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Occurrence of Natural Antibacterial Antibody in Human Parotid Fluid. (30309)

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Although saliva has been reported to contain antimicrobial factors, little attention has been given to the demonstration of the presence of natural bactericidal antibodies in the fluid(1). The designation "natural" antibodies refers to those specificities found in serum of man in the absence of infection or overt stimulation(2). Antibodies other than bactericidal have been shown in both whole saliva and parotid fluid. Investigators have demonstrated the presence in whole saliva of bacterial agglutinins, isohemagglutinins, and diphtheria antitoxins(3). In addition to these studies others have directly demonstrated the occurrence of immunoglobulins γA, γG, and γM† in saliva(4,5).

The reports of Brill and Bronnestam(6) and Mann and Stoffer(7) would indicate that at least some of the antibody found in whole saliva could be contributed by fluid outflow from the gingival pocket. This fluid, rich in serum proteins, would obscure that portion of antibody which might be derived from the salivary glands. Accordingly, it seemed reasonable to examine the fluid obtained from the parotid gland in humans for the presence of natural antibacterial antibody since this secretion could be obtained without contamination directly from the duct in sufficient quantities for assay.

Materials and methods. Parotid fluid and serum. Paired parotid fluid and serum samples were taken from normal, apparently healthy, individuals. The parotid fluid, obtained by paraffin stimulation, was collected aseptically by use of Curby cups and immediately placed in ice. Parotid fluid is normally uncontaminated by bacteria; however, as a precaution against accidental contamination all samples were passed through Millipore® filters (size HA). Aliquots in amounts suitable for each day's assays were frozen at —20°C. Pooled lyophilized parotid fluid was also used in some studies. Serum samples taken at the same time as the parotid fluid were prepared and stored frozen at —20°C without preservatives until used.

Cultures.‡ *Escherichia coli* 0127, *Salmonella typhosa 0901, Shigella dysenteriae* and *Aerobacter aerogenes* were picked from single colonies and transferred to nutrient agar slants. Subcultures were made to Brain Heart infusion (Difco) and grown overnight at 37°C for the bactericidal assay.

Bactericidal assay. The assay method used was described by Muschel and Treffers(8). A modification was made in their procedure by substituting precolostrum calf serum (Colorado Serum Co., Denver) for the adsorbed guinea pig complement recommended (9). Each lot of complement was tested for bactericidal antibody, and if found free, was used in the amounts recommended by Muschel and Treffers.

Precipitin tests. Capillary precipitin reactions were performed using specific antisera to γA, γG, and γM immunoglobulins (Hyland Laboratories, Los Angeles). Gel precipitin

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† The notations used for human immunoglobulins are in accordance with those suggested by WHO Meeting on Nomenclature of Human Immunoglobulins(15).

‡ Obtained through the courtesy of Dr. Anne L. Jackson, Nat. Inst. Allergy & Infect. Dis., N.I.H. Bethesda, Md.

reactions in agar followed procedures given in Crowle(10). Agar-gels were prepared with 1% Ionagar #2 (Consolidated Laboratories, Inc., Chicago) in pH 8.4 barbital buffer. These slides were also used for immunoelectrophoresis which was run for 2 hours at 250 V using the LKB Instruments, Inc. (Stockholm) apparatus.

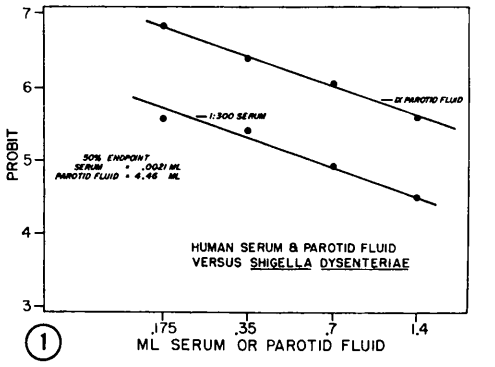
Opsonization. The method used to detect opsonizing factors in parotid fluid was a modification of the procedure of Benacerraf *et al* (11). *S. typhosa* 0901 was grown overnight at 37°C in 250 ml of Brain Heart infusion containing 0.2 mc of uniformly labeled C^{14} glucose. The cells were collected by centrifugation and exhaustively washed in saline until there was negligible radioactivity in the supernatant. The washed cells were resuspended in saline to an optical density of 2.0 and lyophilized parotid fluid was added to the cells to give a final concentration of either 125 μg or 250 μg parotid fluid per 0.1 ml of suspension. A comparable saline suspension of radioactive cells without added parotid fluid served as a control. One-tenth ml of this suspension contained approximately 1.3 $\times$ 10^4 counts per minute. The suspensions were incubated for 30 minutes at 37°C and 0.1 ml portions were injected into the tail vein of 12-16 g NIH female mice. Groups of mice were bled by cardiac puncture at 1, 2, 4 and 8 minutes following injection. One-tenth ml of blood from each mouse was counted for 2 minutes in a low background gas flow counter (Nuclear-Chicago, Des Plaines, Ill.). Results are expressed as the K values of the average counts in the blood samples from 5 mice for each time interval. The formula used for calculation of the K values was $K = \log C_1 - \log C_2/t_2 - t_1$ where C_1 and C_2 are the counts at zero time and 4 minutes later and t is the time in minutes.

Mouse protection. NIH female mice (12-16 g) were injected intravenously with 5 mg high molecular weight dextran (Nutritional Biochemicals, Cleveland) according to the procedure of Shilo(12). The mice were immediately challenged with 0.1 ml of an overnight broth culture of *S. typhosa* 0901. Following challenge, the mice were injected intraperitoneally with either 0.2 ml human serum (pooled), 0.2 ml mouse serum (pooled) or 0.2 ml human parotid fluid (pooled, 20 $\times$ concentrated). Untreated challenged mice served as controls.

Density gradient. Parotid fluid (pooled, 50 $\times$ concentrated) was centrifuged at 39,000 RPM for 16-17 hours in a Model L Spinco ultracentrifuge (SW-39 swinging bucket head). The gradient was 10-37% sucrose formed in normal saline. Twenty-four fractions were collected by piercing the bottom of the tube. Protein content of each fraction was estimated by its absorption at 280 mμ in a Beckman DB Spectrophotometer. Bactericidal and precipitin assays were performed on individual fractions.

Results. Precipitin reactions. Capillary precipitin tests with specific antisera gave evidence of only γA globulin being present in parotid fluid. No evidence of γG or γM was detected even when the parotid fluid was concentrated by lyophilization to 100 times its original concentration. Gel precipitin methods gave comparable results to the capillary precipitin tests. Of note, however, was the band of precipitate formed when 20 $\times$ concentrated parotid fluid was reacted with anti-γA serum produced against γA obtained from human serum. The band gave a similar migration pattern but the immune arc was much shorter and slightly more diffuse. Evidence of a strong cross reaction of serum γA with that of parotid fluid was seen repeatedly on double diffusion gel reactions.

Bactericidal assay. Parotid fluid contains bactericidal antibodies of a low titer when assayed against serum sensitive strains of Gram-negative bacteria. This activity, while not as concentrated as that found in serum, paralleled it to a high degree when plotted using the standard probit method (Fig. 1). Paired serum and parotid fluid showed this parallelism in all instances tested. Table I gives the reciprocal titers from 7 pairs which were tested against *S. dysenteriae.* The titers were 1000- to 2000-fold greater in each instance for serum over that found in parotid fluid. Also of note was the fact that as the serum titer decreased so did that of parotid fluid. This held true for all paired samples except H-5.



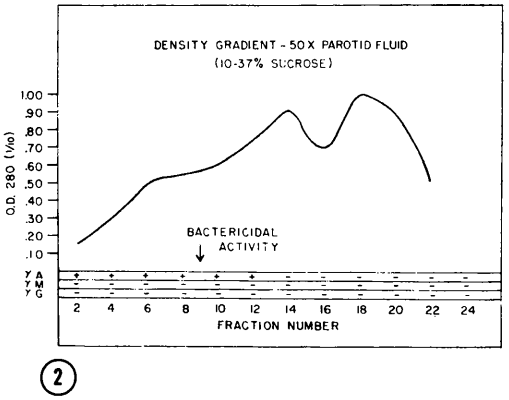


FIG. 1. Typical probit analysis of paired human serum and parotid fluid. Endpoints are read directly from graph and express the amount of whole serum or saliva required to reduce original standard inoculum 50%(8). The titer may also be expressed as a reciprocal of the 50% endpoint.

FIG. 2. Summary of results obtained from sucrose density gradient experiments. The fractions were diluted 1:10 for purposes of estimating protein content.

Only *Shigella* and *Salmonella* exhibited sensitivity to the bactericidal antibody found in parotid fluid although low levels of antibody were found in the serum of these same individuals to *E. coli* as well (Table II).

TABLE I. Comparison of Reciprocal Bactericidal Titers of Human Serum and Parotid Fluid *vs S. dysenteriae.*

Pair No.	Serum	Parotid fluid
H-1	714*	.72*
H-2	588	.37
H-3	467	.22
H-4	467	.21
H-5	357	.51
H-6	232	.19
H-7	217	.18

* Reciprocal of ml of serum or parotid fluid required to kill 50% of the standard inoculum.

One individual was found who exhibited antibody in his parotid fluid to *Salmonella;* however, this parotid fluid was inactive against *Shigella.* No instances were found of antibody to *A. aerogenes* in either serum or parotid secretion.

Mouse protection. Treatment of mice immediately after injection with *S. typhosa* 0901 demonstrated that there are substances in parotid fluid which are capable of conferring protection against dextran-enhanced infection (Table III). Human serum conferred 100% protection whereas parotid fluid (20 ×) gave protection to 60% of the mice. With no treatment there were only 22% survivors.

TABLE II. Summary of Bactericidal Activity in Serum and Parotid Fluid.

	Shigella	*Salmonella*	*E. coli*	*Aerobacter*
Serum	11/11*	11/11	11/11	0/11
Parotid fluid	7/11	1/11	0/11	0/11

* No. positive/total No. of assays.

TABLE III. Protective Effect of Serum and Parotid Fluid Against Dextran Enhanced *S. typhosa* 0901 Infection in Mice.

Treatment	Survivors*	% survival
.2 ml human serum (pool)	15/15	100
.2 " mouse serum (pool)	9/15	60
.2 " human parotid fluid (pool, 20 ×)	9/15	60
None	3/14	22

* No. living 48 hr following infection.

Opsonization. Using the rate of clearance (K value) of C^{14}-labeled bacteria from the circulation of mice as a measure of opsonization, the mice which received bacteria incubated in parotid fluid exhibited an enhanced clearance over those which received bacteria incubated in saline (Table IV). The clearance of the labeled bacteria was rapid during the first 4 minutes and was essentially complete 8 minutes following injection.

Density gradient. Localization of the bactericidal activity in parotid fluid was possible through the use of a sucrose density gradient (Fig. 2). Each fraction recovered was tested for bactericidal activity against *Shigella* and for immunoglobulins γA, γM, and γG using

TABLE IV. Clearance in Mice of C^{14}-Labeled *S. typhosa* 0901 Opsonized with Human Parotid Fluid.

Treatment	K values 4 min	Dose cleared in 4 min
		(%)
Saline control	.089*	46
125 μg parotid fluid	.129	59
250 " " "	.140	62

* Each K value was obtained from average counts of 5 mice.

the double diffusion gel reactions. Bactericidal activity appeared in fraction 9 with some residual activity appearing in fractions 8 and 10. Most of the γA was found in fractions 4 through 10 but traces were also found in fractions 1 through 3 and 11 and 12. No evidence of the other immunoglobulins was found. The bulk of the protein (fractions 13 through 24) was not found to be associated with bactericidal activity.

Discussion. The occurrence of bactericidal antibody in human parotid fluid which is free from contamination with serum products as found in whole saliva of gingival fluid suggests that either the salivary gland is capable of immunoglobulin synthesis or alternatively such antibody is passed from the blood into the parotid gland fluid. Evidence has been presented for occurrence of both events(5,14). The high degree of parallelism between the activity of serum and parotid fluid in the bactericidal reaction indicates, however, that at least in this instance the antibody may have been derived from serum components presumably passed into the parotid fluid *via* the salivary gland. Had the antibody been synthesized by the gland this parallelism probably would not have occurred and the slopes would have been divergent in at least some of the cases encountered(8). In addition, it appeared that the titer of bactericidal antibody found in parotid fluid was a reflection of that found in serum and that a threshold level must occur in serum before the antibody can be detected in parotid secretion.

The question of which class of immunoglobulin is responsible for the bactericidal activity of parotid fluid was investigated with equivocal results. Bactericidal reactions are normally ascribed to the γM class of immunoglobulin with some occurrence in humans of the γG(13). The γA is not normally implicated in this reaction. However, the results from the sucrose density gradient gave evidence that the bactericidal antibody was at least associated with the fractions which contained a heavy concentration of γA. This would tend to exclude participation of γM, a high molecular weight immunoglobulin (19S), thus leaving γG (7S) or γA (7-11S) as possibilities. This would be in line with the results obtained with the density gradient experiment in which the bactericidal reactivity occurred as a sharply localized fraction within the sedimentation range of 7-11S. While the experiments did not furnish direct proof of the class of antibody involved, it would seem that either γA or γG could account for the results obtained. The class of immunoglobulin responsible for the bactericidal activity in parotid fluid awaits further investigation.

Other reactions possibly mediated by antibody observed in addition to the bactericidal reaction were the opsonization-promoting ability of parotid fluid and its protective effect on dextran-enhanced *Salmonella* infection in mice. Because of their low concentration it would be difficult to ascribe a protective role to these antibacterial activities in the oral cavity, but it would seem likely under local conditions, such as found in the parotid gland, they could play a role in maintaining a disease-free state.

Summary. The use of the highly sensitive bactericidal assay has allowed the detection of natural antibody in human parotid fluid to *Salmonella typhosa* and *Shigella dysenteriae*. This antibody activity parallels closely the bactericidal activity found in serum. Bactericidal activity in parotid fluid was localized by sucrose density gradient in fractions within the sedimentation range of 7-11S. Other antibody-like activities in parotid fluid were demonstrated by evaluating its protective effect on dextran-enhanced *Salmonella* infection in mice and phagocytic-promoting ability as measured by the clearance of C^{14}-labeled bacteria. The nature and possible role of such antibody in parotid fluid is discussed.

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Failure of L-Triiodothyronine to Alter Significantly Glucose Utilization by Human Erythrocytes.§ (30310)

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It was demonstrated that parenteral administration of L-Triiodothyronine (L-T3) initially increased and then decreased utilization of glucose by the RBC of rabbits(1). Because of this fact, we became interested in studying the effects of clinically large doses of this compound on utilization of glucose by the human erythrocytes. Certainly, if a peripheral cellular effect could be demonstrated, then there would be a possibility of establishing a clinically useful tool for reflecting total body metabolism and perhaps evaluating thyroid function. The present investigation was therefore designed to study in humans: 1) normal fluctuations in rate of glucose utilization by RBC, and 2) effect of L-T3 on this rate.

Methods and material. Fasting venous blood was obtained from healthy volunteer men and women to insure satisfactory range in age and sex distribution. These individuals were clinically free of any disease and not taking medication. The laboratory procedure followed has been described(1). Blood samples were collected with siliconized equipment, and heparin was used as the anticoagulant. *In vitro* tests were conducted by centrifuging the samples for 20 minutes at approximately 2700 RPM (1000 *g*'s) in an International Clinical centrifuge. The buffy coats and upper red cell layers were carefully aspirated and discarded and the RBC were then resuspended in the subject's own plasma. The final hematocrit was adjusted to approximately 50%. Preparation of RBC suspensions were conducted at room temperature. White blood counts were done on the original sample of blood and on the final suspension of RBC to estimate efficiency of removal of WBC, because of their relatively high rate of glucose metabolism(2,3,4). Glucose determinations were made in duplicate using the Somogyi-Nelson procedure(5). Within one hour after venipuncture, samples in glass-stoppered centrifuge tubes were incubated at 36-37°C in a water bath and equilibrated in air atmosphere since previous experiments did not reveal a significant difference in glucose metabolism under aerobic and

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