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Salivary Immunoglobulin A Antibodies Reacting with Antigens from Oral Streptococci: Longitudinal Study in Humans

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The salivary immunoglobulin A (IgA) activity to antigens from four common oral streptococci was analyzed in samples from five humans. From each individual, parotid and whole saliva were collected 12 times over a period of 4 months. In samples collected at different occasions, the salivary IgA activity varied considerably. The variations showed a covariation with the concentration of total IgA in the saliva samples. A covariation was also found between salivary IgA antibodies of different specificity. It is concluded that reference antigens, in combination with measurement of total IgA concentration, ought to be used when evaluating the salivary IgA response in humans.

Immunoglobulin A (IgA) is the predominant immunoglobulin isotype in human saliva (29). Salivary IgA has been shown to absorb to (4) and affect the adhesion of (31) oral microorganisms. Circumstantial evidence points to a defense function of salivary IgA antibodies against microorganisms associated with dental caries (14, 15, 23, 28).

Although saliva collected from healthy humans is known to contain salivary IgA antibodies reactive with indigenous microorganisms (2, 7, 26), the variation over time of such antibodies has been studied little. However, knowledge concerning this variation is necessary before changes in salivary antibody activity can be evaluated. We therefore studied the variation of salivary IgA antibodies reacting with antigens of four indigenous oral microorganisms in five humans over a period of 4 months.

MATERIALS AND METHODS

Five healthy females, 19 to 21 years of age, volunteered to donate saliva samples 12 times each over a period of 4 months. None of the subjects showed signs of active caries or periodontal disease.

Saliva samples. Ten percent citric acid-stimulated parotid saliva was collected in two fractions, using Lashley cups. Fraction one (PS 1) represented the sample collected during the first 10 min of stimulation, and the second fraction (PS 2) represented that collected immediately thereafter. This was done because, in other studies, salivary IgA has been found to reach a steady-state level after gustatory stimulation (3, 7).

Stimulated whole saliva was collected by having the volunteers chew a piece of paraffin wax directly after the parotid saliva was collected. All samples were collected in ice-chilled glass tubes and immediately frozen. All sampling was performed at the same time of the day, just before lunch. Before use, all samples were thawed, heated at 56°C for 30 min to inactivate degradative enzymes, and centrifuged at 12,500 × *g* for 20 min. A reference pool of citric acid-stimulated parotid saliva from 15 females and 1 male, 21 to 42 and 56 years of age, respectively, was collected before the study.

Bacterial samples. Before freezing, 1 ml from the whole saliva samples was transferred to viability-preserving medium (VMG II; 24) and cultivated by the micromethod described by Westergren and Krasse (30). The colony-forming units (CFU) of *Streptococcus sanguis* and *S. salivarius* were enumerated after cultivation on mitis salivarius (Difco Laboratories, Detroit, Mich.) agar plates incubated aerobically for 48 h at 37°C. The CFU of *Streptococcus mutans* was determined after cultivation on mitis salivarius-bacitracin agar (16), which was incubated in an atmosphere of 96% N_2 and 5% CO_2 for 48 h at 37°C. Serotyping of *S. mutans* was performed according to Bratthall (6). Antisera were kindly supplied by D. Bratthall, Department of Cariology, University of Lund, Malmö, Sweden.

Total amount of IgA. The total amount of IgA was determined by single radial immunodiffusion (SRID) (21), using rabbit anti-human IgA (α-chain specific) serum and stabilized human serum (Beringwerke AG) as the standard.

ELISA. Salivary IgA antibodies reacting with antigens from *S. mutans* strains JC2 (=KPSK2) serotype *c* and B13 serotype *d* (6), *S. sanguis* strain 804, and *S. salivarius* strain 8618 were detected by an enzyme-linked immunosorbent assay (ELISA) technique (12). The ELISA was performed as previously described (7) with the following modifications: microtiter plates (Microelisa plates, M 129 A; Dynatech, Novakemi AB, Enskede, Sweden) were used as the solid phase (32). Additions of antigen, conjugate, and enzyme substrate were made with a variable 50- to 200-μl eight-channel

Titertek pipette (no. 77-859-00; Flow Laboratories, Svenska AB, Solna, Sweden). The anti-immunoglobulin conjugate, anti-human IgA specific for α-chains (Dakopatts A/S, Copenhagen, Denmark) conjugated to alkaline phosphatase ($\simeq$400 IU per mg of protein at 25°C; Sigma Chemical Co., St. Louis, Mo.) according to Ahlstedt et al. (1), was diluted 1:500 in 0.01 M phosphate-buffered saline with 0.05% Tween 20 added. All readings were made at 405 nm with a spectrophotometer (model 24; Beckman Instruments AB, Stockholm, Sweden) equipped with a microinjection system for 200-μl samples. Uncoated wells, coated wells without addition of saliva, and coated wells incubated with parotid saliva from a 45-year-old male suffering from hypogammaglobulinemia served as controls. The hypogammaglobulinemic male was selected from a group of immunodeficient patients studied by J. Björkander, Department of Allergology, First Medical Service, Sahlgren's Hospital, Göteborg, Sweden.

The average optical density for duplicate samples was multiplied by $100/t$, where t is the number of minutes after which the color development was stopped. The salivary IgA antibody activity is expressed as a percentage of the reference pool run on each microtiter plate.

Variation of the ELISA method. The variation of the ELISA method was determined from duplicate samples of the saliva pool run on 10 microtiter plates (Table 1). For duplicate samples, standard errors of the mean (SEM) were 5 to 12% of the mean absorbance for the different antigen preparations.

Antigen preparations. *S. mutans* strains JC2 and B13 were cultivated in a defined medium (10) and used at a turbidity of 75% of 1 optical density unit (650 nm) to coat the solid phase in ELISA as described before (7). Serotype *d* antigen was prepared from *S. mutans* strain B13, grown in a dialyzed yeast extract medium (11), by the method of Linzer and Slade (19). The crude antigen was prepared by boiling the cells in 0.01 N HCl for 20 min. Further purification was done according to Linzer and Slade (19) except that Sepharose CL6B was used in the final chromatographic step. The serotype *d* extract formed a single precipitin band when analyzed immunoelectrophoretically against a rabbit anti-*S. mutans* serum which produced multiple precipitin bands with Rantz-Randall antigen extracts (25) from *S. mutans* B13. Optimal coating concentration for the serotype *d* extract was 6.5 μg/ml in coating buffer (phosphate-buffered saline, pH 7.1, containing 0.02% NaN_3), and the microtiter plates were incubated for 176 h at 37°C. The long incubation time for this antigen preparation was found to be necessary since shorter incubation times gave uneven ELISA values.

TABLE 1. *Variation of the ELISA method*

Antigen prepn	Mean[a]	SEM[b]	% of the mean
S. mutans JC2[c]	2.57	0.13	5
S. mutans B13[c]	1.45	0.11	8
Serotype *d*-AG[d]	0.65	0.06	9
S. sanguis[e]	0.79	0.09	11
S. salivarius[e]	1.37	0.16	12

[a] Mean of assays conducted in 10 microtiter plates using duplicate samples of the parotid saliva pool (diluted one-fourth).

[b] Standard error of the mean (SEM) of duplicate samples.

[c] Whole cells.

[d] Extract of *S. mutans* B13.

[e] Rantz-Randall extract.

Rantz-Randall extracts were prepared from *S. salivarius* strain 8618 and *S. sanguis* strain 804, which had been cultivated in the dialyzed yeast extract medium aerobically for 48 h at 37°C. Optimal coating concentrations for these antigen extracts were 15 and 20 μg/ml, respectively, when incubated for 3 h at 37°C.

A pool of *Escherichia coli* somatic antigens (1) was kindly supplied by B. Carlsson, Department of Immunology, Institute of Medical Microbiology, University of Göteborg, Göteborg, Sweden. The *E. coli* antigen preparation was coated to the microtiter plates at the same concentrations as used by Ahlstedt et al. (1).

Absorption experiments. In one experiment, 10 ml of parotid saliva from a single subject was fractioned on a Sepharose 2B column (18) in order to separate high-molecular-weight non-immunoglobulin agglutinating factors from the salivary IgA. The fractions containing IgA, as detected by SRID, were pooled and divided into five portions of 6 ml each. Before ELISA analysis, four of these portions were absorbed with *S. mutans* B13 serotype *d*, *S. mutans* JC 2 serotype *c*, *S. sanguis* 804, or *S. salivarius* 8618. The microorganisms were cultivated as described above. The pellet from 10 ml of bacterial suspension (optical density of 1,650 nm) was washed three times in phosphate-buffered saline before absorption. After 1 h at 24°C, the bacteria were spun down and the supernatant was analyzed by ELISA. In a second experiment, PS 2 from one subject was absorbed with *S. salivarius* 8618 as described above.

RESULTS

All five subjects harbored *S. salivarius*, *S. sanguis*, and *S. mutans* serotype *c* (10^3 to 10^5 CFU/ml). *S. mutans* serotype *d* could be recovered in low numbers from two subjects only (no. 4 and 5; 0.2×10^3 to 4.8×10^3 CFU/ml).

In parotid saliva, the mean value of total IgA was approximately the same in all five subjects. However, on different sampling occasions, individual values varied from trace amounts to 0.08 g/liter. In two of the five subjects, the total IgA content was slightly lower in whole saliva than in parotid saliva (Table 2).

Table 3 shows the salivary IgA antibodies reacting with antigens from oral streptococci, expressed as a percentage of that of the reference pool. In general, whole saliva showed lower antibody activity than parotid saliva from the same individual. A considerable variation was observed in all persons and for all antigens. Saliva from subjects 4 and 5, who harbored *S. mutans* serotype *d*, showed a considerably higher antibody activity against the purified serotype *d*

antigen than did the saliva from the persons who did not harbor this bacterium.

Figure 1 shows the variation of antibody activity over time for one of the subjects. In addition to a variation of up to 200% (between day 40 and 70), a clear-cut covariation in antibody activity to the different antigen preparations could be seen. The same type of covariation was also observed in the four other subjects, especially in the parotid saliva.

TABLE 2. *Concentration of IgA in parotid and whole saliva samples collected from the five subjects*

Source	IgA concn (g/liter)	
	Mean[a]	Range
Subject 1		
PS 1	0.03	0.01–0.08
PS 2	0.02	0.01–0.03
Whole saliva	0.03	0.01–0.05
Subject 2		
PS 1	0.02	0.01–0.04
PS 2	0.02	0.01–0.04
Whole saliva	0.01	0.01–0.02
Subject 3		
PS 1	0.03	0.01–0.06
PS 2	0.02	0.01–0.03
Whole saliva	0.02	0.01–0.03
Subject 4		
PS 1	0.03	Tr[b]–0.05
PS 2	0.02	0.01–0.03
Whole saliva	0.02	Tr–0.02
Subject 5		
PS 1	0.03	Tr–0.07
PS 2	0.02	Tr–0.05
Whole saliva	0.01	Tr–0.03

[a] Mean of 12 samples.
[b] Tr, Trace amount.

Figure 2 shows analyses of the PS 2 samples from the same subject by SRID. The total IgA showed the same variations over time as the antibody activity to the various antigen preparations shown in Fig. 1.

The covariation between salivary IgA antibodies was further studied by absorption of the PS 2 fractions from subject 5 (Fig. 3). Absorption with *S. salivarius* 8618 dramatically reduced the IgA antibodies reacting with the antigen preparation from this bacterium. Only two samples (no. 4 and 7) showed an antibody activity slightly higher than 20% of the reference pool. Antibodies reacting with whole cells of *S. mutans* JC2 (serotype *c*) could not be reduced by absorption with *S. salivarius* 8618.

Absorption of the IgA fraction from parotid saliva separated on Sepharose 2B with the various strains reduced the antibody reaction to the different antigen preparations (Table 4). The most pronounced reduction was observed for absorption with the homologous strain.

DISCUSSION

In all subjects, a considerable variation in IgA

TABLE 3. *Salivary IgA antibodies reacting with antigens of oral streptococci*

Source	Reaction (% of activity in reference pool of parotid saliva)									
	S. mutans JC2[a]		*S. mutans* B13[a]		Serotype *d*-AG[b]		*S. sanguis*[c]		*S. salivarius*[c]	
	Mean[d]	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Subject 1										
PS 1	145.9	86.4–247.5	148.9	81.6–230.8	69.2	39.1–156.2	68.2	26.5–100.0	64.3	33.3–126.2
PS 2	103.3	35.5–180.2	113.3	22.4–213.5	44.1	<20.0[e]–74.3	36.6	<20.0–77.6	43.6	<20.0–81.6
Whole saliva	124.9	72.7–223.8	94.5	38.8–157.7	24.9	<20.0–42.9	21.6	<20.0–40.6	<20.0	
Subject 2										
PS 1	34.7	<20.0–46.7	39.5	22.0–61.6	126.5	50.1–223.1	28.9	<20.0–62.8	38.2	22.8–53.6
PS 2	34.8	<20.0–61.7	40.3	<20.0–75.9	132.8	64.4–286.4	26.0	<20.0–47.1	40.4	<20.0–61.1
Whole saliva	<20.0	<20.0–23.0	<20.0		28.3	<20.0–57.1	<20.0		<20.0	
Subject 3										
PS 1	50.8	<20.0–111.3	65.9	41.9–147.3	91.1	24.9–312.8	81.2	48.0–171.5	157.2	76.8–375.1
PS 2	35.1	20.1–50.1	54.9	27.3–121.0	68.4	22.4–225.5	59.2	<20.0–144.4	127.7	41.3–282.8
Whole saliva	<20.0	<20.0–26.0	<20.0		<20.0	<20.0–66.8	<20.0	<20.0–22.9	<20.0	
Subject 4										
PS 1	76.4	41.6–104.9	96.0	31.1–180.6	240.4	113.1–399.0	329.7	147.5–625.9	184.1	59.0–433.1
PS 2	59.1	24.5–83.7	71.2	36.6–118.0	189.2	77.9–326.5	237.5	91.5–471.7	137.3	43.2–230.1
Whole saliva	25.6	<20.0–57.1	24.3	<20.0–39.5	56.9	<20.0–133.7	69.2	34.1–175.2	<20.0	<20.0–80.8
Subject 5										
PS 1	75.4	55.6–109.6	185.6	114.6–309.9	316.2	177.7–555.9	103.6	34.1–221.8	143.8	73.0–230.9
PS 2	73.4	57.6–123.7	150.8	112.6–314.6	235.5	168.8–366.9	72.2	42.8–135.1	111.6	69.8–209.7
Whole saliva	32.4	21.2–46.3	33.2	<20.0–48.1	69.6	43.8–146.9	<20.0		<20.0	<20.0–34.2

[a] Whole cells. See Materials and Methods.
[b] Prepared from *S. mutans* B13. See Materials and Methods.
[c] Rantz-Randall extract. See Materials and Methods.
[d] Mean of 12 samples of saliva diluted one-fourth.
[e] Antibody activity less than 20% of the reference was considered as negative, because saliva samples from a hypogammaglobulinemic male occasionally showed an activity of 10% of the reference.

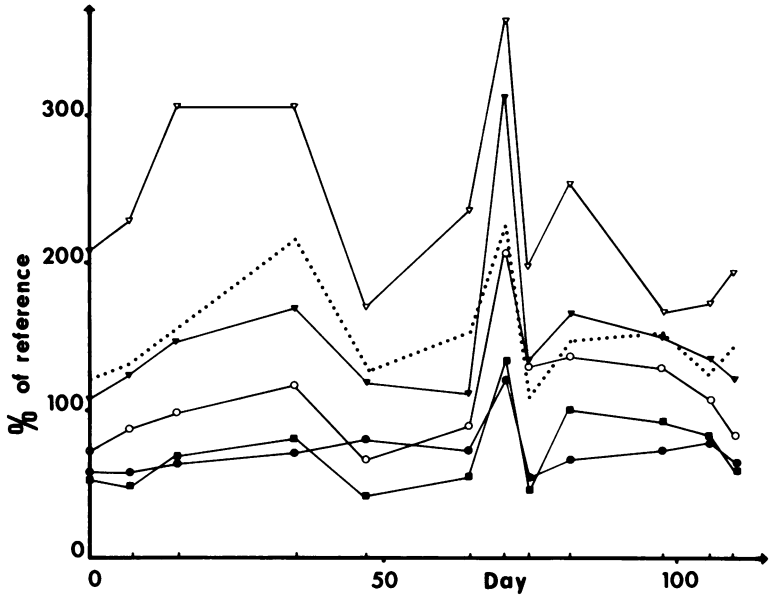

FIG. 1. *Relationship between salivary IgA antibodies reacting with antigens of four oral streptococci and antigens from E. coli in parotid saliva (PS 2, diluted one-fourth) from subject 5. IgA antibodies reacting with* (▽) *a serotype d antigen preparation from S. mutans B13;* (▼) *whole cells of S. mutans B13;* (●) *whole cells of S. mutans JC2;* (■) *a Rantz-Randall extract from S. sanguis 804;* (○) *a Rantz-Randall extract from S. salivarius 8618;* (· · ·) *an antigen pool prepared from eight strains of E. coli (1).*

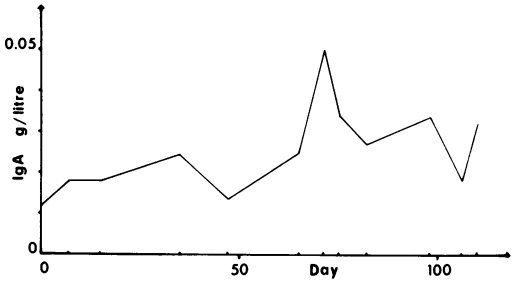

FIG. 2. *Concentration of total IgA (grams/liter) in parotid saliva samples (PS 2, diluted one-fourth) from subject 5.*

antibody reactive with antigen preparations from oral microorganisms was found in saliva samples collected over a period of 4 months. A similar variation was also found for total IgA in both parotid and whole saliva. Whole saliva showed considerably lower antibody activity than parotid saliva to all of the tested antigens, a finding which might be the result from absorption of antibodies by the microorganisms in paraffin-stimulated saliva.

Salivary IgA antibody activity and total IgA in parotid saliva collected during the first 10 min of stimulation (PS 1) were either higher than or similar to the salivary IgA antibody activity and total IgA in parotid saliva collected immediately afterwards. This indicates that salivary IgA may be stored in the parotid gland. However, the variation in salivary IgA activity and total IgA was not reduced in the samples which had been collected immediately afterwards, i.e., PS 2. This suggests that factors other than storage of IgA influence the variation observed in this and other studies (5, 8, 17). One such factor is the secretion rate. Previous investigators (5, 7, 27) have found an inverse relationship between flow rate and salivary IgA concentration. However, this inverse relationship may not be valid for flow rates above 1 ml/min, since there is often a change of slope at high flow rates when concentration is plotted against flow rate (27). In the present study, we used a highly standardized sampling technique, but the flow rate could not be kept below 1 ml/min in all individuals. For this reason, we did not express the salivary IgA in relation to flow rate, i.e., as percentage of activity per minute or micrograms of IgA per minute.

Since a clear-cut covariation exists between salivary IgA antibody reactive with various antigens and total IgA, the salivary immune status in humans would better be expressed as the ratio between a specific antibody activity and total IgA. However, it must be stressed that a varying secretion rate will influence the total amount of antibody secreted in an individual. This fact has

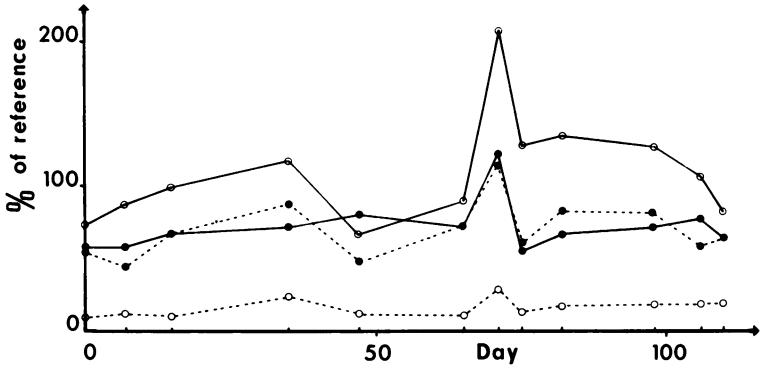

FIG. 3. *Effect of absorption with S. salivarius 8618 on IgA antibodies in the second fraction of parotid saliva (PS 2) from subject 5. IgA antibodies reacting with (—○—) antigens from S. salivarius 8618 in unabsorbed saliva; (—●—) whole cells of S. mutans JC 2 (serotype c) in unabsorbed saliva; (---○---) antigens from S. salivarius 8618 in saliva absorbed with S. salivarius 8618; (---●---) whole cells of S. mutans JC 2 (serotype c) in saliva absorbed with S. salivarius 8618.*

TABLE 4. *ELISA values for unabsorbed and absorbed fractions of parotid saliva from one subject*[a]

Antigen used in ELISA	ELISA value[b]				
		Absorption with:			
	Unabsorbed	*S. mutans* B13	*S. mutans* JC2	*S. sanguis* 804	*S. salivarius* 8618
Serotype *d*-AG[c]	0.74	0.28	0.68	0.60	0.53
S. mutans B13[d]	1.52	0.46	1.12	0.90	0.64
S. mutans JC2[d]	0.90	0.38	0.23	0.58	0.82
S. sanguis 804[e]	0.62	0.57	0.55	0.40	0.52
S. salivarius 8618[e]	1.64	1.13	1.62	1.11	0.17
E. coli[f]	0.45	0.35	0.34	0.32	0.38

[a] Parotid saliva was fractionated to eliminate unspecific absorption of salivary IgA complexed with salivary bacterial agglutinating glycoproteins (13; J. Olsson, D. Bratthall, and A. Carlén, Acta Odontol. Scand., in press).

[b] Expressed as mean absorption of duplicate × $(100/t)$, where t is the time (in minutes) after which the enzyme-substrate reaction was stopped.

[c] Prepared from *S. mutans* B13.

[d] Whole cells.

[e] Rantz-Randall extract.

[f] Somatic antigen pool prepared from eight strains of *E. coli*.

to be considered when the effect of salivary antibodies in the oral cavity is studied.

In this study, single radial immunodiffusion (21) was used to determine total IgA. It has been reported that SRID values for a total IgA in parotid saliva should be multiplied by a factor of 3 when a serum standard is used in order to compensate for the different diffusion rates of polymeric secretory IgA and monomeric IgA in saliva (5). However, recently Mandel (22) has clearly shown that multiplication by a factor of 3 could lead to erroneous results because the standard curves for IIS IgA and 7S IgA are often not parallel. For this reason, our values have not been converted. Since parotid saliva contains both monomeric and polymeric IgA (5), there is need for methods to measure total salivary IgA independent of the different diffusion properties of 7S and polymeric IgA. Preliminary results in our laboratory indicate that the ELISA technique could be used for such measurements. In addition to secretion rate, such factors as hormonal variations and antigenic drift could explain the intraindividual variations over time. However, in this study, no influence of the menstrual cycle was observed either on specific antibody reactions or total IgA. Similar observations with regard to total IgA have been reported by other investigators (20).

It should be observed that the curves in Fig. 1 are not absolutely parallel. One explanation for this could be that antigenic drift of indigenous microorganisms affects the salivary IgA antibody activity, as suggested by Bratthall and Gibbons (8). Another explanation would be simply methodological variations. In our opinion, the most likely explanation is the latter. This assumption is supported by the observation that antigenic changes in microorganisms do not necessarily affect the local antibody response (I. Mattsby Baltzer, Ph.D. thesis, University of Göteborg, Göteborg, Sweden, 1980). The observed covariation could be the result of cross-reacting antibodies, because oral streptococci are known to share common antigens (9). However, absorption of the saliva eliminated the antibodies reacting with *S. salivarius* strain 8618 but did not markedly affect the antibodies reacting with *S. mutans* antigens (Fig. 3). A covariation was also observed for antibodies reacting with an *E. coli* antigen. This antigen prepa-

ration does probably not contain antigens cross-reacting with the oral streptococci, a hypothesis supported by the absorption findings (Table 4). Consequently, cross-reacting antibodies do not explain the covariation between antibodies reacting with antigens from oral streptococci and with *E. coli* antigens (Fig. 1).

Our findings show that salivary IgA antibodies reactive with antigens of oral streptococci may show great variations in saliva samples collected on different occasions. The covariation of specific IgA antibodies and total IgA in saliva suggests that the salivary IgA response to antigens of oral streptococci is fairly constant. These findings imply that reference antigens, preferably in combination with a defined method for measurement of total IgA, ought to be used when studying the salivary IgA response to particular antigens and clearly warrant consideration when interpreting the results of active immunization.

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