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GERMFREE RESEARCH:

A BASIC STUDY IN HOST-CONTAMINANT RELATIONSHIP

I. *General and Theoretical Aspects of the Problem*

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The subject of host-contaminant relationship has many ramifications, which may be focussed where the host and its contaminants first meet. Such a point of view assumes that host and contaminants can be recognized as distinct biologic entities and their association as a dual biologic system is accidental.

If the focal point of discussion is the first meeting of host and contaminants, then it is possible to project a plan of investigation within the framework of a thesis. This thesis is that the pure culture concept may be extended to the animal and on this basis applied to problems in biology and medicine of which the host-contaminant relationship is one. Such an approach is long-range, basic in nature, and exploratory by intent but with advantages of logic and continuity. It presumes that the animal may live as a pure culture free from contaminants.

The conventional animal, after birth, may be considered as a dual biologic system consisting of host (animal) and contaminants (bacteria, worms, ectoparasites, viruses, protozoa, etc.). The investigator may describe this dual system as such and he may, for experimental purposes, add or subtract one or another contaminant, but he has no control as far as contamination in a general sense is concerned. Thus, the conventional animal at any point in its life is the product of continuing accidental microbial experience. As an experienced host, therefore, the conventional animal does not satisfy the demands of the pure culture concept. Unless some logic other than this concept is accepted as the basis for an experimental approach to the problem of host-contaminant relationship, increasing dependency must be placed on the naturalistic approach which is essentially descriptive. The natural approach is foreign to the experimental approach which is essentially artificial, deals with but a single variable, and depends on models to examine or create a phenomenon with the ultimate aim of controlling it to whatever ends are desired.

If the pure culture concept is to be extended to the problems which face us with respect to the host-contaminant relationship, the "host" must be reared free of contaminants, after which they may be brought into association with each other as pure units. Thus, the degree of experimental control necessary to the broad thesis is satisfied at least in theory. In practice, if the host is to be freed from its contaminants, this must be done at the most propitious stage in its life cycle and subsequently reared so as to prevent recontamination.

The host and its contaminants may become associated either 1) before or 2) after

birth or hatching.

1. *Contamination of the host before birth:* The animal, as such, exists first in the fertilized ovum which develops into an embryo and later into the entity capable of an independent life. The period of development prior to birth is one of preparation for independent life during which the animal is endowed with the cells, tissues, organs and systems necessary for carrying on this life and for meeting environmental threats of which microbic contamination is one. It is quite possible that a contaminant may exist as part of the male and female gamete, or at least in this environment in which the gametes develop and in which the fertilized cell grows toward independent life at birth. If this is the situation, then a high degree of synergism must exist; otherwise the association would be reflected adversely during development of the animal. It is, of course, entirely possible that a contaminant may exist as a true symbiont necessary to the economy of the gametes or the developing embryo. This will depend on how the term contaminant is defined and what connotations are implied. If a contaminant is taken to mean life other than that produced by the host protoplasm, the problem is somewhat simplified since there now remains only the problem of identifying this suspected life entity. Entering into this definition is the possibility that genes may act in a foreign cell to its detriment, i.e., changing the course of its development and acting as a virus. These things may call for new definitions; but, if true, such definitions must wait new evidence. On the other hand, viruses may exist as contaminants in the environment of the gametes and remain quiescent until such a time as they can become active in the embryo or animal. Finally, the probability exists that contaminants may pass through the mammalian placenta, or into the egg from the genital tract, and hence grow in the embryo without affecting it, or develop at a later date in the newborn young.

The animal must develop systems and mechanisms during the embryonic period to resist or adapt to contamination at birth and as a fertilized cell these systems may not be present. Moreover, virologists have taken advantage of the embryo to grow viruses in pure culture without contamination. The fact of the matter is that most normal animals develop toward independent life in a protected environment. It would seem illogical that such shielding would not exist while the animal is assuming identity and developing the necessary means for conducting independent existence. As an embryo, it is certainly not capable of meeting the threat of the external environment, much less carrying on independently in this environment. The normal animal at birth shows little detrimental effect due to contamination in the prenatal state. If a contaminant exists, it does not grow in competition with the healthy embryo.

Thus, even though there is a possibility that this animal and contaminants may meet before birth, there are still two separate entities involved and so it should be possible to separate one from the other.

2. *Contamination of the host after birth:* The second point at which the animal and contaminant may meet is at or shortly after birth or hatching. This is fairly evident for the external environment contains varieties of living forms, many of which are capable of growing in or on the animal. Moreover, experience has shown that the animal after birth becomes a host for contamination without exception. These contaminants are in the external environment, not in the animal before birth.

If the two possible points of contamination are considered, it is clear that any start toward separation of the animal from the contaminants must be made at that point where the animal is capable of independent life, i.e., near or at birth. It is possible to eliminate contaminants from the external environment and, if necessary, to devote attention to the elimination of internal contaminants when they can be demonstrated. Unless the threat of contamination at birth is eliminated, the latter problem is impossible of solution if the animal, and not the embryo, is the experimental unit. There is one other possible path to follow and that is to eliminate the contaminants at some point in the life of the animal after birth and after it is conventionally contaminated, but a little consideration will reveal the manifest

difficulties of this situation. Not only must the contaminants within the animal be eliminated but also those in the environment as well. Moreover, this approach complicates the problem tremendously with respect to the possible contaminants brought in with the germ cells, or added to the animal from its mother during intra-uterine life or from the egg during external incubation of the oviparous embryo.

It is the animal taken at or shortly before birth and reared in a sterile environment which we presently call "germfree"—a term which has the sanction of usage. We define a germfree animal as one which is free from all life other than that produced by its own protoplasm within the limitations of available methods to detect living forms. This animal may be considered free from bacteria, yeasts, molds, Rickettsiae, viruses, protozoa, worms and ectoparasites. Further, we mean that not only is the animal free of these contaminants so far as we know, but so is the environment in which it passes its life and goes into successive generations. We also refer to a germfree animal as "pure animal" when it is used experimentally. Thus, for the purposes of the host-contaminant problem, we have available a host which will answer the requirements of the pure culture concept and an experimental approach is open on this basis. The germfree animal may be studied as a pure animal or a pure culture of animals, or it may be brought into association experimentally with other pure cultures (microbes, etc.) in order to explain some phase of the broad problem of host-contaminant relationship.

Obtaining germfree animals is one thing, but rearing them germfree through successive generations depends on the proper apparatus and techniques. Such apparatus will not satisfy the main thesis if it is designed to merely contain a germfree animal, e.g., the hatching of a chicken in a jar. The problem of design is more basic than this and broader in implication. The basis lies in recognizing the philosophy of isolation which is a corollary of the pure culture concept. According to this philosophy, an isolate must be contained within neutral barriers and the space they enclose be expanded or contracted as needed. The environment so formed must be freed from all contaminating factors. For example, while the wall of a test tube might be considered as a barrier and the pure culture the isolate, the passage from tube to tube of the isolate, no matter how careful the technique, results in a break in the barriers because the isolate is brought into contact with a contaminated environment even though for a brief interval. This admits to possible contamination.

The design of the Reyniers Germfree System meets the requirement of the basic design because it permits complete control of the environment. The individual units (Germfree Cages) can be freed of all living contaminants by sterilization with steam under pressure. Food and water can be taken into the unit or debris removed by sterilization in situ. Air can be sterilized or filtered by devices which are a part of the unit and objects can be handled within the unit through flexible gloves or mechanical devices which are an integral part of the wall. Units can be combined in any usable numbers so that one unit can be brought to the same degree of environmental control as an adjacent unit to which it is attached, thus making it unnecessary to expose an isolated object to the external environment during a transfer or at any other time. In short, the units comprise a system for exact control of the environment whether that be microorganisms, dust, gas composition, pressure, temperature or humidity.

While we are here concerned with rearing germfree animals, it should be remarked that the system is also being used to contain dangerous pathogens in air-borne and other studies as well as for machining objects in an atmosphere free of oxygen, or running chemical studies during which manipulation is necessary in an atmosphere of known composition, pressure and humidity. In short, it is possible with this system to exactly and conveniently control the environment to a degree consistent with the experimental demands. While the shape and size of these units may change in time for convenience or some other purpose, we believe the system is adequate in principle for the purpose for which it is intended and will remain so because the principles of design around which it has been built are consistent with

the basic theories of isolation and the concept of pure culture.

Quite apart from the specific questions that might be asked about the host-contaminant relationship, and they are many, it is necessary, in our judgment, first to study the animal free from contaminants through successive generations even before it is used for experimental purposes. There are many reasons for this but the main reason is that we know scarcely anything about life under these conditions. It is necessary to establish a base line against which the action of a contaminant can be measured. Therefore, in 1930 this approach was set down as part of the long range program in Lobund Institute, contingent, of course, on being able to obtain and rear animals free from contaminants and on the development of a suitable system for controlling the environment in which this could be done with certainty. In essence this phase of the program consists in describing the germfree animal morphologically, biochemically, physiologically, and serologically, assuming of course the necessary degree of standardization with respect to health of the animal. Unless this is done experience has shown us that many mistakes will be made when the animal is used as a tool to answer specific questions about some phase of the broad problem.

If we pursue this line of thought further in relation to the problem set before us, two directions are possible: 1) backward, so to speak, in an attempt to discover and study host-contaminant relationships arising from possible prenatal contamination; and 2) forward, on the assumption that the animal contains no contaminants at all and, therefore, has no experience with them.

1. If we work backwards into the problem, there are several approaches — one through genetics, another through observations over the entire life of the animal and its progeny. There is also the direct approach in which an attempt is made to break apart any symbiotic relationships by placing stress on the animal through the use of radiation, carcinogens, etc., or to deliberately contaminate it with a known virus.

2. If we work forward in the problem on the assumption that the animal is free from contaminants at birth and after, the approaches are just as varied. The problems of nutrition and of the biochemistry involved are of considerable interest inasmuch as there is no intestinal flora to complicate the study. Quite apart from the more specific question of "intestinal synthesis" lies the possibility of studying many other things such as transport systems for chemical groups. The nutrition of the pure animal may be studied for the first time since it cannot be so investigated as a contaminated system. There are also the problems of longevity, resistance to non-viable contaminants, the many problems of physiology, inflammation, and tissue repair, to name only a few.

But quite apart from these demands is the need to establish a healthy germfree animal brought to a standard which makes it possible to use it as an experimental tool in direct approaches to specific problems. The conventional animal can never be brought to the same degree of standardization, nor can it serve in the same areas as the germfree animal with the same degree of certainty. This is so because the conventional animal cannot fill the requirements of the pure culture concept and is always a contaminated or dual biologic system in which the host through its experience with uncontrolled contaminants, presents activated systems with respect to contaminant association. Thus, we must always start in this study with the association already underway and never with a host system not activated, or ready to be experimentally activated, as is the situation with a germfree animal per se, i.e., to describe its natural history as fundamental to any challenge which might be submitted to it and always relating the study to the healthy, standard, normal, germfree individual. The pressure to apply these animals as tools in specific studies has made it difficult to adhere to these basic studies on the animal itself. Nevertheless, we think the study of the germfree animal per se is essential to the main problem of host-contaminant relationship if we are properly to evaluate experimentally induced host-contaminant associations.

Having considered briefly the need for studying the germfree animal as a pure sys-

tem, we now pass to the problem of bringing it into association with pure contaminants. The concept is simple. What we are trying to explain is the situation which exists in the normal conventional animal and this serves as a point of reference. Since the conventional animal represents an extremely complex situation, it is theoretically necessary first to describe it. This can be done in some instances with sufficient clarity as in the case of disease or the production of antibodies specific to a known antigen, but there are areas, e.g., intestinal synthesis, aging, etc. which are not so clearly describable. At any rate, experimental theory permits the factors involved to be analyzed and described before recombination under controlled conditions until the original complex is re-established. In addition, the procedure mentioned also allows synthesis of new situations not leading to a reconstitution of the natural complex, but this is another path.

In the use of the germfree animal, there is a tendency to consider the conventional animal as a control, in other words, to compare the conventional animal to the germfree animal. In some instances this is possible but in the matter of introducing a pure culture to the germfree animal the proper control is the germfree animal unassociated with a contaminant. This may seem like a minor point but again experience has shown the need for emphasizing it. The conventional animal is at any time the result of an indiscriminate experience with contaminants, whereas the germfree animal has no such experience and when a pure culture is introduced to it the effect is not experimentally the same. It is obvious that basic biologic reactions and patterns are alike in all instances, because the host, whether it is reared germfree or in a conventional manner, is endowed with the same systems and responses; it is just that in the germfree host some systems have not been activated and some responses not awakened.

The germfree animal does not show antibodies against bacteria, which are the commonest contaminants, unless it is brought into contact with bacteria. When a pure culture of bacteria is introduced, the response is specific and there are also definite tissue responses to the association. Using the germfree animal and knowing that it does not have antibodies against bacteria of any kind, that the lymphatic tissue is in general reduced, and that the wandering lymphocytes are not trained, it is possible to elicit controlled activation to the degree necessary for a step by step study of the general phenomena underlying these activities. This is not possible with the conventional animal simply because activation has started at birth.

If we attempt to associate the specific responses brought about by introducing a pure culture of bacteria into the germfree animal to resistance against infection, or to other general biologic phenomena such as aging, experiments may be set up step by step with a degree of certainty. Or, on the other hand, if the interest is in tumors or some other pathologic process, these phenomena may be studied with the knowledge that we are not confusing the picture through external contaminants which, even though they may not be specifically involved, do have an effect on the host which effect, in turn, may be related to the problem and confuse the interpretation of results.

We have tried herein to present a broad and basic approach to the host-contaminant relationship. By extending the pure culture concept to higher animals, by proper instrumentation of the principle of isolation as it involves the control of environment, and by studying the germfree animal in many species and through many generations in the absence of contaminants, it is possible systematically to set up a program for the study of host-contaminant relationship as this may be manifested both in basic and applied problems. It is obvious that in this limited space, and considering that this work has been going on since 1928, only a fragment of the results can be brought to your attention, but they will indicate the planning of the long-range program and approach. The host-contaminant relationship with its many manifestations in basic biology and medicine has a focus and that is at the point where the host and contaminants first come into contact with each other. The problem is basically that of rearing the host germfree. The methodology has been worked out and is operating. The road from this point is well marked but long.

II. *Serologic Observations in Germfree Animals**

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The production of animals which can be reared and maintained in the germfree state[1-3] has presented the opportunity by which many observations regarding host-contaminant relationships can be made. This paper deals with some of the serologic reactions that have been observed in germfree and conventional laboratory-reared animals.

The existence of higher animal life in the germfree state poses the question as to whether antibody exists in animals reared devoid of microbial life. The literature is replete with reports that the serum of normal (non-injected and non-clinically infected) animals contains so-called "natural" antibodies against a variety of bacteria. These "natural" antibodies are demonstrable with bacteria which can be isolated from the animals' own digestive tract as well as with organisms which in many cases can not be demonstrated in the gastrointestinal tract nor in the environment at the time isolation procedures are attempted.

Proponents of the Hirszfeld[4] school propose that such antibody is physiologic, arising naturally through a postnatal gradual physiologic ripening of the serum proteins and is thus independent of any external stimulus. This "serogenesis" viewpoint includes the assumption that "natural" antibacterial bodies arise during the normal production of serum proteins simply through a fortuitous chemical affinity between the normally produced serum protein and certain bacterial antigens.

Others propose that this type of antibody is not natural but rather has been produced in response to external stimulation, either through contact with the specific bacterium in question or through contact with a closely related organism or antigenic substance. The external stimulation view does not necessarily negate the accidental chemical affinity idea as one means of producing antibacterial antibody since it is at least possible that some antibody may be produced in this way. However, it is generally believed that the usual way in which such antibacterial antibody arises is through some external antigenic stimulation.

Considering now the germfree animal which has spent its entire life free from viable microbial associates, it was of interest to determine whether such animals actually do possess antibacterial antibody in the absence of any prescribed procedure, such as parenteral injection, which might stimulate antibody production. Thus, if the antibacterial antibody found in the serum of conventional animals is truly a "natural" antibody, it should also appear in the germfree animal independent of any external stimulus. However, if these so-called "natural" antibodies are in reality induced via external stimuli, they probably should fail to arise in the germfree animal.

Normal conventionally-reared animals are also known to produce hemagglutinins and hemolysins reacting with certain foreign species' erythrocytes. Again there exists some controversy as to whether such hemagglutinin is physiologic or induced. Most investigators accept the theory that heterohemagglutinins and isohemagglutinins are developed under genetic control as a gradual physiologic ripening of the serum proteins and that external antigenic stimuli are not necessary in this case for their development.

However, Wiener[5] has expressed the idea that the alpha and beta isohemagglutinins in normal human blood serum arise via

* The data reported herein will be presented in greater detail in Lobund Reports No. 3 under the title "A Survey of Germfree Animals: I. The White Wyandotte Bantam and White Leghorn Chicken" by James A. Reyniers, Philip C. Trexler, Robert F. Ervin, Morris Wagner, Thomas D. Luckey and Helmut A. Gordon. These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Notre Dame, NR, 131-067.

stimulation from the external milieu by exposure to organisms, food or other materials containing compounds identical or similar to the human blood group substances A and B. If this is true, then certainly heterohemagglutinins could also arise through some similar process, e.g., horses immunized with Type XIV pneumococci give rise to anti-human RBC antibody.[6]

With regard to such heterohemagglutinins, we should be able to predict that if such antibodies are truly physiologic, then the presence or absence of viable bacteria per se in an animal should not influence formation of antibodies of this type unless the germfree state itself were to leave the animal in a serologically immature or underdeveloped state.

It should be noted that unless otherwise indicated, the following results were obtained using sera from normal chickens which were not injected or otherwise purposely treated in any way with antigenic substances. Comparisons are made between chicks raised germfree in Reyniers Germfree System and chickens reared by conventional laboratory brooder-room methods. Both germfree and conventional chicks were fed the same autoclaved semi-synthetic diet.

"Natural" Heterohemagglutinins for Rabbit Erythrocytes

Sera from chicks ranging in age from 0 to fifteen days failed to agglutinate rabbit erythrocytes. However, at the age of thirty days and older, both germfree and conventional chick sera agglutinated these erythrocytes to titers ranging from 1:4 to 1:64. There was no significant difference in titers observed in germfree vs. conventional chicks at comparable ages ranging up to approximately one year. A slight increase in titer was observed in both groups with increase in age.

The negative reaction in the young age groups was expected since it is well known that very young animals either fail to produce antibody or at best give poor responses even when treated parenterally with antigenic substances. The presence of the heterohemagglutinins in the thirty day and older germfree chicks would indicate that at least *viable* microorganisms are not responsible for heterohemagglutinin production. The question as to whether such antibody arose through "serogenesis" or by stimulation with some unknown dietary substance is still unanswered. However, the ability to eliminate the influence of viable microorganisms by use of germfree techniques offers a valuable tool for the future study of the possible role of external stimuli in the origin of such antibody.

"Natural" Antibacterial Agglutinins in Germfree and Conventional Chickens

Paracolobactrum Aerogenoides Test Tube Antigen: This organism was isolated from the feces of a conventional laboratory-reared chick and represents one of the more predominant bacteria in the digestive tract.

Young conventional chick sera failed to agglutinate this antigen in the test tube. However, conventional chicks ranging in age from thirty days to approximately one year produced titers ranging from 1:4 to 1:128. There was a gradual increase in titers observed with an increase in age.

The germfree chicks failed to agglutinate P. aerogenoides at all ages up to approximately one year.

Similar data were also obtained with a chicken strain of Escherichia coli. These results obtained with the paracolon and coliform organisms offer evidence that the so-called "natural" antibacterial antibodies are not natural but are produced as a result of exposure to external stimuli.

Micrococcus Epidermidis Test Tube Antigen: Conventional chicks 0 to fifteen days of age failed to agglutinate this organism. However at ages of thirty days to approximately one year, titers ranging from 1:4 to 1:64 were observed. A gradual increase in titer occurred with an increase in age.

The germfree chicks failed to agglutinate M. epidermidis in ages up to 170 days. However germfree chicks at the over-200-day-age level did show positive agglutination at 1:8 titer.

While M. epidermidis can be isolated from the feces of conventional chicks (which in itself might account for the presence of agglutinins in the conventional sera) it was also found that this same organism is one of the more predominant bacterial forms

that can be isolated from the diet prior to sterilization. The autoclaving of the diet fed the germfree birds destroys the viability of the bacteria intrinsic to the ingredients that make it up, as well as those introduced through handling during diet preparation. However, loss of viability after autoclaving does not preclude retention of antigenicity. The delayed appearance of anti-Micrococcus epidermidis agglutinins is probably a function of low concentration of organisms in the diet plus low antigenicity incurred either through the nature of the organism itself or as a result of the vigorous heat treatment during autoclaving.

The failure to demonstrate anti-Paracolobactrum aerogenoides or anti-Escherichia coli agglutinins in germfree chicks up to one year of age was correlated to the failure to demonstrate such organisms in the diet. We have never been able to detect coliform organisms in periodic tests on the preautoclaved ration.

However, the presence of dead M. epidermidis in the diet may account for the production of agglutinins in the older germfree chicks by a process analogous to immunization with oral vaccines.

Antibody Response to Large Numbers of Dietary Organisms: In the case of Micrococcus epidermidis, we were dealing with dietary organisms present in small numbers as adventitious "impurities" of the other dietary ingredients.

The following are isolated observations on chicks fed large numbers of dead bacteria which were purposely incorporated in the ration. The source of bacteria was from "BY-21," a fermentation product of the Commercial Solvents Corporation. It was incorporated in the diet at a 2 per cent level. Direct microscopic examination of the diet showed large numbers of bacteria to be present. The antigen used for testing the presence of agglutinins was prepared from a bacillus species isolated from the BY-21 before autoclaving.

Two germfree chicks receiving large numbers of dead bacteria in the BY-21 diet produced agglutinins to titers of 1:16 and 1:32 against the BY-21 bacillus when observed at ages of forty-two and sixty-nine days respectively (well prior to the 200 day Micrococcus epidermidis agglutinins previously referred to). The anti-E. coli and anti-M. epidermidis titers for these sera were negative. A germfree chick not receiving dietary BY-21 failed to agglutinate the BY-21 antigen at age thirty-nine days.

The Response of Germfree and Conventional Chicks to Parenteral Injection of Antigen

The question is often asked, "How will the germfree animal, which has had no immunologic experience with living microorganisms, react to non-viable antigenic substances when introduced by parenteral injection?" Germfree and conventional chickens were given intravenous injections of Salmonella pullorum bacterin or sterile beef serum.

In this experiment, no attempt was made to produce maximum titers in these animals since it was feared that over-stimulation might overcome or mask initial differences in the two groups. The reactions recorded here were in response to three small intravenous injections given on alternate days.

The germfree and conventional chicks reacted almost identically to parenteral injection, producing average agglutinin titers of 1:128 against the Salmonella pullorum and average precipitin titers of 1:4096 for beef serum. The non-treated germfree and conventional chick sera gave negative reactions for anti-Salmonella pullorum agglutinin and anti-beef serum precipitin. It remains to be seen whether single small dose injections may show more subtle differences. At this stage, it appears that the germfree state has not handicapped the antibody-producing apparatus.

Summing up, it was shown that germfree chickens produce heterohemagglutinins in time and titer quite comparable to conventional chicks. It has also been shown that antibacterial antibody apparently arises in response to external stimuli. Conventional chicks, harboring coliform bacteria as part of the intestinal flora, produce agglutinins which react with these intestinal isolates. Germfree chicks, on the other hand, were not exposed to viable coliforms nor could any evidence be found that they were exposed to dead coliforms in the diet. Germ-

free chicks failed to develop anti-coliform antibody.

That germfree chicks can produce antibacterial agglutinins in response to external stimuli was demonstrated by the fact that Micrococcus epidermidis could be isolated from the diet prior to sterilization and that germfree chicks fed such sterile diets eventually produced agglutinins against this organism. It was also shown with the BY-21 diet that feeding large numbers of dead bacteria shortened the time at which antibacterial antibody could be detected in germfree chicks.

Finally, it was shown that intravenous injection of antigenic substances into germfree and conventional chicks resulted in the production of comparable antibody titers in the two groups.

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III. *Morphologic Characterization of Germfree Life**

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The aim of this study was to characterize the germfree animal with data concerning growth, organologic status, reproduction and other general criteria of health. Subsequently, the role of the lymphocyte was taken up in some morphologic and functional detail and from this viewpoint a comparison was drawn between the germfree and conventional animal.

The materials forming the basis of this report were chickens and rats distributed in groups of mixed sexes, aged from the newborn to one year.

Morphological Description of the Germfree Animal: On first approximation, it can be stated that higher organisms living in the absence of germs do not change in macroscopic appearance. Growth rate, red cell and hemoglobin concentration of the blood are close to normal, or somewhat higher in the germfree. However, this difference never reaches the degree of statistical significance. The germfree animal matures and begins its cycle of reproduction at the same age as, or a little earlier than, the normal conventionals. At present there are rats in Lobund's germfree colony which represent the eighth generation after the initial litter born by cesarean operation from a conventional mother animal. In respect to aging, our observations are only spotty; however, they permit us to state that the germfree animal lives at least as long as the conventionals of the stock colony. Thus, from the viewpoint of general health, the germfree animal appears normal, when measured with

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the yardstick of conventional laboratory standards.

However, a systematic survey of our animals has also revealed the existence of some vitally irrelevant, but biologically essential, differences between the germfree and the conventional. These differences can be briefly summarized. Organs which in normal conditions are in direct contact with a bacterial flora, e.g., the gastrointestinal tract, weigh significantly less in the germfree than in the conventionals (expressed in weight per 100 gm. body weight). This characteristic is also shared by the lymph follicles and nodes which are dispersed in or adjacent to the intestinal canal, such as: the Peyer patches, mesenteric lymph nodes and notably the ileocecal tonsil of the chicken. Other organs of the germfree animal which in the contaminated animal have no flora-contact, show identical weight conditions, duplicating the normal controls. This seems to apply also to the more remote lymphocyte-bearing organs, such as the spleen. Thus, in this comparative study between the germfree and the conventional, there have been established two clearly different groups of organs. One group faces the exterior and has a significantly reduced weight in the germfree; the other, which is harbored in the interior of the organism, shows no difference in the two animal categories.

In an effort to interpret this difference, only partial answers can be offered. It has been established that the lesser weight of the external environment organs of the germfree is not paralleled by dry-weight differences, thus ruling out the possibility of a difference in hydration. Similarly, it has been found that the mass of lymphocytes, though actually reduced in these organs of the germfree animal, could not account for the rather substantial weight-deficit observed. Preliminary evidence shows that certain differences in the amount of connective tissue are mainly responsible for this phenomenon.

At this point an additional detail should be emphasized. One of our previous statements implied that in terms of endocrine organ weights there is no difference between germfree and conventionals. Indeed, all our animals appeared endocrinologically normal. This fact, combined with the reduced weight of some lymphopoietic organs in the germfree, merited special attention, particularly in view of some current theories concerning the relationship between the endocrines and lymphocytic mobilization. Therefore, in order to contribute additional evidence to this question, parallel determinations of adrenal cholesterol and ascorbic acid were run, in order to gauge, presumably, the functional status of the adrenal cortex. The results showed that there was no difference from this viewpoint between these two animal categories. It is believed that this evidence of endocrine stability is an added support in proving the normalcy of the germfree animal.

Another interesting difference between the germfree and the conventional animals concerns the degree of variability in some of their morphologic and functional characteristics. A comparison of the coefficients of variability of organ weights in the two animal categories clearly demonstrates that in most organs of the external environment, the germfree animal shows a lower scattering than the conventional, as exemplified in the case of the small intestine and the ileocecal tonsil. In respect to the organs of the internal environment, there is no difference in scattering between the two groups. At present only a speculative explanation is offered for this evidence. It is plausible that organs unexposed to the flora-stimulus, such as in the germfree, should respond with a higher degree of uniformity. Experimental details of this question are presently under elaboration.

Observations Concerning the Lymphocyte in Germfree Animals: The discussion concerning the role of the lymphocyte in this field of investigation is best introduced by way of the previously mentioned, greatly reduced weight of the ileocecal tonsil in the germfree chicken. For example, between the sixty day old germfree and conventional White Wyandotte Bantam chicken the ratio in the weight of this organ is approximately 1 to 2. With increasing age, this ratio can reach even 1 to 3 or more. Histologic observation has shown that the reduced weight of this organ in the germfree animal is paralleled by a considerable reduction in its lym-

phocyte content. Following this lead the concentration of lymphocytes was determined in various organs where this was feasible and appeared to be of some importance. Our results showed, for example, that the ileocecal tonsil, an organ of the external environment, shows a drastically lower lymphocytic content in the germfree (germfree-conventional ratio 1:10). The spleen, a representative of the organs of internal environment, failed to demonstrate any differences in this respect. The blood, which probably draws its lymphocyte supply from both the organs of external and internal environment, occupies a mid-position between these two extremes (germfree-conventional ratio, 1:2). The combined results unquestionably demonstrate the importance of the bacterial flora in determining the size of the lymphocytic stock of exposed organs.

In an effort to elucidate these findings from the functional viewpoint only preliminary observations can be offered. Actually, a common treatment of both rat and chicken lymphocyte data appears permissible for this purpose because the basic response of these cells to bacterial stimulation always showed an identical pattern in spite of the fact that two such distant and unrelated species were involved.

The first question investigated was the antigenic sensitization of the lymphocyte. Is it possible to demonstrate a parallelism between the spectacular lymphopoietic stimulation of the ileocecal tonsil and a sensitization of the involved lymphocytes against intestinal bacterial antigens? If so, is this sensitization absent in the instances when there is no direct flora-contact, such as is the case in all lymphopoietic centers of the germfree or in the remote internal environment centers of the conventional animal? Using the lymphocytolysis technique of Favour,[1] lymphocytes of various origins have been tested in vitro against a number of intestinal and other antigens. So far, the evidence is not conclusive and therefore only the progress of the experiments is reported herein.

Our work has revealed some more detail in the studies concerning the production and utilization (release and removal) of the lymphocytes by various organs. These experiments were performed mainly in rats and should be regarded as preliminary tests only. The procedure consisted of taking blood samples from the vein of different organs; simultaneously samples were secured from the arterial system. Thus, the concentration of white blood elements was established before and after the blood flowed through the organ. This indicated the positive or negative contribution which occurred in the course of the passage. The values have been corrected for arterio-venous hemoconcentration but not, as yet, for flow. Generally, the procedure was similar to that described by Ambrus and others.[2]

Blood samples taken from the upper, mid and lower small intestine of the conventional rat indicated that lymphocytes were being removed from the blood at all points of this organ. As far as the number of the lymphocytes removed from the blood was concerned, an inverted relationship seemed to exist between this value and the bacterial content of the intestine: i.e., the lower the segment of the intestine, the less lymphocytes were removed from the blood. This finding appears to be somewhat paradoxical if it is accepted that the lymphocyte participates and is used up in the defense against bacteria. However, the paradox is dissolved if it is considered that the blood, as a conveyor of lymphocytes, is gradually relieved by the increasing lymphopoietic resources of the lower gut. Splenic blood showed that this organ, as is well known, is a major source of lymphocytes in the conventional animal.

A test case for the correctness of these data and conclusions is offered by the germfree animal, where 1) no bacteria are present in the intestinal canal, 2) presumably no lymphocytes need to be removed from the blood for defense purposes and finally, 3) the lymphopoietic centers in the lower gut are functioning only at a certain idling speed. The results show the following pattern. The upper part of the small intestine which presumably produces and utilizes only few lymphocytes, affects only little the blood's arterial lymphocytic level during the course of the passage. In the lower portions of the intestine, an increasing amount of lymphocytes was found in the venous blood and it is conjectured that this is the result

of the reduced, but still present, lymphopoietic activity of the lower gut in germfree conditions. The spleen of the germfree animal appears to release significantly fewer lymphocytes than that of the conventional animal. This observation is in apparent contradiction with a previous statement in which it was maintained that in this organ there is no difference in lymphocytic stock between the two animal categories. The explanation of this discrepancy probably is that stock and production of a cell type, in a given organ, do not necessarily go hand in hand.

Thus far, this paper has dealt only with animals that were fully adapted to their bacterial or bacterium-free environment. Some data have been obtained, however, on the changes in lymphopoietic status which accompany the transition from germfree to contaminated life. An illustration of this is the lymphocyte production of the spleen as it was observed in one germfree and in three other ex-germfree rats which were sacrificed one, two and three weeks after a gross bacterial contamination. A complement of the picture is given by the concomitant data concerning one conventional rat. The net difference between the germfree and the conventional, i.e., the initial and the terminal state in this case, is in the threefold production of spleen-lymphocytes. During the three weeks of transition, however, the spleen-lymphocyte output was as follows: 1st week 15-, 2nd week 30-, and 3rd week 7-fold increase over the original germfree value. These data appear to be a good general example of the progress of adaptative phenomena. It is interesting to note that the appearance of antibodies usually coincides with the subsiding of this spectacular lymphocytic response.

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