GNOTOBIOTIC PIGS: PROCUREMENT, MICROBIAL FLORA, SERUM PROTEINS AND LYMPHATIC TISSUES

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Introduction

Gnotobiotic pigs have been procured and reared for research purposes in North America since the early sixties (4, 7, 12). They have many features similar to those of the common gnotobiotic laboratory animals, but their larger size and specific species characteristics make them a preferable animal for many types of research (4). The gross morphology of the internal organs and the quantitative values of the formed elements of blood of the gnotobiotic pig, are reported to be essentially similar to those of the conventionally reared pig (7, 14). Being colostrum deprived, the newborn gnotobiotic pig possesses no significant immunoglobulins or maternal antibody (3, 7). Since the pig has been used in gnotobiology for a relatively short time, many of its characteristics have not yet been investigated.

In view of the potential value of the gnotobiotic pig as an experimental animal, facilities were established at the Ontario Veterinary College in 1966 for rearing these animals. One or two litters have been procured monthly. This report describes the equipment and the techniques used and includes studies of the microbial flora, serum proteins and lymphoid tissue of the first six litters.

Materials and Methods

Rearing Equipment and Sterilization Techniques

The equipment consisted of three main components, hysterectomy hood, transfer isolator and eight rearing isolators which were similar to those used at the Ohio State University (7, 8).[1]

Prior to use, the isolators were thoroughly cleaned and disinfected by spraying with a 2% peracetic acid solution. The internal attachments of the isolators, such as false floors, shelves and partitions were autoclaved at 15 pounds per square inch pressure for 45 minutes and then sprayed with peracetic acid. Items that could not be sterilized by chemical means (e.g. gauze, swabs, hypodermic needles and syringes) were autoclaved and then sealed in stainless steel canisters and resterilized in a hot air oven at 150° C for four hours. The latter method was adopted because no vacuum autoclave was available during the initial studies, precluding the use of a supply cylinder. The surface of the canisters and the metal cans containing liquid sterile diet were sprayed with peracetic acid and introduced into the isolators. The interiors of the isolators containing all the items were sprayed again with peracetic acid and the unit was then sealed. After a minimum of two hours fumigation, filtered air was allowed to circulate through the isolators for at least 48 hours or preferably longer, prior to introduction of pigs.

The Pigs

A total of 28 Yorkshire pigs from six different litters were included in the studies. They were obtained from sows on the 113th or 114th day of gestation by the hysterectomy method as described by Meyer *et al* (7), and reared in sterile isolators. The temperature of the room was kept at 90° F to 100° F for the first week and then reduced gradually to 75° F. A sterile canned milk substitute[2] was fed to the pigs beginning on the day following delivery. The initial amount was 15 ml three times daily. This was increased by 8 ml per feeding each day until a maximum of 240 ml three times daily was reached when the pigs were 28 days old. Thereafter, the level was kept constant.

Blood samples were collected for serology from the anterior vena cava of ten of the pigs at weekly intervals. Twenty of the pigs were killed for microbiological and histological studies at different ages from birth to 12 weeks of age.

Tests of Germ Status

The pigs to be examined were anaesthetized inside the rearing isolator with intravenous pentobarbitone sodium BP then exsanguinated through the axillary arteries. Tests were then

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[1]Manufactured by Partsco Inc., 2977 Lamb Avenue, Columbus, Ohio 43219.

[2]SPF Lac-Borden Co., 350 Madison Ave., New York 10017.

performed for the presence of bacteria, fungi, mycoplasma and viruses.

The kidneys were removed first to provide cells for tissue cultures. Then small pieces (approximately 0.5 cm diameter) of tissue (spleen, liver, lung, rectal wall and contents, turbinate bone and adjacent nasal mucosa and skin, subcutaneous tissue and hair from the ear) were placed in tryptic soy broth,[3] thioglycollate broth,[3] liquid Sabouraud's medium,[3] and liquid PPLO medium.[3]

After two week's incubation at 37° C, the cultures were inoculated onto blood agar plates, solid PPLO medium, and McConkey agar and incubated overnight aerobically and anaerobically. In addition tests for Haemophilus were made by inoculating blood agar plates with lung tissue. The medium was cross streaked with a culture of haemolytic staphylococcus to provide a source of co-enzyme I.

About 0.5 gm of each of the tissues listed above were used for virological study, by passaging them four times in porcine kidney cell cultures. In the final passage they were inoculated into tubes containing cultures on coverslips. The coverslip preparations were stained with hematoxylin and eosin, and examined for cytopathic effects as compared to uninoculated control preparations.

Serology

In addition to the sera of the gnotobiotic pigs, serum samples were obtained from 15 two-week-old and 12 eight-week-old conventionally reared pigs for comparison.

The serum proteins were separated by electrophoresis on cellulose acetate strips which were then stained with Ponceau S and cleared. The cleared strips were scanned in a Photovolt Densitometer with an automatic integrator to determine the percentage of gamma globulin. The total protein in the serum samples was measured by the biuret method as described by Henry (1). These two figures were then used to calculate the concentration of gamma globulin in the serum.

Histology

Samples of the following tissues were taken at necropsy for histological studies: turbinate bone and adjacent epithelium, thymus, adrenal gland, lung, liver, spleen, duodenum, jejunum, ileum, large coiled colon, cecum, and mesenteric lymph nodes. The turbinate and lung were fixed in Bouin's fluid and the remainder were fixed in 10% formalin. Sections were stained with hematoxylin and eosin. In the histological examination of the various organs special attention was paid to the lymphatic tissue.

[3]Difco Laboratories, Detroit, Michigan, U.S.A.

Results

Survival rate and general health

Of the 28 hysterectomy-derived pigs eight died and twenty survived until they were killed. Two of the deaths occurred within 48 hours of delivery in pigs that were weak and pale at birth. Another two pigs died after bleeding for serology. The other four deaths occurred in pigs that were left together in pairs in one compartment of the isolator. In each case the larger pig rapidly attained dominance over the smaller pig, consumed most of the feed and finally killed it. After being left in the isolator for as long as 25 days, the dead pigs possessed a faint odor, and had only minor changes in the internal organs.

The remaining pigs appeared alert and healthy throughout their lives. Their growth rates were slower than those of conventional pigs and their feces were softer and darker. During subsequent work (not included in these studies), some pigs have been maintained in isolators for as long as six months. In some cases, for economic reasons, SPF lac was substituted by canned condensed cow's milk.[4] This was found to be satisfactory for maintaing the animals in good health and in a germfree state.

Microbiological Findings

No bacteria, fungi or mycoplasma could be detected in any of the pigs when the system was functioning properly. In a single incident *Escherichia coli* and *Clostridium perfringens* were isolated from the feces and large intestine of a nine-week-old pig following accidental damage to the isolator. No cytopathic effects were observed on the coverslip preparations.

Serology

The mean gamma globulin levels in the various age groups are presented in Table I. For comparison, the mean gamma globulin levels in the serum of 15 two-week-old and 12 eight-week-old normal pigs were determined and the results are presented in Table I. Two-week-old germfree pigs had a gamma globulin level of 0.256 ± .11 gm per 100 ml serum, whereas naturally reared swine of the same age had a level of 1.099 ± 0.32 gm per 100 ml. The levels for eight-week-old pigs were 0.243 ± 0.09 gm per 100 ml of serum in germfree animals and 1.019 ± 0.17 gm per 100 ml in naturally reared pigs.

[4]Farmers Wife 1—Cow and Gate Ltd., Brockville, Ontario.

TABLE I

SERUM GAMMA GLOBULIN IN GERMFREE AND NORMAL SWINE

Age in Weeks

Pig No.	1	2	3	4	5	6	7	8	9	10	11	12	13
7/4 B	0.315*	0.461	0.301	0.090	0.223	0.222	0.314	0.300					
7/4 C	0.370		0.190	0.240	0.199	0.233+	0.325	0.480	0.531+	0.240+			
7/5 A				0.237									
7/5 B						0.140							
7/5 C	0												
10/2 G	0.125	0.138	0.127	0.158	0.162	0.267	0.375	0.305	0.266	0.161	0.397		
11/1 E		0.236	0.156	0.356	0.149	0.144	0.253	0.265	0.130	0.116	0.280		
12/3 D		0.267	0.288	0.296	0.148	0.125	0.232						
13/2 A		0.310	0.156	0.136									
13/2 B		0.120	0.244	0.303	0.305	0.106	0.120	0.100	0.120	0.266	0.122	0.267	0.105
14/3 A	0.273	0.260	0.236	0.253									
Mean of Germfree Group	0.222	0.256	0.212	0.229	0.198	0.167	0.259	0.243	0.172	0.168	0.266		
Mean of Normal Group		1.099						1.019					

*gms of gamma globulin per 100 ml serum.

+This animal was contaminated sometime after week 5 and therefore the last five serum samples were not included in the determination of the mean.

Histology

Liver—Hemopoietic cells were evident in four pigs aged from 11 days to nine weeks, distributed in diffuse groups containing two to 15 cells in each. They appeared to be absent from the livers of the remaining 11 pigs. One pig, two weeks of age, had discrete vacuoles of fat in the liver cells diffusely throughout the tissue.

Spleen—The spleen of the 27-hour-old pig was devoid of white pulp but hemopoiesis was active throughout the red pulp. Groups of normoblasts were not apparent but individual normoblasts were spread throughout. One pig aged one week and three pigs aged two weeks had active hemopiesis in the red pulp and had detectable white pulp (Figure 1). Normoblasts were present in groups. The cells making up the white pulp appeared to be large lymphocytes and reticulum cells. Hemopoiesis was not evident at four weeks or thereafter. At five weeks there was a suggestion of germinal center formation in that primary follicles were vaguely formed and the centers of some were pale. Obvious secondary follicles were not present until eleven weeks. In two pigs eleven weeks of age and one twelve-week-old pig secondary follicles in the splenic white pulp were numerous (Figure 2). However, the total amount of white pulp was only slightly greater in the twelve-week old pigs compared to those aged four to nine weeks. Plasma cells were not apparent in any spleens examined.

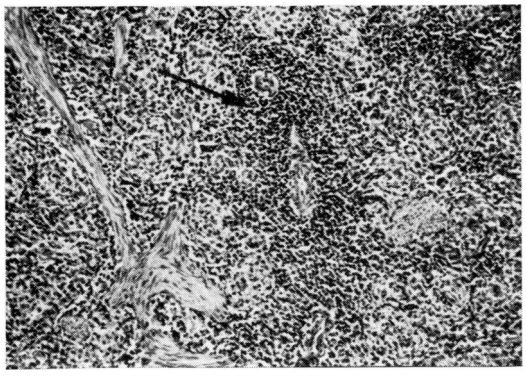

FIGURE 1. Spleen of one-week-old pig 7-5-C. A thin rim of white pulp surrounds the arteries in the center and to the upper right.

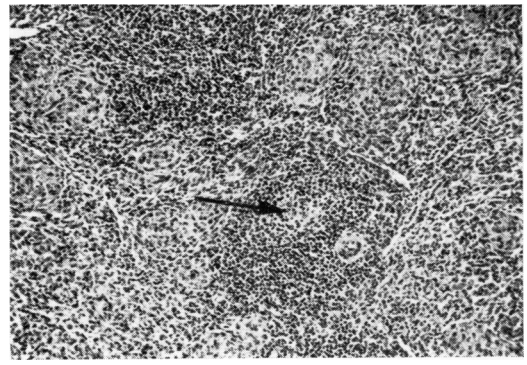

FIGURE 2. Spleen of eleven-week-old pig 12-2-G. The white pulp in the center contains a pale area to the right of the artery. The pale area represents early secondary follicle formation.

Mesenteric lymph nodes—There was very little variation in the mesenteric lymph nodes of the eleven pigs examined. The one-week-old pig had several primitive, sparsely populated germinal centers. The cortex of the lymph node of the eleven-week-old pigs had more but essentially similar germinal centers. The medulla was sparsely populated in all the nodes examined. In most pigs there were numerous neutrophils and eosinophils in the medulla of the lymph nodes. Many eosinophils were not heavily granulated and had round nuclei. Plasma cells were not evident in any of the lymph nodes.

Thymus—In the cortex of the two-week-old pig's thymus there was an outer zone of uniform large round nuclei resembling lymphoblast nuclei. Inside this zone there was more variation in cell type between large and small dark lymphocytes. In the cortex of the thymus of four pigs five weeks of age, there was considerable variation in the relative numbers of large and small lymphocytes. Little variation occurred between six and 12 weeks; the cortices were composed of several-sized cells with the medium size predominating.

The medulla of the thymus varied as did the cortex from pig to pig. If the cortex varied greatly in cell type so did the medulla; whereas, if large uniform cells predominated in the cortex, then large uniform cells predominated in the medulla. Within this variation the medullary cells resembled histiocytes whereas the cortical cells resembled lymphocytes.

Duodenum—There were no appreciable morphological changes in the duodenum of 14 germfree pigs ranging in ages from one to 12 weeks. Goblet cells were present at one week and were almost as numerous as in the twelve-week-old pigs. Brunner's glands were normal. Mature plasma cells were not evident in the lamina propria of four pigs two weeks and under, but were present in very small numbers in the pigs five and six weeks of age. At eight weeks and above, mature plasma cells were easily found but were not numerous. One

eleven-week-old pig had an active Peyer's patch in the duodenum.

Jejunum-Ileum—Sections of jejunum and/or ileum were examined from twenty pigs aged twenty-seven hours to twelve weeks. Peyer's patches were present in the twenty-seven-hour pig but the lymph tissue was diffuse and rather sparse within the patches. At two and four weeks the Peyer's patches were larger and cellular activity was indicated by increased density of cells and by the presence of large blast-type cells in areas suggestive of early germinal centres (Figure 3). Mature plasma

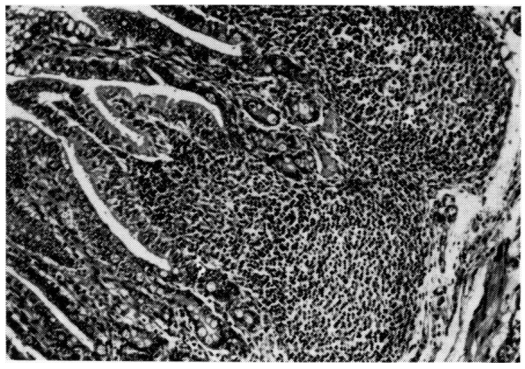

FIGURE 3. Small intestine of five-week-old pig 10-2-E. Note the pale centers in the lymph follicles of a Peyer's patch.

cells were not evident in the lamina propria until four weeks and then only very few were present. Eosinophils were numerous both in the lamina propria and in Peyer's patches. As in the lymph nodes, these were variable in appearance. The epithelial cells in many sections of this region of the intestine were extremely vacuolated especially toward the tips of villi (Figure 4). The vacuolation was still marked in pigs seven weeks of age but gradually reduced with age; however it was apparent at eleven weeks. Germinal centers were quite discrete in Peyer's patches of pigs from five to 12 weeks of age. There was considerable variation in the activity of the Peyer's patches and the numbers and sizes of germinal centers from pig to pig. Plasma cells were easily found but were not numerous in pigs eight weeks and over.

Large intestine—The degree of cellularity of the lamina propria was quite variable in all age groups and even in different areas of the large intestine of the same pig. In some pigs there appeared to be a complete lack of reticuloendothelial cells in the lamina propria, whereas others had a diffuse scattering of such cells both in the lamina propria and in the sub-

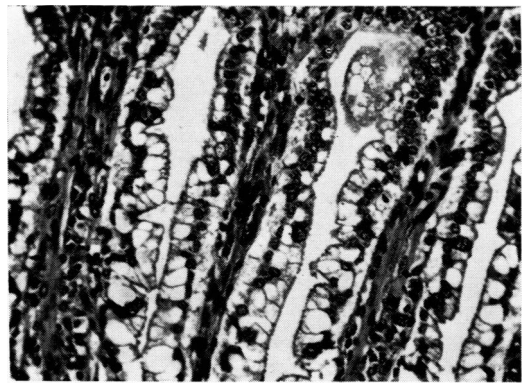

FIGURE 4. Small intestine of five-week-old pig 12-3-B. Note the vacuoles in the cytoplasm of the lining epithelial cells.

mucosa. Lymphoid follicles were present usually in the submucosa but often extended into the lamina propria. Germinal centers were not seen. About half the pigs starting at two weeks of age had a golden-colored pigment in macrophages in the lamina propria. The pigment was present in variable quantities and in some pigs was very pronounced. A material of similar color was often present in the lumenal content.

Lung—Evidence of inflammation was not present in any of the lung slides examined. In addition, lymph follicles were not present around any of the air passages or blood vessels. In most lung slides large bronchi and smaller bronchioles were represented.

Other tissues—Adrenal glands from nine pigs and parts of the central nervous system of five pigs were examined and all were normal. Small areas of the turbinate bones were sectioned from thirteen pigs and lymph tissue was not evident in any. Numerous large osteoclasts were present in the turbinates of most of the pigs of all ages.

DISCUSSION

Hysterectomy has provided an efficient and reliable means for procuring viable and healthy germfree piglets. The disadvantage of hysterectomy as compared to the more refined and time-consuming closed hysterotomy technique (4, 8, 12) is that the sow has to be sacrificed. However difficulties associated with anesthesia and aftercare of the dam following hysterotomy often make the latter impractical as a survival operation (12). Development of a suitable general anesthesia would be an important contribution in this field.

The methods of sterilization of the equipment that were employed and the feeding of canned feed, made possible the rearing of germfree pigs without the use of a vacuum autoclave and proved to be simple and practical. The type of feed that was fed, had the limitation that it was comparable to the diets consumed by conventional pigs only until weaning, e.g. a maximum of eight weeks of age. This factor along with the size and design of the isolators tend to restrict the application of the methods which have been described to work with young animals, particularly in the case of comparative studies in digestion and nutrition. At the present time the establishment and maintenance of germfree pig colonies which would reproduce in isolation appears to be technically difficult and impractical.

Even though the tests for microbial status covered a wide range of organisms they do not prove conclusively that no infectious agents were present. With regard to viruses, the proportion that grow in porcine kidney tissue cells and produce observable cytopathic effects is relatively small, and, in the present studies, those that would not do so would not have been detected. Again, the tests for bacteria employed would not have detected all bacteria, nor would the media used for culture of mycoplasma have supported the growth of all mycoplasma, some of which are notoriously fastidious in their requirements. In spite of these limitations, the fact that the results obtained with all except one of the pigs were negative, suggests that the pigs were indeed germfree. Further confirmatory evidence of this was that the pungent odor normally associated with pigs was absent in the isolators, that dead pigs did not decompose when left in the isolator, and that milk contained in cans which were left open in the isolators for long periods of time appeared normal in appearance, taste and smell.

If the pigs obtained and reared by these techniques are indeed free of infectious agents, they have a unique potential value to research, particularly in the study of infections with single agents in organs which normally harbor a variety of organisms, for example, the intestines, skin and respiratory tract.

The germfree pigs were comparable to other gnotobiotic animals in that their gamma globulin levels were much lower than those of their conventionally reared counterparts (15). In the gnotobiotic rat and the mouse there is a variation in the gamma globulin levels according to the diet fed (16). This parameter has not been studied in the pig. Aside from antigens which the feed probably contained, there were dead bacterial cells, as detected by direct microscopy. Presence of the antigenic material apparently stimulated the development of lymphoid tissue associated with the digestive tract, but was insufficient to cause an increase in the circulating gamma globulin levels between one and 12 weeks of age of the germfree pig. The relatively constant gamma globulin levels, the fact that the germfree pig is immunologically competent very early in its life (3) and a body size permitting frequent collections of blood, should make it a useful animal for immunological studies.

Variation in the histological features of the same tissue between individual pigs, even of the same age, was quite apparent. For example, the germinal centers varied in activity in the spleen and Peyer's patches even in one age group and the cellularity of the intestinal lamina propria was similarly variable. Within this variation a definite progression was apparent in the formation of white pulp in the spleen. Lymph nodes, on the other hand did not seem to change significantly with age. The fact that plasma cells were not found in the spleen and lymph nodes but were present in the lamina propria of the intestine is not easily explained. If germinal centers were present in the spleen and lymph nodes, the formation of some plasma cells would be expected. Antigens in the feed may account for the presence and activity of lymph follicles in the intestine. Such antigens may also account for the follicles in the spleen and lymph nodes. Although originally denied (9), the presence of lymph follicles in the spleens of some gnotobiotic animals is now accepted (11).

The significance of lymph follicles in the lung is argumentative and even after an extensive review of the subject, Jericho (2) was not prepared to state whether their presence in the lungs of pigs was normal or pathological. None were found in the lungs of the germfree pigs although the amount of tissue examined was not sufficient to make a conclusive statement in this regard. Probably lymph follicles in lungs are the result of irritation or antigenic stimulation and are non specific in nature.

To the authors' knowledge, this is the first detailed description of the histology of the lymphatic tissues of gnotobiotic pigs. Personal observation (R.T.) indicates that gnotobiotic pigs are similar to other gnotobiotic species (6, 11) in having reduced activity and volume of lymph tissue as compared to their conventionally reared counterparts. In addition, the cellularity of the intestinal lamina propria is much reduced in gnotobiotic pigs compared to conventional pigs. The description of the

pig's intestinal tract by Sloss (10) was for 125-lb pigs and the only variation with age recorded was that the amount of lymphoid tissue increased with age. The vacuolation of intestinal epithelial cells which was observed in the gnotobiotic pigs has not been described in conventional pigs nor in germfree animals of other species (6).

Summary

A method for procuring, rearing and testing of gnotobiotic pigs and the histology of their lymphoid tissue is described.

The animals were obtained by hysterectomy and kept in isolators up to 12 weeks of age. In all but one pig, no bacteria, fungi, mycoplasma or viruses were detected. The gamma globulin levels were significantly lower than those of conventional pigs of similar ages, and did not increase until at least 12 weeks of age.

There were variations in histological features of tissues including the lymph tissues between individual germfree pigs, even of the same age. Within this variation a definite progression with age was apparent in the formation of white pulp in the spleen but not in the lymph nodes. Plasma cells were not seen in these organs, but some were present in the lamina propria of the intestine. Lymph follicles were not observed in the lungs.

The possible applications of these animals for research purposes have been discussed.

Résumé

Les auteurs décrivent une méthode pour obtenir, élever et évaluer des porcs gnotobiotiques. Ils décrivent également l'histologie de leur tissu lymphoïde.

On obtint les animaux en pratiquant une hystérectomie et ils furent gardés dans des isolateurs jusqu'à l'âge de 12 semaines. On ne trouva de bactérie, fungus, mycoplasme ou virus que chez un seul porcelet. Les taux de gamma-globuline des porcs ainsi obtenus étaient sensiblement inférieurs à ceux qu'on recontrait chez d'autres porcs de même âge. En outre, ces taux demeuraient stationnaires, du moins jusqu'à l'âge de 12 semaines.

On nota des différences dans les caractéristiques histologiques des tissus, y compris le tissu lymphatique, des porcs gnotobiotiques, même pour des sujets du même âge. On observa une progression marquée, en rapport avec l'âge, dans la formation de la pulpe blanche de la rate, mais non au niveau des ganglions lymphatiques. On ne décela aucune cellule plasmatique dans ces organes, même si on en identifia dans la lamina propria de l'intestin. On n'observe pas de follicule lymphoïde au niveau des poumons.

Les auteurs envisagent l'utilisation éventuelle de ces animaux à des fins de recherches.

Acknowledgments

We are pleased to acknowledge the conscientious work of Mr. D. Jol, who took care of the pigs, Mrs. P. Brown who prepared the tissue cultures and carried out the microbiological tests and Mrs. J. Boyle for preparing the histological slides. We wish to thank Drs. E. H. Bohl, E. M. Kohler, and R. D. Henthorne for their help and advice. The photographs were provided by the Audio Visual Services Department of the University of Guelph. The work was supported by a grant from the Canadian Medical Research Council.

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BOOK REVIEWS

The Biology of Animal Viruses, Volume I: Molecular and Cellular Biology. Frank Fenner. Published by Academic Press, New York. 1968. 474 pages. Price $18.50.

This two volume text was initially intended to be a revision of the well-known "Principles of Animal Virology" by F. M. Burnet. However, the tremendous advances in this field since 1960, as well as the author's differences in emphasis, necessitated an entirely new book. In the preface to Volume I, the stated aim is to deal "in a comprehensive way with the broader biological principles of animal virology". The designated audience is the research worker, the teacher and the graduate student in the areas of animal virology and molecular biology. The subject is limited to the viruses of warm-blooded vertebrates. Volume I deals with characteristics of these viruses and their interactions with animal cells. Volume II will deal with the interactions of viruses and the host animals.

In Volume I the current classification, morphology and chemical composition of viruses is well-documented in the first three chapters. Then, a review of the structure and function of animal cells is followed by four chapters on virus-cell interactions. The related areas of viral genetics, mixed infections and interferons are covered very adequately in the last three chapters. The pleasing format followed for all topics is the description of a model system to which representative viruses of the twelve virus groups are compared. Where pertinent, new procedures or theories are described. For example, under virus classification, a new group name, Encephalovirus is proposed to replace the name Arbovirus, and justification for this change is documented in detail.

The material is presented in an organized fashion and even those topics that are generally difficult to explain are handled skillfully in simple terms. The book is printed on good quality paper with excellent illustrations, charts and figures. Errors in editing are very infrequent. The comprehensive list of over 2,500 references effectively gives entrance to the scientific literature. This book meets the stated aim of the author and will be very useful to anyone interested in animal virology or molecular biology. *J. R. Saunders.*

Otters—A Study of the Recent Lutrinae. C. J. Harris. Published by Weidenfeld and Nicholson, London, and The Ryerson Press, Toronto, Ontario. 1968. 397 pages. Price $21.

This book, in the World Naturalist series, is highly readable and amply illustrated, and deserves a place on the bookshelf of anyone with any interest in or connection with otters, either in the wild state or in captivity.

For the veterinarian, zoologist, and naturalist —or other interested amateur or professional— it offers a very complete description of the various species of Otter, a simple key to their classification, their occurrence in nature, feeding habits, etc.

For anyone interested in breeding otters in captivity, the chapter dealing with this subject is quite thorough and detailed, including the probable pitfalls, proven diets, construction and location of cages, and much other pertinent information.

This book is, in the main, a compilation of most of the available material published on the otter, containing many direct quotations, and with a full and detailed list of references. But as well, it presents the personal experience of the author who, as one of the few to have bred otters in captivity, is well qualified to speak on the subject and does so in an entertaining and often amusing manner. *E.R.F.*