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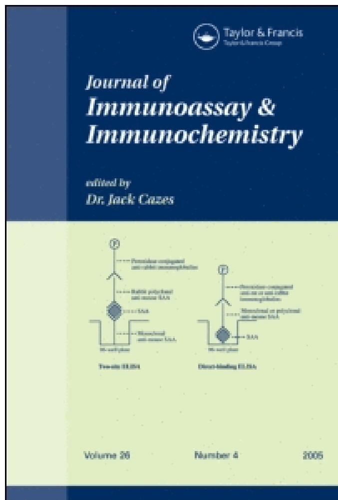

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Evaluation of Mouse Salivary IgA Directed Against Indigenous Oral Bacteria

Harold Marcotte[a]; Marc C. Lavoie[a]
[a] Groupe de Recherche en Écologie Buccale, Faculté de Médecine dentaire Université Laval, Québec, Canada

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EVALUATION OF MOUSE SALIVARY IGA DIRECTED AGAINST INDIGENOUS ORAL BACTERIA

Harold Marcotte and Marc C. Lavoie
Groupe de Recherche en Écologie Buccale, Faculté de Médecine dentaire
Université Laval, Québec, Canada G1K 7P4

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ABSTRACT

We are developing an ELISA to follow the evolution of specific salivary IgA directed against the indigenous oral bacteria of the BALB/c mouse. To reduce the variability of the IgA levels detected between different mice, we standardized the method used for sampling saliva and the method used for bacterial cell fixation. Incubation of whole bacteria for one hour at 4°C in poly-L-lysine-treated plates followed by glutaraldehyde fixation increased ELISA reactivities by improving cell fixation. Our results also indicate that salivary IgA concentrations in BALB/c mice peak at the age of three months and that biweekly carbachol-stimulated saliva sampling does not significantly affect the amount of salivary IgA detected.

(KEY WORDS: ELISA, Bacterial fixation, Poly-L-lysine, Salivary IgA, IgA variability, Indigenous bacteria)

INTRODUCTION

Immunoglobulin A is the major species of immunoglobulin found in external secretions, particularly in saliva (1,2). In mice, total salivary IgA (s-IgA) concentrations and the reactivities of IgA specific to indigenous oral bacteria were found to vary greatly

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from one individual to another and between different samples from the same individual (1). In humans, salivary flow (3), age (4), hormonal changes (5), genetic background (6), diet (7), stress (8) and oral infections (9) have been identified as sources of variability in the concentrations of s-IgA. In rodents, most of these factors can be controlled and salivary flow, sampling frequency and age are recognized as the major sources of variability in s-IgA concentrations (10,11). The detection technique used can also introduce variability. Antibodies specific to bacterial antigens have been detected and quantified by different methods such as immunofluorescence (12), agglutination (13), radioimmunoassay (RIA) (14), and enzyme-linked-immunoassay (ELISA). The detection of specific antibodies by indirect ELISA can be carried out using whole bacteria, bacterial membrane extracts and purified antigens (15). The use of whole bacterial cells avoids lengthy antigen purification procedures and probably more closely duplicates the antigen-antibody interactions which occur in vivo. The adsorption of whole bacteria to ELISA plates is, however, unstable and different strategies have been used to increase bacterial fixation (1,14-19).

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In this work, we show that a technique usually used to fix human cells (20), and not often used for bacterial cell fixation (17,18), was more effective in fixing indigenous oral bacterial cells from the BALB/c mouse than a previously reported technique (1). Results also indicate that s-IgA concentrations peak at the age of three months in BALB/c mice and that biweekly carbachol-stimulated saliva sampling does not significantly affect the amount of s-IgA detected.

MATERIALS AND METHODS

Mice

BALB/c male mice, from Charles River Canada Inc. (St-Constant, Québec, Canada), were housed individually in plastic cages on wood chip bedding, fed a

standard diet (Charles River Rat Chow RMH 4020, St-Constant, Québec, Canada) and tap water ad libitum. All the experiments started one week after the mice arrived at the Université Laval animal care unit. All animals were cared for in accordance with institutional guidelines.

Bacterial preparations

The standard strains (Lactobacillus murinus 2D2.05, Enterococcus faecalis M2.01, Staphylococcus aureus S0.6, Staphylococcus epidermidis 2S3.03, Staphylococcus sciuri S3.11, Staphylococcus warneri M1.01, Proteus mirabilis 2L3.22, Escherichia coli 2D4.05, Enterobacter cloacae 2D1.18, Clostridium bifermentans 2D1.04, Bacillus sp. L2.09), representing the major species previously isolated from the oral cavity of the BALB/c mouse (21), were grown overnight from frozen or freeze dried stock cultures in Trypticase Soy Broth (BBL, Becton-Dickinson and Co., Cockeysville, MD, USA) enriched with 0.3% yeast extract (BBL), at 37°C under anaerobic conditions (80% N_2: 10% H_2: 10% CO_2, Coy Glove Box, Coy Manufacturing Co. Ltd., Ann Arbor, MI, USA). The bacterial cells were harvested by centrifugation (5000 x g, 15 min) and washed twice in sterile phosphate buffered saline (PBS-phosphate buffer 100 mM, pH 7.2, 128 mM NaCl). The bacterial cells were then resuspended in PBS and this suspension was used for testing the three fixation methods. As they form aggregates, the suspensions of L. murinus, E. faecalis, S. epidermidis, S. sciuri, and S. warneri were dispersed by ultrasonication before being used (10 seconds, 1/8 inch special microtip , 50% duty cycle, output 5, Sonifier® cell disruptor, Model W350, Heat Systems-Ultrasonics Inc., Plainview, NY, USA).

Fixation methods

Bacterial suspensions were adjusted to an O.D. 660 nm of 0.5 (Spectronic® 20 spectrophotometer, Bausch and Lomb Inc., Rochester, NY, USA) and the cell

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concentration was evaluated using a Petroff-Hausser counting chamber (C.A. Hausser and Son, Philadelphia, PA, USA). Different dilutions of the standardized cell suspensions of each bacterial species were deposited in duplicate in round bottom wells of polystyrene Immulon II ELISA plates (Dynatech Laboratoires Inc., Chantilly, VA, USA)

In method A (1), the bacteria were resuspended in carbonate buffer (pH 9.6) and 50 µl of the bacterial suspension was added to the wells. The plates were dried overnight at 37°C. To fix the bacterial cells, a solution of 0.5% (v/v) glutaraldehyde (Sigma) in cold PBS was added to the wells (50 µl/well). The plates were incubated for 15 minutes at room temperature (22).

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Method B was modified from Carroll et al. (20). The Immulon plates were pretreated with a solution of poly-L-lysine (MW 70 000-150 000, Sigma) (0.01% (v/v) in PBS) for 90 minutes at 37°C. After three PBS washes, the bacterial suspensions were added (50 µl/well) and incubated for 1 hour at 4°C. The cells were then fixed with 0.5% glutaraldehyde (50 µl/well) for 15 minutes at room temperature and the plates were washed with PBS.

In method C, the bacterial cells were resuspended in PBS and 50 µl/well was added to plates pretreated with poly-L-lysine (as in Method B) and dried overnight at 37°C. The bacteria were then fixed with glutaraldehyde.

In all three methods, the glutaraldehyde residue was blocked with 100 mM glycine buffer containing 0.1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO, USA) (150 µl/well) for 30 minutes at 25°C (22). The plates were then stored at -20°C until used.

ELISA protocol

Rabbit antisera against each bacterial species obtained as previously described (23) were used to evaluate the three methods of cell fixation as too much mouse saliva

would have been needed to determine optimal experimental conditions. A dilution of the specific antisera that gave a satisfactory reading (that did not exceed an O.D. 414 nm of 2.0 on the EIA reader) in preliminary experiments was used. The dilution to be used varied from 1:5000 to 1:80000 depending of the antiserum.

The Immulon plates to which the bacteria were fixed were brought to room temperature, washed three times with 10 mM PBS containing 0.05% Tween 20® (ICN Biomedicals Ltd) and blocked with a solution of 2.5% (v/v) BSA in PBS-Tween 20® (200 µl/well). After three rinses with PBS-Tween 20®, the appropriate dilutions of the specific antiserum were added (50 µl/well) to the corresponding plates which were then incubated for 1 hour at 37°C. After three washes with PBS-Tween®, peroxidase-labelled goat anti-rabbit IgG serum (1:3000) (Bio-Rad Laboratories, Mississauga, Ontario, Canada) was added to the wells and the plates were incubated for 1 hour at 37°C. After two washes with PBS-Tween 20® and one wash with PBS, 100 µL of 2,2'-azino-di-3-ethylbenzthiazoline sulfanate (ABTS) substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was added and the plates were incubated for 30 minutes in the dark at 37°C. The reaction was blocked with a 2% (v/v) sodium dodecyl sulfate (SDS, Sigma) solution (50 µl/well). The optical densities were read at 414 nm (EIA Reader model 2550, Bio-Rad Laboratories). Negative controls included wells without bacteria and wells without added antisera.

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Reproducibility of Method B for the detection of mouse immunoglobulins

Lactobacillus murinus 2D2.05 cells were fixed to the plates as described in method B. A pool of carbachol (Lentol®, MTC Pharmaceutical, Cambridge, Ont., Canada)-stimulated saliva, obtained as previously described (1), from 30 BALB/c male mice (6-8 weeks old) was added to 14 wells. The serum from a mouse subcutaneously immunized with L. murinus was also used (1:1000-1:10000) in duplicate to test variations using three separately prepared plates. The reactions were visualized either

with peroxidase-labelled goat anti-mouse IgA serum (1:2000) or with a peroxidase-labelled goat anti-mouse IgG serum (1:6000) (Kirkegaard and Perry Laboratories).

Effect of age on salivary IgA concentrations

Carbachol-stimulated saliva samples were obtained from three groups of six BALB/c male mice aged 2, 3 and 4 months. Two months later, the same mice were resampled.

Influence of the sampling frequency

Carbachol-stimulated saliva was obtained from four groups of five BALB/c male mice (6-8 weeks old) sampled every 1 (group I), 2 (group II), 4 (group III) and 6 weeks (group IV) respectively.

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Total salivary IgA concentrations

Total s-IgA concentrations were determined by ELISA. The wells of the Immulon II plates (96 round bottom wells) were coated with goat anti-mouse IgA (Kirkegaard and Perry Laboratories) diluted (1:2000) in carbonate buffer pH 9.6. The saliva samples were tested at dilutions of 1:10 and 1:100 and the reactions were visualized using peroxidase-labelled goat anti-mouse IgA (1:8000) after the addition of the substrate as described above. Standard IgA curves were determined simultaneously using a reference mouse serum (lot 0022, ICN).

Statistics

The Mann-Whitney test was used to compare s-IgA concentrations and volumes of saliva sampled. The correlation between saliva volumes and s-IgA concentrations was determined by the Spearman rank test. The level of significance was fixed at $P < 0.05$.

RESULTS

Comparison of the three methods of bacterial cell fixation

The number of bacteria required to obtain maximal reactivities varied according to the bacterial species and the method used (Table 1). For most of the eleven species fixed using method C, fewer bacterial cells had to be added to achieve an optimal reaction and more had to be added for method B. Method A gave lower peaks of reactivitiy than methods B and C except for Bacillus sp. and S. warneri. Method C gave higher maximal reactivities for all species except L. murinus, S. aureus and S. sciuri (the predominant species encountered in the oral cavity of mice) which were better fixed by method B. Comparisons of the methods in relation to the number of bacteria added are presented in Figures 1a and 1b for L. murinus and P. mirabilis. Similar results were obtained with the other bacterial species. Methods A and C, in which the wells were allowed to dry, resulted in the formation of cell deposits. We adopted fixation method B to further improve our ELISA technique.

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Reproducibility of method B

As good fixation of L. murinus was obtained with method B, we first determined if specific s-IgA against this predominant species (60% of the total cultivable flora) of the oral cavity of the BALB/c mouse could be detected before proceeding with the standardization of our method. Specific s-IgA and serum IgG were detected (Table 2) and low variability coefficients were obtained in the reactivities. The controls without bacteria gave no reading and the optical densities of controls without saliva or serum gave 0.08 for the peroxidase-labelled goat anti-mouse IgA and 0.045 for the peroxidase-labelled goat anti-mouse IgG.

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TABLE 1

Comparison of the Three Methods of Bacterial Cell Fixation to Polystyrene ELISA Plates

Bacterial species	Optimal number of added bacteria (X 10^8 bacteria/ml)			Maximal reactivity[a] (O.D. 414 nm)		
	A[b]	B[c]	C[d]	A	B	C
L. murinus	0.9	0.9	0.9	1.223	1.772	1.563
E. faecalis	4.8	2.4	4.8	1.015	1.137	1.196
S. aureus	0.1	0.4	0.2	0.779	1.274	0.987
S. epidermidis	7.1	7.1	7.1	1.008	1.112	1.340
E. coli	6.8	6.8	0.2	0.935	1.328	1.496
E. cloacae	3.2	6.3	0.8	0.892	1.665	1.735
P. mirabilis	0.4	1.6	0.4	0.806	1.391	1.563
C. bifermentans	1.8	1.8	0.9	1.484	1.493	1.544
S. sciuri	1.5	3.0	3.0	0.462	0.797	0.783
S. warneri	3.9	3.9	3.9	0.785	0.773	1.035
Bacillus sp.	1.7	1.7	0.9	1.383	1.359	1.660
mean	2.9	3.2	2.1	0.979	1.282	1.355

[a] For a serum dilution which gave satisfactory readings in preliminary experiments.
[b] Drying of bacteria suspended in carbonate buffer.
[c] Incubation of bacteria for 1 h at 4°C in pretreated poly-L-lysine plates.
[d] Drying of bacterial suspensions in pretreated poly-L-lysine plates.

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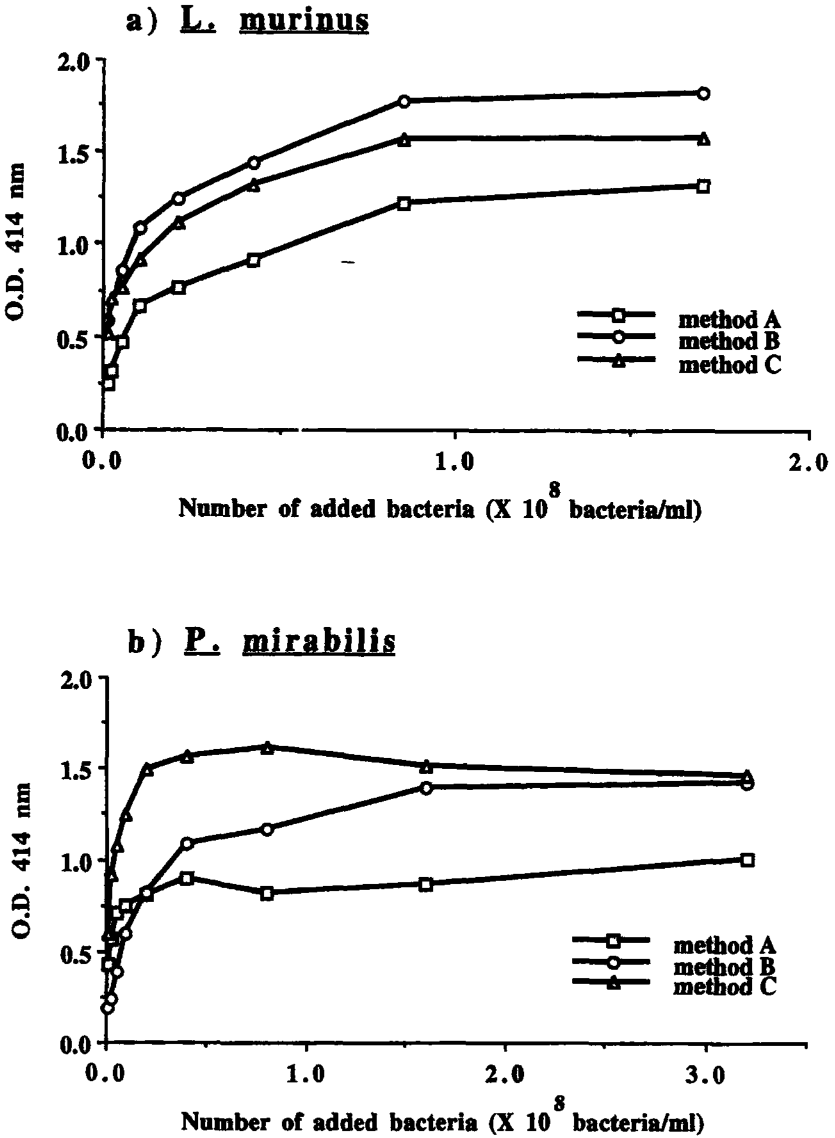

FIGURE 1: Comparison of the three methods of bacterial cell fixation for a) L. murinus and b) P. mirabilis.

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TABLE 2

Variation of Mouse Salivary IgA and Serum IgG Reactivities towards L. murinus Using Method B

Sample		O.D. 414 nm	Coefficient of variation	
			intra-ELISA	inter-ELISA[a]
saliva	(undiluted)	0.297	9.57[b]	-[c]
serum	1:100	1.589	3.07 (2.59-4.91)[d]	8.47
	1:1000	0.912	2.64 (0.87-6.49)[d]	4.46
	1:5000	0.389	3.44 (1.76-9.02)[d]	6.94
	1:10000	0.240	1.35 (0.27-4.82)[d]	11.26

a Measured in three different assays.

b Measured in 14 wells.

c Not measured.

d Median (and range) of the coefficient of variation obtained from the duplicates of three different assays.

Effect of age on salivary IgA concentrations

The volumes of carbachol-stimulated saliva were similar ($P > 0.05$) for mice from different age groups. The mean volume obtained per mouse was 48 ± 5 µl. The s-IgA concentrations generally increased with age but reached a peak at the age of three months as noted by the only statistically significant difference ($P < 0.05$) observed between the two-month-old group and the other groups (Figure 2). The median of s-IgA concentration calculated from the three- to six-month-old groups was 3.55 µg/ml (range, 1.52-20.52 µg/ml) and the average concentration was 7.18 ± 6.46 µg/ml. No significant correlation ($P > 0.05$) between salivary volumes and s-IgA concentration was observed.

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Influence of the sampling frequency

Salivary IgA concentrations generally increased with time (Figure 3). The increase was more significant for groups III and IV. For the samples taken at week 12, s-IgA concentrations were significantly ($P < 0.05$) higher for groups III and IV as compared to groups I and II.

Salivary volumes generally decreased after multiple stimulations (Figure 3), even for groups III and IV. The decrease was greater in group I in which some mice were not stimulated at all at the third sampling (week 2). Significant differences were observed only at week 6 between groups II and IV and at week 12 between groups I and II. A significant negative correlation ($P < 0.05$) was observed at week 12 between s-IgA concentrations and salivary volumes for the four groups.

No significant differences in the total s-IgA (s-IgA concentration x volume sampled) were observed between groups II, III and IV. However, for group I, total s-IgA levels fluctuated between samples, and occasionally (weeks 8 and 12), were significantly ($P < 0.05$) lower than for samples obtained from the other groups.

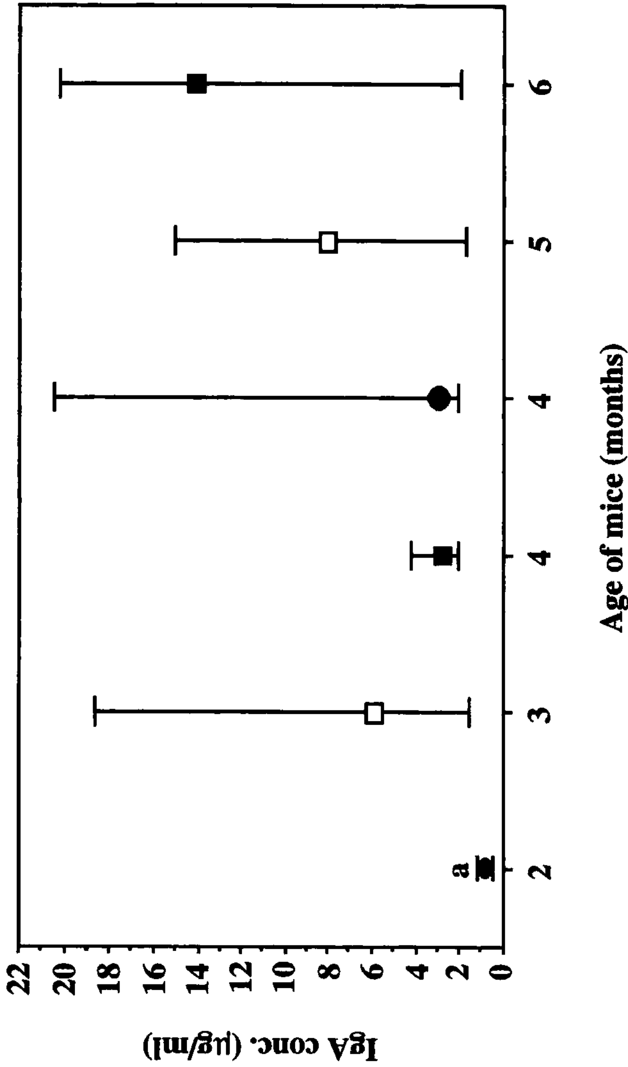

FIGURE 2: Effect of age on salivary IgA concentrations. Values are given as median and range. (a) Significantly lower ($P < 0.05$) than the other groups.

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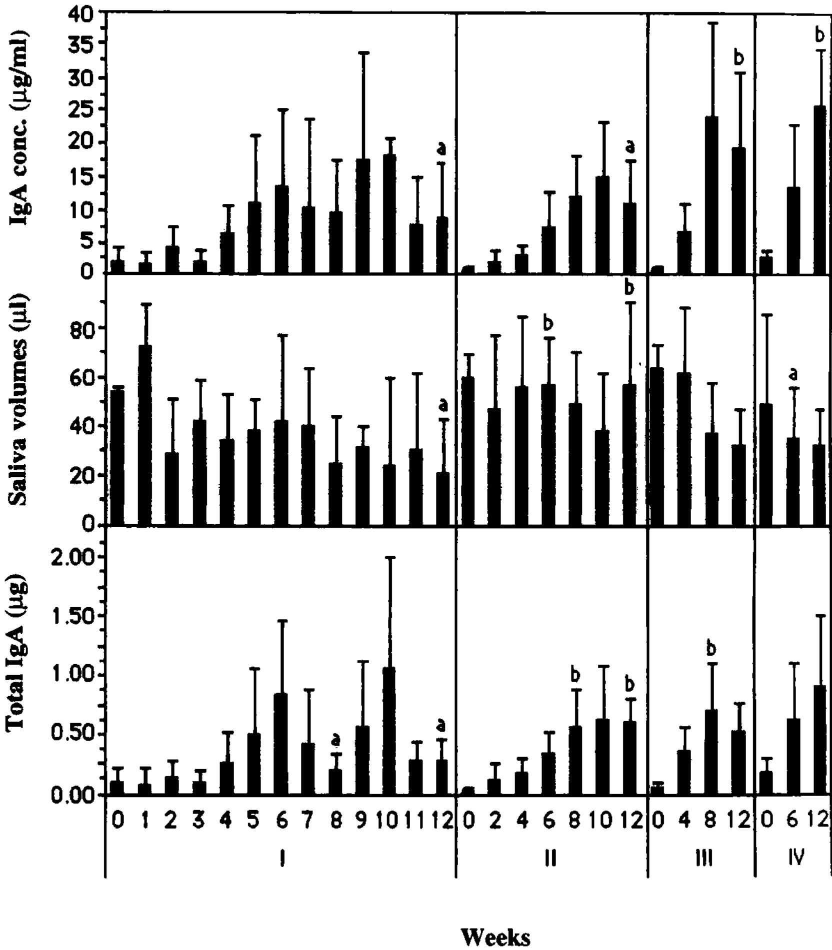

FIGURE 3: Influence of the sampling frequency on salivary IgA concentrations. For corresponding weeks, (b) significantly higher than (a).

DISCUSSION

It has already been shown that poly-L-lysine is very effective in promoting the binding of human cells to ELISA plates (20), but, to our knowledge, poly-L-lysine has been rarely used to fix bacterial cells (17,18). Although Verschoor et al. (18) found no advantage in using poly-L-lysine as an undercoat for the absorption of whole cells of mycobacteria, probably due to the unique cell wall of these bacteria (24), our results demonstrate the effectiveness of this technique for fixing cells from various bacterial species. This method gave highly reproducible results for evaluating the reactivities of mouse s-IgA and serum IgG specific to an indigenous bacteria (L. murinus) of the oral cavity of BALB/c mice. The coefficients of variation inter-assay and intra-assay were lower than 12%, which is comparable with other published results (15,16).

Poly-L-lysine is a cationic polymer used to coat solid surfaces and enhance the adherence of negatively charged molecules (25). As bacterial cells have negatively charged surfaces, they bind to the poly-L-lysine. The amount of bacteria to obtain maximal reactivities varied according to the bacterial species indicating different degrees of cell binding. This may be due to the different surface charges, different cell wall structures and appendages (26) and different cell volumes.

It has been observed that, in carbonate buffer (method A), hydrophobic interactions between bacterial cells and polystyrene are unstable (15). By increasing the number of interactions between the bacterial cells and the polystyrene, the poly-L-lysine probably stabilized the attachment process and increased cell fixation which resulted in the detection of higher specific reactivities. Drying bacterial suspensions overnight in the precoated well (method C) results in a higher level of cell fixation for most of the bacterial species tested than a 1-hour incubation at 4°C (method B). However, undesirable deposits were formed (method C) which could affect the detection of

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specific s-IgA. We adopted method B because it gave higher reactivities with the predominant bacterial species of the oral cavity of mice (L. murinus, S. aureus and S. sciuri), no deposit is formed in the bottom of the wells and it takes only one hour to fix the bacteria. Although bacterial cell fixation to Immulon plates was improved with method B, the sensitivity of the method cannot be determined using one dilution of antisera. Furthermore, optimal conditions for use with antisera are not necessarily the same as would be required when using saliva. The technique will have thus to be further improved for use with saliva by testing different types of plates, peroxidase substrates and the number of bacteria added.

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Glutaraldehyde fixes bacterial cells by interacting with the amino groups on the cell surface (27). This polyaldehyde can sometimes fix nonspecifically to serum components or to the conjugate used in the ELISA (20). In our experiments, no such nonspecific reactions were observed, confirming previous observations (1,19).

The average total s-IgA concentration detected in BALB/c mouse saliva in the present work (7.2 ± 6.5 μg/ml) was generally lower than the previously reported concentration (24.3 ± 12.5 μg/ml) (1) for similar salivation rates but agrees with recent results reported by Russell and Wu (28) (7.26 ± 4.27 μg/ml). A modification in the proportions of the indigenous oral bacterial flora of the BALB/c mice from Charles River could, in part, explain this discrepency. As compared to previous results (21), we recently observed that the oral cavity of this mouse strain was predominently (usually more than 60% of the total cultivable flora) colonized by Lactobacillus murinus (29). It has been reported that the development of the mucosal immune system is dependent on the indigenous bacterial flora (30). The immunogenicity of bacterial species varies considerably and a modification of the oral or intestinal microflora could result in a change in s-IgA concentrations.

As has been reported previously (1), the concentration of s-IgA varies considerably from mouse to mouse. In humans, this variability can be attributed to

many different factors (3-9). In the present study, most of these variables could be controlled as mice with the same characteristics (genetic background, age, sex, specific pathogen free, diet), housed and sampled under controlled conditions, were used. Different rates of salivary flow could contribute to the variability observed between individual mice. We also observed that under apparently the same conditions, the indigenous oral bacterial flora could vary considerably from mouse to mouse (21) and, for the reasons mentioned above, could affect salivary IgA concentrations.

Levels of s-IgA in BALB/c mice peaked at 3 months of age (Fig. 2). These results are similar to those obtained by Van der Heijden et al. (31) who reported that the increase in intestinal secretory IgA paralleled an increase in intestinal antibody-producing cells which could be explained by a greater stimulation of the Peyer's patches by intestinal bacteria. A similar increase in s-IgA (Fig. 3) was observed during the evaluation of sampling frequency on the variability of s-IgA concentrations and could be attributed to the age of the mice, as it was observed whether they were sampled every 2, 4 or 6 weeks. As previously reported (1), carbachol stimulation every two weeks does not seem to significantly influence saliva volumes or total s-IgA concentrations (Fig. 3). More frequent sampling did, however, reduce saliva volumes and the amount of s-IgA fluctuated from week to week. With less frequent sampling, the amount of s-IgA tended to increase steadily (Fig. 3). Contrary to the cholinergic and adrenergic actions of pilocarpin which, after multiple injections, result in an increase in salivary volume, thus reducing the s-IgA concentrations (10), cholinergic stimulation alone, with carbachol, can reduce salivary gland secretions. Our results could also be explained by an increased tolerance by the mice, which after multiple injections, no longer react to carbachol stimulation.

The present results indicate that adherence of bacterial cells to poly-L-lysine-treated ELISA plates and fixation with glutaraldehyde constitute an adequate method for fixing indigenous bacterial species from BALB/c mice for the quantification of specific

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s-IgA. Optimal s-IgA concentrations are obtained by sampling no more frequently than every two weeks and by beginning the sampling period at the age of 3 months.

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