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PATHOLOGICAL STUDIES OF MALPEQUE DISEASE

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PATHOLOGICAL STUDIES

OF

MALPEQUE DISEASE

A Report on Laboratory Investigations

made for the

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during

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by

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- C O N T E N T S -

PART I: INTRODUCTION.

In May, 1937, I was asked by Dr. A. W. H. Needler, of the Biological board of Canada if I would be willing to co-operate in the study of the "Malpeque disease" among oysters in Prince Edward Island. After receiving a general description of the deseuse, and in view of the fact that it seemed to be an epidemic disease, I agreed to make such laboratory studies as my University duties would permit.

It was then arranged that material should be collected during the summer months when the diseuse is most in evidence and forwarded to my laboratory. Thirty-six bottles containing about one hundred and eighty diseased oysters taken from various areas and preserved for histological study in formalin-acetic-alcohol and Bouin's fluid were brought to my laboratory at the opening of the fall term. In addition, several lots of dead or dying oysters were brought in without preservation, for bacteriological and virus studies.

The work naturally fell into three divisions:

(1) Bacteriological cultures.

(2) Inoculation experiments with bacteria isolated from diseased oysters and Seitz filtrates which might

contain virus. The inoculation experiments were carried on under the supervision of Dr. A. H. Leim at the Atlantic Biological Station at St. Andrews, New Brunswick.

(3) Histological studies, chiefly by the paraffin method and frozen sections.

PART II: BACTERIOLOGICAL STUDIES

Diseased oysters were brought from Prince Edward Island to the laboratory at Sackville as quickly as possible. They were scrubbed clean with a stiff brush to avoid carrying contamination inside when opening. After opening, the area in which lesions occurred was seared with a spatula to avoid confusing the normal external bacterial flora with micro-organisms that might be present within the lesions or tissues. Sterility check cultures were made from the seared areas as a further precaution, and all proved sterile. The lesion was then opened and cultures were made from the contents, either with a sterile swab or with a platinum loop.

For primary isolation, most of the cultures were made on plain agar, Loeffler's blood serum, and plain broth. Thereafter the usual routine methods for identification were followed.

An adequate series of cultures were made from fresh healthy oysters, using the same methods and areas as in the diseased oysters, to use as controls.

The results of the studies on both diseased and healthy oysters are as follows:

Four lots of oysters, diseased and normal, were examined, and a total of 164 primary cultures were made, half of each lot being incubated at 15° to 18° C. and the other half at 37° C. Very little growth was obtained at 37° C.

First Lot, October 20: 108 cultures made from diseased and normal.

Diseased: 21 species of bacteria were present. Many thousands of colonies were examined. All the bacteria present were apparently the usual flora of soil and sea water, but the author does not pretend to know the bacterial flora of sea water very thoroughly. Only one organism which we will call "184" was a repeater, appearing in 8% of the cultures. It proved to be of no pathogenic significance.

Normal: 8 species of bacteria were present. Very few colonies, and they were probably from the outside, for four-fifths of the plates from inside normal tissues were sterile or contained only a colony or two of air sarcinae. "184" was not present.

Second Lot Later: These were normal oysters. 16 plate cultures gave only a total of 7 colonies, representing 4 species, and showing nothing of interest. We may safely say that normal oysters from the beds represented have very few bacteria present, - so much so that I feel that a healthy oyster must possess a

strong phagocytic or bacteriostatic power.

One of the most marked evidences of Malpeque disease is the loss of that bacteriostatic power.

Third Lot: November 16, from Dr. Leim.

Dead: 16 plate cultures were made, 12 from dead oysters and 4 from normals. "184" appeared in only 1 out of the 16.

These oysters died quickly after inoculation with one of the cultures I prepared, but showed no evidence of Malpeque disease or of localized lesions of any sort. They turned a dark even gray in color. Apparently they died from toxic doses of the massive inoculum or from oxygen depletion of the water.

Normals: Nothing of interest, except that sections showed a great increase in phagocytes, but a normal distribution of them.

Fourth Lot, December 10, from Dr. Needler.

Diseased: These were Malpeque oysters from Rustico, showing yellow-green blotch lesions, but no pustules of the sort seen in summer.

24 plate cultures were made, using the lesions only.

12 of the lesions were sterile.

The other twelve gave rather small growth, a total of 15 species being observed.

"184" appeared in 8 (33 1/3%) of the cultures,

but it is apparently a common and widely distributed saprophyte. I have not found it in normal oysters, and it seems to be present only where tissue degeneration has taken place. I do not intend to spend any more time on "184".

I am not satisfied with my section studies of this lot. They were frozen sections, and should have been repeated in permanent preparations, but time did not permit. Let it suffice to say that they did not show any evidence of bacterial or virus infection.

For critical study of the histology of the lesions, particularly of phagocyte mobilizations, they were not satisfactory.

Interpreting the foregoing results, we find that:

(1) There was no bacterium consistently present in the lesions or anywhere else within the tissues.

(2) That while there were "repeaters" in a certain percentage of the cultures from diseased oysters, there was still no definite and coherent evidence of any specific bacterial infection. (This has been also borne out by section studies and by the inoculation experiments)

(3) The bacteria found within the tissues were not obligate parasites, nor could they be identified as pathogens at all.

(4) We feel that from the nature and variety of the organisms found in the lesions and elsewhere in the tissues, that they were merely secondary invaders which had entered

debilitated tissue or in the wake of some degenerative disease.

PART III: INOCULATION EXPERIMENTS

Bacteria

From the "repeaters" in the cultures from diseased oysters, three were selected and massive broth cultures of from 500 c.c. to 1,000 c.c. were made. In addition to these, a similar large broth culture was made by direct inoculation with pustule fluid and pieces of diseased tissue, thus giving a mixed culture of several species.

These cultures were forwarded to Dr. Leim at St. Andrews where, under his supervision, healthy oysters were placed in troughs containing sea water and maintained under optimum conditions. The troughs were divided into inoculation troughs and control troughs. (At this stage of this report, the reader is referred to bibliography references 1 to 4 inclusive).

Inoculations varying from very heavy initial inoculations to smaller inoculations regularly repeated were made.

After one very heavy inoculation, the water grew turbid and deaths began to occur. Samples of these oysters returned to my laboratory for examination did not show any resemblance to Malpeque disease either in their

general appearance, or in bacteriological studies made on them. The deaths were apparently due either to the toxic effect of such heavy inoculation or to oxygen depletion.

Virus.

Seitz filtrates were prepared from diseased oysters as follows:

Entire oysters were run through a sterile meat grinder, and an equal volume of fresh sea water was added and the whole mixture was thoroughly stirred, pressed, and stirred again. The remaining thick and somewhat mucoid liquid was passed through Seitz filters operated by a strong electric air pump. Filtration was very slow and difficult, but a plentiful amount was obtained in several days of constant operation. The filtrate was tested for bacterial sterility and was then transferred to brown glass bottles, sealed, and shipped without delay to Dr. Leim.

All oysters in the inoculation troughs remained perfectly healthy, there being not the slightest evidence of disease resulting from massive inoculation with the filtrate.

PART IV: HISTOLOGICAL STUDIES

To begin with, it must be emphasized that our knowledge of the histology of the oyster is very incomplete, and what we do have is scattered throughout a number of journals. I have tried to obtain literature citations from several sources, but the only material of any immediate value was Yonge's paper (bibliography reference 6) provided by Dr. Needler. It need hardly be pointed out that pathological studies require a very thorough knowledge of the normal histology of the subject before they can be of very much value, and I must make it clear at the outset that I consider the studies described hereinafter as being badly handicapped by such lack of knowledge.

I have, however, made cateful comparisons between the diseased areas and the corresponding areas in healthy oysters, and am at least able to give some description of the outstanding characteristics of the disease.

Photomicrographs made from typical diseased areas were made by myself and accompany this report.

In addition to the report, I shall be glad to lend the collection of seventy-seven permanent preparations totalling three hundred and seventeen sections to anyone

wishing to use them in a further study of the disease. It is quite possible that someone with a better knowledge of molluscan histology may be able to give a better interpretation.

Technique.

All material used in these studies was fixed in formalin-acetic-alcohol. The material in Bouin's fluid mentioned at the beginning of the report was not used for paraffin sections, as we find considerable shrinkage (See bibliography reference 5) when this fixative is used on oysters. We are not satisfied with any fixation tried so far. (See Part 5, No.8).

In order to minimize shrinkage, aniline oil was used for de-alcoholization, replaced by xylol, and followed by the usual infiltration and imbedding. Most of the paraffin sections were cut at ten microns, but some were cut at five microns for comparison. Ten microns give the best general pictures. Paraffin was removed by xylol, followed by chloroform, followed by the usual descent through the alcohols.

A number of stains were used, namely: Heidenhain's iron-alum hematoxylin, Delafield's hematoxylin, Harris' hematoxylin, Van Gieson's picro-fuchsin, Loeffler's alkaline methylene blue, Goodpasture's stain for bacteria in tissues, Chlorazol Black E, aurantia Orange G, Biebrich scarlet, Scarlet R, and several others.

It was found that Heidenhain's iron-alum hematoxylin gave by far the best results on this material and that a counterstain was hardly necessary. Loeffler's alkaline methylene blue was of about equal value and had the additional value of being an excellent stain for demonstrating bacteria in tissues, for which latter purpose we used it in addition to the special technique of Goodpasture for that purpose. We did not find that Goodpasture's method gave us any more information than did Loeffler's method. Loeffler preparations tend to fade, however.

The permanent slides are about evenly divided between Heidenhain and Loeffler staining, and also some Goodpasture slides. Sections stained in hematoxylin were mounted in green euparal which intensifies detail in hematoxylin stain, and sections stained in Loeffler's were mounted in neutral balsam.

Frozen Sections.

The great bulk of the sections, amounting to considerably over seven hundred, were cut on a freezing microtome operated by solidified carbon dioxide ("dry ice"). The usual gum-and-syrup mixture was used. Most of the frozen material had been fixed in formalin-acetic-alcohol, but some of it was fixed in 5% or 10% formalin either in water or physiological saline or sea water.

Large numbers of sections were cut and graded up to 70% alcohol and were stored at that strength until their turn came for examination.

The bulk of our studies were made on these frozen sections, as it would be utterly impossible for us to have done a similar number by the paraffin method. Moreover, there is a great advantage in frozen sections for such pathological studies as it obviates many changes and artefacts which may occur in the paraffin method.

For the general appearance of the disease, see Dr. Needler's summary report for 1937 (Bibliography reference 7).

The microscopic appearance of the disease is quite characteristic in one respect, but much less definite in another respect. In examining sections of "pustules" one is immediately struck by the presence of dense groups of mobile cells which have collected at one or more foci. Usually there is one large group but not infrequently we find a number of smaller groups.

These mobile cells are the phagocytes illustrated and described in pages 315 - 317 of Yonge's monograph. They consist, according to DeBruyne, of seven different types of blood cells but "it is doubtful how many of these represent different stages in the same type". (Yonge)

Despite long and careful study, we have been unable to contribute any new interpretation of the different

types, nor is it easy to distinguish more than three or four kinds of phagocytic cells in any of our preparations.

It is obvious however that there are different types of phagocytes present in these pustular cell-clusters.

There are also present in these groups certain cells which do not appear to be phagocytes nor even oyster cells at all. They may be algae, diatoms, or protozoa. We are unable at this stage of the investigation to identify these cells, or even to recognize their general classification. We are quite aware, of course, that diatoms, green algae, and other plankton organisms are always found in the organs of feeding and digestion, but we can only account for the presence of these organisms outside of the digestive tract by the fact that the walls of the diverticula degenerate and rupture. In some of our frozen sections we observed large numbers of green or brown granules, both inside the phagocytes and also lying outside of the phagocytes. These are probably the enterochlorophyll granules described by MacMunn. From the foregoing it will be seen that we have in the lesions a melange of cells, granules, and probably degenerate cells or cellular detritus very difficult to sort out, and that will require prolonged and intensive study if we are to

interpret, the pathology of the "pustules" correctly.

Experiments at the Atlantic Biological Station, For which we provided bacterial cultures and filtrates which might contain a virus, have given no evidence whatever that this is a communicable disease of bacterial or virus etiology. Our chief interest now lies in the possibility that some of these foreign cells in the cell groups may possibly be protozoa, and that if the disease is communicable it may have a protozoan etiology which the bacterial and virus experiments would not demonstrate at all. This points to the need of direct inoculation experiments by injection and by contact, preferably under the already established conditions in the set-up at St. Andrews.

Suggestions for further experiments follow in Part 5.

PART V: SUGGESTIONS FOR FURTHER STUDIES

(1) As the communicability of the disease has only been studied from the bacterial and virus standpoints, the possibility of protozoan infection should be explored experimentally. We have found it very difficult to say whether or not some of the cells present in the pustules are protozoa, and it would be shorter and surer to perform direct inoculation experiments by the injection of pustular liquid hypodermically or otherwise into the body of disease-free oysters. Controls should be made, of course, by injection with sterile physiological saline.

(2) Further contact experiments in trays similar to those already made should be continued and extended, but I would suggest that they be transferred entirely outside of Prince Edward Island waters.

(3) I would recommend that both for direct inoculation and contact experiments that the out-door and supervision at Saint Andrews would be very desirable, but that is a matter for the officers of the Board to decide on.

(4) The possibility should also be considered that oysters in the diseased areas are ingesting some toxic substances, organic or inorganic. While the lesions

of this disease exist in several different parts of the body, we think it significant that they greatly predominate in the so-called "liver" region, properly called the digestive diverticula. Many of our sections have shown abnormally large mobilization of phagocytes bordering the lumen. In water bacteriology we are beginning to find that certain species of Trichomonas and Anabaena are toxic to higher animals, if not in their natural condition at least when decomposed thus liberating toxic substances. This is only a suggestion and I do not know whether it is worth anything or not.

(5) It might be valuable to use sediment from beds in diseased areas as an inoculum in the St. Andrews troughs.

(6) Fresh living oysters, both diseased and normal, should be studied by dark-field methods in the diverticula region, to determine (a) the activity of the phagocytes and (b) the possible presence of protozoa in the lesions.

(7) Other molluscs in the diseased areas should be examined for the presence of anything comparable to Malpeque disease in oysters, or even that they might harbor an organism non-pathogenic to them but possibly pathogenic to oysters.

(8) I am not at all satisfied with the fixatives, and I would recommend:

(a) Small blocks instead of whole oysters, and very gently handling to avoid traumatic injury and rupture of the diverticula.

(b) New fixatives, trying (1) Carnoy's acetic alcohol, with or without chloroform and sublimate (2) Gilson's mixture plus iodine and

(3) Benker's fluid.

(c) Getting into paraffin as soon as possible.

(d) Frozen sections from fresh unfixed oysters, to avoid chemical fixation altogether.

PART VI: SUMMARY

While these studies have not demonstrated any definite etiological agent, we feel, however, that substantial progress has been made and that a great deal of spade work has been done toward limiting the field of possibilities. We may summarize our findings up to the present as follows:

(1) Extensive cultural studies together with intensive microscopical examinations of the lesions do not reveal any bacterium to be the cause of the disease. The "repeaters" observed in culture series were evidently secondary invaders or ordinary saprophytes, and were of no etiological significance.

(2) It was remarkable both in the cultural studies and in the sections that bacteria were relatively scarce, many of the cultures proving sterile and many of the sections showing few or no bacteria, even when stained by special methods for demonstrating bacteria in tissues.

(3) No evidence of a virus was found, and we have been unable to find a single instance of inclusion bodies in any of the material examined.

(4) The experiments at the Atlantic Biological Station were painstakingly set up and supervised, and yielded no evidence whatever of bacterial or virus communicability.

(5) After examining many hundreds of sections, both temporary and permanent, we have a fairly clear picture of the pathological processes involved, but as yet we are unable to reach a satisfactory interpretation of the picture. The "pustules" are evidently composed of great numbers of mobile phagocytic cells together with degenerative tissue in varying stages of disintegration and colliquative necrosis. There are also present in the pustules and in the diverticula several types of cells which we are unable to identify with any certainty. Some of them might prove to be protozoa or other organisms of possible pathogenic power. The most difficult part of the whole research has been the attempt to determine the nature of the cells present not only in the pustules but in other parts of the tissues as well. We may or may not be able to determine their nature by further study.

(6) Suggestions for further experiments as to communicability have been made.

(7) A collection of seventy-seven permanent slides accompanies this report and are at Dr. Needler's service. It is requested that the slides be returned to the Department of Biology and Bacteriology, Mount Allison

University, Sackville, N. B., when they have served their purpose.

ACKNOWLEDGMENT

I am indebted to Dr. A. W. H. Needler, Dr. A. H. Leim, Mr. Ross Homans, and the staff members of the Atlantic Biological Station for their courtesy and co-operation, and to my Assistant, Miss Margaret Miller, for technical assistance.

(Signed) Roy Fraser

Professor of Biology and Bacteriology.

PART VIII: MISCELLANEOUS NOTES

1. No studies were made of nerve structures.
2. Sections were treated with dinitrosoresorcinal to see if iron had anything to do with the disease, but the results of the several tests were negative.
3. About 15 slides were made of bits of diseased tissue crushed between slides ("crush smears") and stained in a variety of ways, but very few bacteria were noted and practically no information was gained thereby.
4. After communicability is settled, the cause, inception, and progress of the degeneration of tissues should be the first study. I am somewhat inclined to think that that process comes first in the pathological order.

PART VII: PHOTOMICROGRAPHS AND PHOTOMICROGRAPHIC TECHNIQUE

Camera: A Beck ocular camera, coupled with a Spencer microprojector, "bellows" length, 15 inches: L. P., H. P., and O. I. objectives with 10x ocular.

Ray Filter: None

Plates: 3¼ x 4¼ Eastman Process

Paper: Velox No.4 Contrast, glossy.

Exposures: Two to six seconds.

REFERENCES

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2. Leim, A. H., letter to R. Fraser, Nov. 15, 1937.
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4. Fraser, R., letter to A. H. Leim, Jan. 11, 1938.
5. Orton, J. H. "Note on shrinkage of Oyster Tissues in Bouin's Fixative." Journal of the Royal Microscopical Society, Vol. 57, Dec. 1937, p. 255.
6. Yonge, C. M. "Structure and Physiology of the organs of Feeding and Digestion in Ostrea edulis." Journal of the Marine Biological Association of the United Kingdom, Vol. 14, No. 2. Aug. 1926.
7. Needler, A. W. H., "Oyster Mortality in the Charlottetown Region." Summary Report for 1937 to the Biological Station.

We append also the following references given to us by Dr. Paul Bartsch, Curator of Mollusks, Smithsonian Institution, Washington, D. C., through the courtesy of Mr. A. S. Windsor of the General Biological Supply

House of Chicago, Ill. Dr. Bartsch stated that there is no comprehensive treatise on the histology of the oyster available. Although we were unable to consult these references, we feel that they might possibly be useful in further work:

Grace, C. and Schmitt, F.O. Journ. Morph. Vol. 40, 1925, pp.479-515 (Ciliated Cells in Oyster).

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Kellog, J. L. Bull. U. S. Fish Commission, Vol. 10, 1890, pp.389-486, pls. 79-94.

Dahmen, Peter, Jenaische Zeitschrift fur Naturwissenschaft, Vol.59, 1923. pp.575-626. Good bibliography.