OFFICE OF NAVAL RESEARCH

ANNUAL PROGRESS REPORT

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For Period 2/1/53 to 6/30/54

NR: 180 030

CONTRACT: NORN 494 (07)

ANNUAL RATE: $6,480.00 (9,150.00 for the period indicated)

CONTRACTOR: Trustees of Tufts College

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TITLE OF PROJECT: STUDY OF SPECIFIC ANTIHYALURONIDASES IN SERUM

Objectives: See Page 1

ABSTRACT (OR SUMMARY) OF RESULTS See Page 1 - 3

a. Since start of project:

b. During current report period:

PLANS FOR FUTURE: See Page 4

Immediate:

Long Range:

REPORTS AND PUBLICATIONS

(During current report period)

OBJECTIVES:

This investigation originated from the demonstration of a high correlation between the hyaluronidase titre of saliva and oral disease; and the bacterial origin of the salivary hyaluronidase. First reported by Lisanti (ONR Project-NR 183005 reports), these observations have been checked and corroborated. There are three possible explanations for these results: (1) hyaluronidase plays a part in the production of oral disease, (2) the bacteria producing the hyaluronidase, but not necessarily the hyaluronidase itself, are involved and (3) neither the bacteria nor the hyaluronidase are involved and the correlation merely represents an increase in the oral flora. To obtain the evidence necessary to choose among these possibilities, one may use a direct approach such as the testing of the action of the bacterial enzymes on tooth structures, or seek further biological evidence of the involvement of these enzymes in oral disease. It is this latter type of evidence with which we have been concerned. If bacterial hyaluronidases, or the organisms producing them are in any way involved in oral disease, then it should be possible to find some evidence of this involvement in the serum. This can be readily done by testing the serum for the presence of specific antihyaluronidases.

Whole saliva samples from 50 subjects from the Chelsea Naval Hospital were studied for hyaluronidase activity. Sera from these subjects were studied for antihyaluronidase activity against four types of streptococci, Lancefield types A and K and two strains of St. mitis, designated as #19 and #14. Parotid saliva samples from these same subjects were studied for inhibitor activity against the #19 enzyme. The hyaluronidase activity of some samples of whole saliva was studied with a specific inhibitor against the #19 enzyme. Rabbits were actively immunized against the #14 and #19 enzymes to test the specificity of the antihyaluronidases thus produced and to use the specific inhibitors produced to identify the sources of the hyaluronidase in the saliva.

SUMMARY OF RESULTS:

Salivary Hyaluronidase: The salivary hyaluronidase activity was determined by measuring the change it produces in the viscosity of a purified hyaluronic acid solution in 45 minutes. Two ml. of saliva were used in 4 ml. of a 0.3 gm./l solution of the acid. The other conditions of this method have been specified elsewhere by the author (Biochem. J. 42, 260 and 269, 1948; J. Gen. Physiol., 36, 361, 1953). The change in the flow-time of the hyaluronic acid solution in 45 minutes is expressed as per cent of the viscosity increment due to hyaluronic acid (the flow-time of the hyaluronic acid solution minus the flow-time of the buffered saline in which the acid is dissolved). Controlled experiments have shown that this can be taken as a measure of the hyaluronidase concentration in the saliva. In the fifty samples of saliva tested, this value ranged from 2.4% to 63%. Preliminary calculations show that, in this series as in previous studies, a good correlation exists between the hyaluronidase concentration of saliva and the extent of oral disease. Further statistical analysis of this and other data is in progress and will be submitted when completed.

Ten of the saliva samples were tested with a specific inhibitor against St. mitis #19 which had been prepared in a preliminary experiment.

The details of the preparation of such inhibitors will be given in a later section. The relevant fact at this point is that there was very little inhibition of the hyaluronidase activity by this inhibitor, indicating that at the most a very small fraction of the salivary hyaluronidase was derived from #19 in these samples.

Parotid Saliva: Parotid saliva has no hyaluronidase activity, but in many instances has a slight inhibitory action on the #19 enzyme. The results would warrant an intensive study of a few selected samples of parotid saliva to determine the nature of this inhibition.

Specific Hyaluronidase Inhibitors in Serum: When serum is heated at 60°C for 15 minutes the non-specific inhibitor which is normally present in all sera is inactivated as shown by a lack of inhibitor activity against the testes hyaluronidase. Serum treated in this manner, however, shows considerable inhibitor activity against bacterial enzymes. The inhibitor activity of serum is measured in terms of the amount of hyaluronidase it will inactivate. The unit of hyaluronidase activity was chosen for this work as that amount which will give a half-time of 100 seconds under conditions of testing specified in the references given above. The unit of inhibitor activity is defined as that amount which will inactivate 50% of one unit of hyaluronidase.

Serum samples from all of the subjects were tested against the #19 enzyme. The inhibitor levels ranged from 3.3 units/ml. of serum to 133 units/ml. There is no immediately apparent relationship between the inhibitor levels and the hyaluronidase titres in saliva. Such data, however, may prove of some value in the further analysis of the inhibitor activity of parotid saliva. Twenty-four of the samples were also tested against enzyme from #14, Lancefield type A, and Lancefield type K streptococci. The results are summarized in Table I.

TABLE I

Inhibitor levels in 24 samples of sera against #14, A and K enzymes expressed as % of the inhibitor levels against #19 enzyme.

	#14	A	K
Range	0-122	120-1800	0-169
Average	58	680	76

It is not possible to say whether there is a different entity inhibiting each of these enzymes in serum. We know from animal experiments that antihyaluronidase produced against the #19 enzyme will not inhibit enzyme from #14 or A, and that antihyaluronidase produced against the #14 enzyme will not inhibit enzyme from #19, A or K. We do not know now, but are in the process of determining, whether an inhibitor produced against the enzyme of Lancefield A will inhibit the others. We cannot therefore say with certainty, in view of the very high inhibitor activity against the A enzyme, that there are specific inhibitors against the #14 and #19 enzymes until the animal experiments are completed.

Classification of Streptococci Isolated from the Oral Cavity

About thirty organisms have been isolated by Lisanti and associates from the oral cavity (saliva, periodontal pockets, carious lesions, etc.) and classified as St. mitis. The hyaluronidase produced by these organisms was tested with the specific inhibitors against the #14 and #19 enzymes obtained as described in a subsequent section. Without exception all of these enzymes fell into two categories—in one category those inhibited by the #19 inhibitor but not by #14 and in the second category those inhibited by the #14 inhibitor but not by #19. This clear cut classification greatly simplifies the study of the activity of these organisms in relation to oral disease.

Production of Specific Antihyaluronidases Against Bacterial Enzymes:

Rabbits were injected subcutaneously with 50 units/kg. of partially purified bacterial enzyme twice a week for four weeks (method of immunization described by Harris and Harris, J. Immunol. 65, 255, 1950). Four rabbits were given the #14 enzyme and four #19. Control serum samples before immunization showed a total lack of specific inhibitor activity against both of the enzymes. Saliva samples were obtained by injecting the animals with 1 mg. of pilocarpine. All of the control saliva samples showed a non-specific type of inhibitor activity—i.e. approximately equal inhibitory activity against both #14 and #19 enzymes.

At the end of four weeks all of the rabbits showed specific inhibitor activity. Those injected with the #19 enzyme showed inhibitor activity ranging from 20 u/ml. to 200 u/ml. of serum against the #19 enzyme but none against the #14 enzyme. Those injected with the #14 enzyme showed inhibitor levels ranging from 34 u/ml. to 63 u/ml. against the #14 enzyme but none against the #19 enzyme. Saliva samples obtained at this time showed no evidence of the presence of a specific inhibitor. Some of the rabbits were given further injections and in one instance, with an animal given enzyme #19, it was possible to raise the inhibitor level to 450 u/ml. A saliva sample obtained at this time gave some evidence of the presence of a specific inhibitor. Its inhibitory activity against the #19 enzyme was 3-4 times as great as that against the #14 enzyme. Some three months after the beginning of the immunization the inhibitors had not lost specificity. At this time two animals which had been immunized against the #19 enzyme were given a single injection of the #14 enzyme, and two immunized against #14 were similarly injected with the #19 enzyme. One of these animals which had been initially immunized against the #19 enzyme responded with a tremendous rise in the inhibitor level against both #14 and #19 enzymes (740 u/ml. against #19 and 1400 u/ml. against #14). The others showed no change in the inhibitor level. One rabbit initially immunized against the #14 enzyme showed massive hemolysis 5 days after the injection of the #19 enzyme. To determine the degree of specificity of the inhibitors produced by immunization, the sera were tested for inhibitory activity against enzyme from Lancefield type A and type K streptococci. There was no inhibitory activity against the type A enzyme. Only those animals which had been immunized against the #19 enzyme showed any inhibitory activity against the type K enzyme. The identification of the organism from which the type K enzyme was obtained is being rechecked. At the end of three months the animals were bled and the sera freeze-dried and stored for future use.

PLANS FOR FUTURE WORK:

Immediate: Our work has shown that most human sera inhibit hyaluronidase from St. mitis #19 and#14. Furthermore, we have shown that a large number of streptococci isolated from the oral cavity are immunologically identical with one or the other of these organisms with respect to the hyaluronidase they produce. The animal experiments have shown that the antihyaluronidases produced in response to the injection of bacterial enzymes are highly specific. If it can be proven definitely that specific antihyaluronidases exist in the sera of a large number of individuals against hyaluronidase from #19 and #14, it can be assumed that these organisms are generally and actively involved in infections, and their presence in the oral cavity will assume added importance. To obtain such proof, it is necessary (1) to immunize animals against Lancefield A and K enzymes and test the specificity of the anti-hyaluronidases thus produced and (2) to study the sera of a number of individuals intensively to ascertain whether their inhibitory action against streptococcal hyaluronidase is due to a single entity or to number of different, specific, antibodies. It will also be necessary to determine whether the active enzyme is essential for the production of the antibodies, or whether immunization can be obtained with inactivated enzyme (e.g. enzyme inactivated in 0.5% formaldehyde).

The saliva of a number of subjects should be studied with the four specific inhibitors which will be available to determine the source of the hyaluronidase in saliva. If a high correlation is found between one type of oral disease and the #14 enzyme and another type of oral disease and the #19 enzyme, then it will be difficult to argue that the correlation between the salivary hyaluronidase titres and oral disease reflects nothing more than an increase in the oral flora.

Long Range: If the results of the experiments show convincingly that the organisms St. mitis #19 and #14 are actively involved in oral disease, then we shall proceed to find out how. Perhaps the most straight-forward approach would be to study the action of these organisms or their enzymes on various tooth structures; but the results of such studies may not be conclusive and other means must be explored. A thorough study should be conducted to find out what enzymes besides hyaluronidase (and β-glucuronidase in the case of #14) these organisms produce.

If the evidence should lead us to believe that the increased hyaluronidase titres associated with oral disease are purely coincidental with an increase in the oral flora, or that the hyaluronidase is of non-streptococal origin, then we must look for other sources of the hyaluronidase in saliva, or other organisms in the oral flora which contribute activity to oral disease.