.

[35] Pathfinder™®, Kallestad Diagnostics, Austin, TX.

and resuspended with HBSS to dilutions of 10^{-6}, 10^{-7} and 10^{-8}. Two piglets were each orally inoculated with 1 ml of 10^{-6} dilution of the viral suspension, three piglets with 1 ml of the 10^{-7} dilution and three piglets with 1 ml of the 10^{-8} dilution. Piglets were observed 3 times daily for signs of diarrhea. Fecal samples were collected just prior to inoculation and 48 hours after inoculation for ELISA testing. Piglets that had diarrhea and a positive ELISA test 48 hours after inoculation were considered infected. The pig infectious dose fifty (PID_{50}) was defined as the dilution of the stock virus that caused diarrhea and a positive ELISA test in 50% of the piglets.

Sixty-six CDCD and four naturally born (NB) piglets were used to determine the dose of virus inocula that would allow the infected piglets to survive until 8 DPI. The NB piglets used in this study were taken from the sows at two days of age. All the CDCD and NB piglets were infected with a serial 10-fold dilution of stock rotavirus (10^{-8} to 10^{-10} dilutions) at five days of age. Piglets were observed for signs of diarrhea, vomiting and dehydration. Piglets were euthanatized at 1, 2, 3 or 8 DPI, and intestinal sections were collected and examined microscopically. The presence of rotaviral antigen was detected either by ELISA using the fecal material or by an immunohistochemistry technique on the intestinal sections.

RESULTS

Virus infectivity titration

All eight CDCD piglets inoculated with 10^{-6}, 10^{-7} or 10^{-8} dilutions of stock rotavirus showed clinical signs of diarrhea. Fecal samples collected at 2 DPI were positive for the presence of rotavirus using the ELISA. Since all of the piglets were positive for rotavirus infection, the PID_{50} of the stock rotavirus was not determined, but was considered to be less than 10^{-8}.

Study using 10^{-8} dilution of stock rotavirus in CDCD piglets

Of the 18 CDCD piglets that were inoculated with 10^{-8} dilution of stock rotavirus, 13 piglets were euthanatized as scheduled on 1, 2 or 3 DPI. Five piglets which were scheduled to be euthanatized 8 DPI did not survive. All piglets showed clinical signs of rotaviral infection, including diarrhea, vomiting and severe dehydration. In addition to severe villous atrophy, some of the piglets also had colonies of rod-shaped bacteria adherent to intestinal villi and crypts. Rotaviral antigen was detected in all piglets.

Study using 10^{-9} dilution of stock rotavirus in CDCD piglets

Of the nine piglets that were inoculated with 10^{-9} dilution of stock rotavirus, five piglets were euthanatized as scheduled on 1, 2 or 3 DPI. Four piglets did not survive until 8 DPI. The clinical signs and lesions were similar to those of CDCD piglets infected with 10^{-8} dilution of stock rotavirus.

Study using 10^{-10} dilution of stock rotavirus in CDCD piglets

Of the 14 piglets that were inoculated with 10^{-10} dilution of stock rotavirus, seven piglets were euthanatized as scheduled on 1, 2 or 3 DPI. Seven piglets did not survive until 8 DPI. The clinical signs and lesions were similar to those previously described.

Study using CDCD piglets not infected with rotavirus

Of the 25 piglets observed in this study, 10 piglets were euthanatized as scheduled when they were six, seven or eight days of age (1, 2 or 3 DPI). Fifteen piglets did not survive until 13 days of age (8 DPI). Clinical signs were mild to severe diarrhea and dehydration. Intestinal lesions in most of the piglets were severe villous atrophy with the presence of rod-shaped bacteria adherent to the intestinal villi and crypts. Both the ELISA with the fecal material and the immunohistochemistry technique with the intestinal

sections were negative for the presence of rotavirus antigen.

Study using 10^{-10} dilution of stock rotavirus in NB piglets

Four NB piglets were infected with 10^{-10} dilution of the stock rotavirus. Diarrhea were observed in all piglets 2 and 3 DPI. Fecal samples from all piglets collected at 3 DPI were ELISA positive, indicating a rotaviral infection. The clinical signs of diarrhea disappeared at 5 DPI. All piglets looked healthy after 5 DPI. One piglet died at 7 DPI with a ruptured stomach. Rotaviral antigen was not detected in fecal material nor the intestinal section from this piglet. The remaining three piglets were euthanatized 8 DPI. Rotaviral antigen was not detected in piglets euthanatized 8 DPI.

DISCUSSION

All of the CDCD piglets, whether infected or not, did not survive until 8 DPI. It was very difficult to maintain piglets that had not consumed colostrum in a relatively germfree environment. Villous atrophy and the presence of rod-shaped bacteria adherent to the small intestinal villi and crypts were consistently seen in the CDCD piglets, indicating the possibility of a dual infection with *E. coli*.

Colonies of rod-shaped bacteria were observed in the intestinal lumen of the NB piglets, but no bacteria were

adherent to the intestinal villi and crypts, indicating a protective role of colostrum against the bacterial infection seen in CDCD piglets. However, rotavirus infection was established in the NB piglets. Since the infection and remission were established in the NB piglets inoculated with 10^{-10} dilution of rotavirus, the NB piglets and 10^{-10} dilution of stock rotavirus were used for the studies in Chapters 1, 2 and 3.

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VITA

The author was born in Surabaya, Indonesia in June 20, 1953. He received the degree of Doctor of Veterinary Medicine from University of Airlangga, Indonesia in 1978. Since 1979, the author has worked at the Department of Pathology, Animal Disease Investigation Center in Maros, Indonesia. In 1985, the author received a scholarship from Australian International Development Assistance Bureau and spent 2 years (1985-1986) as a graduate student in the Department of Pathology, James Cook University, Australia. He received the degree of Masters of Science in 1989. In the fall of 1991, the author received a scholarship from Indonesian Development Planning Agency (BAPPENAS) and was admitted into a Ph.D. program in the Department of Pathology, Michigan State University.