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Rabbit polymorphonuclear leukocyte migration in vitro in response to dental plaque

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Human dental plaque extracts in Gey's medium and whole human saliva were tested in the Boyden Chamber system for chemotactic activity against rabbit peritoneal polymorphonuclear leukocytes. Positive controls were a semipurified bacterial chemotactic factor and a complement derived chemotactic factor. Gey's medium alone served as a negative control. The method appeared to be very sensitive as concentrations of less than one mg plaque per ml medium gave a clear chemotactic response. Whole human saliva was less chemotactic than dental plaque. Incubation of equal concentrations of plaque extract in both the upper and lower compartments of the Boyden Chamber did not result in a large increase in cell migration, indicating that most cell migration in reaction to dental plaque extract in this system depends on a concentration gradient. The chemotactic factor or factors were present in plaque at the time it was collected, they were readily dissolvable in water, and they were heat stable. The dose range in which dental plaque extract would evoke a chemotactic response was variable and usually very narrow. Replicate tests yielded highly variable results indicating that this method is not suitable for quantitative comparisons. Extracts of the same dilution were prepared from dental plaque samples from a small population with varying degrees of gingivitis. Differences in chemotactic response to those samples could not be related to the intensity of gingivitis.

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Introduction

The concept of chemotaxis was first formulated in botany to explain the locomotion of plant cells in reaction to chemical stimuli (Pfeffer 1884, 1888). These experiments indicated that a concentration gradient of a chemotactic agent would cause a motile cell to assume a directional path. Chemotaxis of leukocytes was demonstrated *in vitro* (Comandon 1917, 1919, McCutcheon, Wartman & Dixon 1934, McCutcheon & Dixon 1936), but evidence for chemotaxis *in vivo* is only circumstantial. Some investigators claim to have seen directional migration of cells in reaction to localized irritants (Clark & Clark 1920, 1922, Leber 1891). Injection of substances known to be chemotactic for eosinophil granulocytes *in vitro* resulted in accumulation of the same cell type *in vivo* (Cohen & Ward 1971). However, if strict criteria are applied, no conclusive proof for the existence of chemotaxis *in vivo* is present (Harris 1953).

A new technique to study chemotaxis was introduced by Boyden (1962). It consists of a device in which two compartments are separated by a porous membrane. The upper compartment receives a suspension of polymorphonuclear leukocytes (PMN), and the

lower a test agent in solution. If the test agent is chematactic, its concentration gradient at the separating membrane will result in migration of the PMN's through the membrane pores. Since the introduction of this technique, considerable progress has been made in the understanding of sources and mediators in chemotaxis. Bacteria are an important source of exogenous leukotaxins (Ward, Lepow & Newman 1968). Complement is a major (although not the only) source of endogenous leukotaxins since these are formed when the complement system interacts with a variety of agents (Sorkin & Stecher 1971).

A possible connection between gingivitis and chemotaxis has been postulated (Tempel et al. 1970). Inflammatory cells, mainly neutrophil granulocytes, migrate from the connective tissue into the gingival crevice with the number of PMN's increasing with the degree of inflammation (Attström 1971). Some oral bacteria have been shown to produce a factor directly chemotactic for neutrophil granulocytes in vitro (Tempel et al. 1970). The same authors indicated that dental plaque would also have this effect. Whole saliva was chemotactic for neutrophils, but not pure parotid or submaxillary-sublingual saliva. This suggests that salivary chemotaxis may be dependent on the oral flora. Lindhe & Helldén (1972) found migration of PMN's in response to dental plaque in Boyden Chambers. They did not, however, include the appropriate controls in their experiments and so the migration may have been a random movement of the cells rather than true chemotaxis.

Dental plaque contains factors which could be indirectly chemotactic as well. Plaque endotoxins, enzymes, and antigens could conceivably react with plasma to release leukotaxins. Some mechanisms in complement activation have been discussed with reference to the possible relation to gingival inflammation (Mergenhagen, Tempel & Snyderman 1970). The leukotaxigenic potential of plaque with fresh plasma was further demonstrated (Mary, Haggan, & Folke 1972).

In the present investigation, two problems were investigated. First, the direct leukotactic potential (without serum mediators) of pooled dental plaque was studied. Secondly, since variations in the increase of crevicular leukocyte migration with the development of gingival inflammation between persons have been noted (Schiött & Löe 1970), the leukotactic potential of individual dental plaque samples was also examined to detect possible differences between plaques from patients with various degrees of gingivitis.

Material and Methods

Boyden Chamber Method

The upper and lower compartment of Boyden Chambers were separated by membranes with an average pore size of 1.2 micrometer and 100 micrometer thickness* In some cases 10 micrometer thick membranes were used.** The lower compartment was filled with 1.8 ml test solution and the upper with 0.6 ml cell suspension. After incubating the chambers for three hours in humid air with 5 % CO_2 at 37°C, the membranes were removed, fixed in alcohol, stained with hematoxilin and mounted on microscope slides upside down (Boyden 1962). Cells which had migrated all the way through the membrane were counted at 450X magnification in fields defined by an ocular grid. For the Nuclepore membranes, the counts were performed at 1000X magnification using the same grid. Five fields, evenly distributed across the membrane in one direction and five fields in a direction perpendicular to the former were counted.

* Sartorius SM 11303 or Schleicher and Schuell Selectron Grade B2–1.

** Nuclepore, 1 micrometer pore size.

The total cell count of these ten fields served as the measure for chemotatis. In each experiment replicate chambers were incubated together. With exception of the experimental variables, the treatment of the chambers and their contents was identical.

Polymorphonuclear Cells

Polymorphonuclear leukocytes were harvested from the peritoneal cavity of white New Zealand rabbits 8 to 12 hours after injection I. P. of 0.1 % glycogen in 0.9 % saline with 50 mg/ml neomycin. The cells were harvested by an intraperitoneal rinse of 50 to 100 ml 0.9 % saline with 2 U/ml heparin. This was then centrifuged at 250 g for 10 minutes at 4°C and resuspended in Gey's medium to give a cell concentration of approximately 2.5 X 10^6 PMN's/ml. This cell suspension was used immediately so that all chambers in each experiment contained PMN's from the same batch. Gey's medium contained Gey's balanced salt solution, 2 % bovine serum albumin, penicillin 100 U/ml, and fungizone 0.25 microgram per ml. The medium was filter sterilized and the pH was adjusted to 7.2 $\pm$ 0.04 with sterile sodium hydroxide. Unless otherwise indicated, this medium was used for all cell suspensions as well as the various dilutions of the chemotactic agents.

Test Agents

Plaque was collected from all accessible tooth surfaces from clinic patients with care taken to avoid food debris, calculus, carious dentin and blood. These patients had a variety of periodontal problems varying from early gingivitis to advanced periodontitis. The plaques from several subjects were weighed immediately, pooled, added to a tube containing ice-cold Gey's medium, and stored at −20°C until needed. Some plaque samples were dried (100°C overnight) before adding them to the medium. The plaque samples were dispersed by shaking, although occasionally (indicated in the figures and tables) dispersion by ultrasound (1 min, 60 watt) was performed. The plaque suspensions were centrifuged at 3200 g, at 8000 g or the gross debris was allowed to sediment. The supernatants were retained and diluted to the desired concentrations, which are indicated as reduced to wet weight of plaque per volume medium before discarding the sediment.

Unstimulated whole saliva was collected from laboratory personnel and treated similarly to the plaque suspensions. Semipurified preparation of a bacterial chemotactic factor (BF) and a complement derived chemotactic factor (C5a) were kindly supplied by the National Institute of Dental Research. These preparations in a suitable concentration in Gey's medium served as positive controls for chemotactic activity. The medium alone served as a negative control.

Experiments with Pooled Plaque

Serial dilutions of plaque extract were made in order to test the dose-response relationship. The effect of a concentration gradient was evaluated by mixing the plaque extract with the PMN suspension so as to equal the concentrations of the extract in both upper and lower compartments. The concentration of the PMN's was kept at 2.5 X 10^6 PMN/ml for all chambers in the experiment.

The effect of the antibiotics in Gey's medium was investigated by dividing a plaque sample into two parts which were tested in identical manner with exception of the deletion of the antibiotics from the medium for half of the sample. In this experiment the presence of cultivable bacteria in the Boyden Chambers was also investigated. For this purpose one loopful of the contents was taken from the lower compartment of each Boyden Chamber after incubation and plated on MM10 sucrose agar (Syed & Loesche 1972).

To investigate the variation in outcomes,

tests with five replications were done, using different membrane types and brands. In these experiments, the control substances as well as dental plaque were used as test agents.

Experiments with Individual Plaque Samples

For those experiments in which a comparison of the chemotactic activity of plaques from different individuals was attempted, the plaque samples were not pooled. The subjects for these comparisons were children or young adults, who were either patients of the University of Michigan School of Dentistry, patients of the Washtenaw Community College Dental Clinic, or residents in the Plymouth State Home for the mentally retarded (Northville, Michigan). The persons selected from the latter institution had severe gingivitis with soft and swollen marginal gingivae and papillae which bled easily. Reddening extended into the attached gingiva. The other subjects in these experiments exhibited only slight gingivitis despite moderate to heavy plaque accumulations. There was little evidence of caries in any of the subjects used. The individual plaque samples were diluted to the same concentration of 1 mg/ml and only samples sufficiently large to allow at least triplicate determinations were used.

Table 1

Cell migration in response to plaque, whole saliva, and control substance+

Test substance	PMN*	
Control (BF 2.5 mg/ml)	2454	2234
Plaque extr. 4 mg/ml	1066	634
Plaque extr. 2 mg/ml	2504	2386
Plaque extr. 1 mg/ml	1084	1090
Plaque extr. 0.5 mg/ml	1092	1040
Plaque extr. 0.25 mg/ml	711	386
Plaque extr. 0.12 mg/ml	297	253
Plaque extr. 0 mg/ml	177	22
saliva dil. 1:4	2316	1434
saliva dil. 1:8	1288	832

* cell counts from duplicate tests
\+ with Sartorius membranes, samples sonicated, debris allowed to sediment

Table 2

Cell migration in response to dental plaque and control substance°

Test substance	PMN*		
Control (BF) 2 mg/ml	192	159	122
Control (BF) 1 mg/ml	139	127	106
Control (BF) 0.5 mg/ml	30	24	12
Control (BF) 0.25 mg/ml	12	9	8
Plaque extr. 1 mg/ml	10	5	6
Plaque extr. 0.5 mg/ml	7	2	2
Plaque extr. 0.25 mg/ml	8	8	3
Plaque extr. 0.12 mg/ml	11	7	6
Plaque extr. 0 mg/ml	1	–	–

* cell counts from triplicate tests
° Sartorius membranes; samples vortexed; debris allowed to sediment

Statistical Analysis

Regression equations were computed with PMN count as dependent and plaque extract concentration as independent variable. Exclusion of zero from 95 % confidence intervals computed for linear regression coefficients indicated significance. Regression coefficients were compared using the t-test. Significance of curvilinearity was evaluated with the F-statistic. The data from the experiments with plaque extract on both sides of the membranes were subjected to multivariate analysis (Allan 1973). Homogeneity of variance was examined with Bartlett's test (1937). Duncan's multiple range test (1955) was used for evaluation of differences between outcomes of individual samples.

Results

Experiments with Pooled Plaque

Dental plaque exhibited chemotactic activity in the Boyden Chamber. As little as 0.25 mg plaque per ml medium evoked a distinct

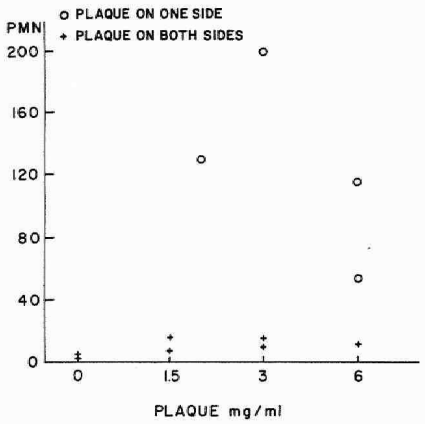

Fig. 1. Cell migration in response to plaque with and without a concentration gradient. Schleicher and Schuell membranes.

response from the PMN's. The dose-response relationship was often linear only over a narrow range. Usually there appeared to be a maximum concentration beyond which the chemotactic activity diminished. The linear regression coefficient was not significant, whereas curvilinearity was significant at the 0.01 level. (Table 1). This table also shows the response to dilutions of whole saliva which possessed chemotactic activity as well. Occasionally plaque extract would not result in PMN migration, even though control substances in the same experiment had a positive effect. An example is given in Table 2. The linear regression coefficient for Bacterial Factor in this experiment was significant at the 0.05 level.

The presence of similar concentrations of plaque extracts in both upper and lower compartments of the Boyden Chambers greatly reduced PMN migration when compared to tests where a concentration gradient was present, thus indicating the existence of true chemotaxis (Figure 1 and 2). This difference was not so pronounced when thinner nuclepore membranes were used. Multivariate analysis of these data indicated that plaque extract on the test side as well as cell side of the membrane had a significant but different effect on the cell counts in these experiments. This confirms that dental plaque could both induce random and directional migration.

Samples from Boyden Chambers after incubation in which no antibiotics were employed in the medium, showed abundant bacterial growth. No growth could be obtained from chambers in which antibiotics were used. Figure 3 shows that there was a similar chemotactic response in both situations. The regression coefficients calculated for plaque with and without antibiotics were not significantly different. The chemotactic response to extract from dried plaque was of approximately the same magnitude as from fresh plaque. Sonication and sedimentation with different gravitational forces did not seem to affect the chemotactic activity of the samples.

A great variation between and within experiments was noted. Although chemotactic

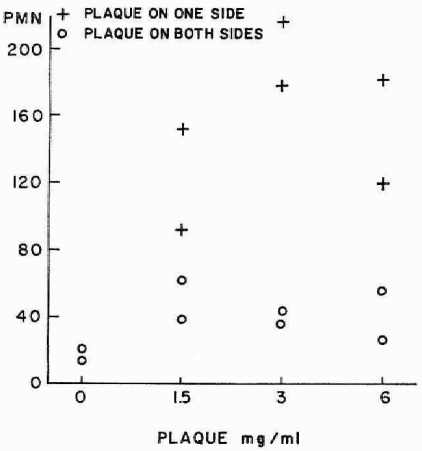

Fig. 2. Cell migration in response to plaque with and without a concentration gradient. Nuclepore membranes.

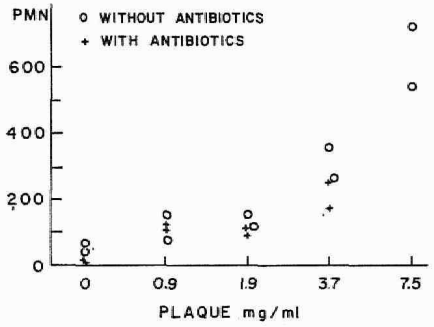

Fig. 3. Cell migration in response to plaque under conditions favoring bacterial activity and conditions suppressing bacterial activity.

agents could generally be distinguished from negative controls, the quintuplate experiments demonstrated a wide range of outcomes for each test substance. The variation was significantly different for each set of replicates ($P < 0.0001$). (Table 3). The method error of the counts of the cells on the membranes was apparently not responsible for this variability: When 44 membranes were recounted at a different occasion, the difference between the counts of the same membrane was never larger than 10 (mean 3.6) for outcomes under 101 and never larger than 24 (mean 9.9) for outcomes greater than 100.

Table 3

Cell migration in response to several substances for different membranes with replicate tests°

Test substance with Sartorius membranes	PMN*				
Bacterial factor 1 mg/ml	377	290	188	84	76
C5a dilution 1:4	725	344	277	90	10
C5a dilution 1:8	368	355	319	11	5
Plaque extract 1 mg/ml	136	102	100	80	32
Medium alone	14	11	–	–	–
Test substance with Nuclepore membranes	**PMN***				
C5a dilution 1:8	94	86	81	57	
Plaque extract 1 mg/ml	292	218	183	0	
Medium alone	24	22	15	–	

* cell counts for each membrane. The numbers on the same line are for identical replicates
° samples vortexed and debris allowed to sediment N.B. The magnification used for counting cells on the two types of membranes is different.

Table 4

Cell migration in response to plaque samples from different persons (1 mg/ml)°

	PMN*			
Slight gingivitis				
Person A	365	287	233	–
Person B	643	112	76	–
Person C	74	45	42	–
Severe gingivitis				
Person 1	399	221	184	15
Person 2	299	195	145	–
Person 3	110	108	1	–
Bacterial factor 1 mg/ml	318	163	112	–
Medium alone	7	7	7	–

* cell counts from individual membranes. Numbers on the same line represent replicate tests from the same sample
° Sartorius membranes; samples vortexed and centrifuged at 8000 g

Experiments with Individual Plaque Samples

Individual plaque samples were compared on two occasions for a total of twelve persons. It was difficult to obtain plaque from each person sufficient for at least triplicate tests. None of the differences between the outcomes for each individual plaque sample were statistically different at the 5 % level and, therefore, could not be related to the differences in severity of gingivitis in these individuals. The results of one such experiment are seen in Table 4.

Discussion

The results of the present investigation indicate that dental plaque contains compounds which are chemotactic for rabbit PMN's in

the Boyden Chamber. When membranes were incubated with plaque extracts on either side, significantly higher cell counts were observed than with the negative controls. This indicates that the plaque extract also increased the random movement of PMN's. However, this effect was small when compared with membranes incubated with a concentration gradient. In these instances, a three to five fold increase in PMN migration was observed. Therefore, the measurements reflect for the most part directional migration or true chemotaxis of PMN's for plaque extract. It has been suggested that the thinner nuclepore membranes are as good or better as the thicker membranes for chemotaxis determinations (Horwitz & Garrett 1971). The differences in cell counts in situations with and without a concentration gradient are however, more pronounced for the thicker membranes, indicating that the latter would be the more suitable for chemotaxis determinations.

One may assume that the chemotactic factors were present at the time of plaque collection as the plaque was manipulated in a manner which prevented bacterial growth, i.e. kept frozen and incubated in the presence of antibiotics. Also, dried plaque possessed chemotactic activity. Further, omitting the antibiotics from the medium, thereby permitting plaque bacteria to grow and metabolize during the incubation period, did not alter the PMN response.

Whole saliva evoked cell migration in a dilution of 1:8 or roughly 125 miligram saliva per ml medium. Dental plaque showed chemotactic response with quantities less than 1 mg/ml medium. An increased migration of PMN's follows the accumulation of dental plaque in vivo (Schiött & Löe 1970). The observation that dental plaque is a stronger chemotactic agent than saliva in vitro supports the hypothesis that plaque factors induce this increased migration in vivo.

Since no plasma or serum was employed, the responsible chemotactic factors should be classified according to present standards as a direct cytotaxin. (Keller & Sorkin 1968).

Chemotactic activity of the peritoneal fluid of the rabbit at the time of collection of the PMN's in this study is low (Snyderman, Phillips & Mergenhagen 1971). The washing and subsequent dilution of the cell suspension would further reduce the concentration of any factor present. Moreover, such a factor, being on the cell side of the membrane would most likely have an inhibitory effect on cell migration. Yet, since it is impossible to obtain a PMN suspension free from other cell types, the possibility of mediators of some other kind playing a role cannot be discarded entirely.

Since plaque chemotactic factors are apparently heat stable, one would expect them to be of bacterial origin. Of course, contributions from other sources such as crevicular fluid and disintegrating cells cannot be excluded completely. However, further support for the hypothesis that factors elaborated by dental plaque rather than complement derived chemotactic agents induce the crevicular leukocyte migration in vivo was found by Attström & Larsson (1972) who noted that carrageen complement inhibition of dogs did not affect this crevicular leukocyte migration.

The dose-response relationship approached linearity at low concentrations (Table 1). However, at high concentrations often no further increase in PMN migration was observed. This is at variance with previously reported observations (Tempel et al. 1970). The curvilinear dose-response relationship may be a property of the chemotactic agent, but it is equally well possible that dental plaque also contains inhibiting substances. Another explanation might be that the cell adherence to membranes diminishes with increased plaque extract concentrations causing cell loss from the surface of

the membranes during incubation and staining procedures. Since the determinations in the present study were already made at the lowest possible level of cell migration which might be distinguished from a negative response, an increased number of replicate determinations would not necessarily improve the quantitative aspect of the method when used on whole dental plaque extract. This conclusion is not in agreement with a recommendation previously made (Second Internat. Confer. Periodont. Res. 1972).

Previous reports have not mentioned the difficulty in obtaining good results with the Boyden method. In the present investigation, it was not until many failures that cell migration through micropore membranes could be obtained consistently. The number of PMN washings seemed to be critical as increased handling of the cell suspension impeded PMN migration. This is at variance with the observations by Schade and Mayr (1930). However, a comparison of the results with another investigation (Tempel et al. 1970) in which essentially the same procedures were followed shows that the sensitivity of the method in the present investigation was at least as good or better.

The results of the experiments indicate a great variability. The large range in the outcomes in the tests with different types of membrane in which special effort was made to have all conditions in the quintuplets identical showed that the poor reproducibility was intrinsic to the method. The reproducibility of the counts of the cells on the membranes was good and apparently contributed little to the observed variations. Analysis of variation with Bartlett's Test showed that these variations were different with each change in experimental variable. This indicates that quantitative comparisons cannot be made until the error of the method is eliminated or thoroughly characterized.

Lack of all migration in response to dental plaque extracts while controls were positive, was noted on two occasions .This might be explained by contamination of a plaque sample from one of the patients with a cytotoxic or inhibitory substance, possibly originating from food or a mouthwash. Obviously the chance for such an occurrence will be higher in pooled than in individual samples.

If dramatic differences in chemotactic activity exist between dental plaques from one person to another, these differences would have shown up in the experiments designed to investigate this possibility. Results from triplicate tests on different plaques overlapped each other. Since we purposely assembled test groups of persons with either extremely mild or severe gingivitis and as there was no obvious relation between chemotactic activity and intensity of gingivitis, we could not assign any biological significance to the slight variations between chemotactic activity from dental plaques from one person and another.

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