INFECTION AND IMMUNITY, Dec. 1995, p. 4695–4703 Vol. 63, No. 12
0019-9567/95/$04.00+0

An Antigenic Peptide Inducing Cross-Reacting Antibodies Inhibiting the Interaction of *Streptococcus mutans* PAc with Human Salivary Components

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Received 13 March 1995/Returned for modification 27 April 1995/Accepted 12 September 1995

A 190-kDa surface protein antigen (PAc) of *Streptococcus mutans*, in particular the A region of this molecule, may be implicated in the induction of dental caries via an interaction with salivary components. For this reason, it was probably used successfully as an antigenic component for experimental vaccination to prevent dental caries in animals. While developing a synthetic peptide vaccine for dental caries, as reported herein, we have identified a unique peptide, TYEAALKQYEADL, as a candidate vaccinal immunogen. The amino acid sequence of this peptide completely corresponds to the sequence of a B-cell epitope in the A region of PAc and additionally contains its own T-cell epitope for B10.D2 mice within the molecule. This peptide strongly induces the production of only cross-reacting antibodies against PAc. In addition, as demonstrated by surface plasmon resonance analysis using the BIAcore system, these cross-reacting antibodies inhibit approximately 50% of the binding of fluid-phase salivary components to immobilized recombinant PAc.

A 190-kDa surface protein antigen (PAc) (27) of *Streptococcus mutans* serotype c, which has been variously termed streptococcal antigen I/II (31), B (32), P1 (6), SR (1), and MSL-1 (5), may be involved in the induction of dental caries and so was successfully used as an antigenic component for experimental vaccination to prevent dental caries in animals (15, 16, 40). The two main criteria for the acceptance of a vaccine are its safety and efficacy. For dental caries in particular, which is fortunately not a lethal disease, the vaccine must be absolutely safe; i.e., it should not possess any epitope that elicits an autoimmune response or present any other danger to human health. With regard to these properties, studies aimed at developing synthetic vaccines against viral infections (2, 24) indicate that a peptide vaccine may be among future vaccines for prophylactic dentistry.

Recently, in terms of the design of synthetic vaccines, an alanine-rich repeating region (A region; amino acid residues 219 to 464) of the PAc molecule may be an effective antigenic region for the immunological prevention of dental caries (40), given the strong antigenicities of this region (29, 36) and probably the interactions with salivary components (3, 4, 8, 21, 23). Takahashi et al. (40) investigated the immunogenicities of several synthetic peptides designed speculatively from the nucleotide sequence of the gene for PAc (28) and demonstrated that intranasal immunization of mice with a PAc(301-319) peptide corresponding to a part of the A region elicits strong suppression of the colonization of murine teeth by *S. mutans*. On the basis of these findings, to determine antigenic peptides effective for the immunological prevention of dental caries, the many antigenic determinants in the PAc molecule, including the A region, have been determined by epitope scanning analysis using multiple pin-coupled peptide technology (19, 29, 39). However, the immunogenicities of the peptides containing those antigenic determinants are still unclear.

Using a complete set of purified sequential overlapping 19-mer peptides covering the entire sequence of the A region of PAc instead of the previous pin-coupled peptide technology, we examined not only antigenic determinants (B-cell epitopes) but also T-cell epitopes in this region and the immunogenicities of several selected peptides in B10.D2 mice. As a result, we selected one of the effective antigenic 19-mer peptides containing T-cell and B-cell epitopes for the production of cross-reacting antibodies to the recombinant PAc (rPAc) protein and succeeded in the truncation of this peptide to a 13-mer peptide which still maintained the desired immunological properties. In addition, the inhibitory effect of the antiserum raised against this peptide on the binding of fluid-phase salivary components to immobilized rPAc was determined by real-time biospecific interaction analysis with the BIAcore system (10, 39).

MATERIALS AND METHODS

Mice. B10.D2 mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). Female mice, 6 to 8 weeks of age, were used in all studies.

Human saliva. Whole saliva was collected from a male donor 31 years old by paraffin stimulation into a chilled container and was clarified by centrifugation at 10,000 × *g* for 10 min at 4°C.

rPAc and rPAc fragments. rPAc protein was obtained from the culture supernatants of transformant, serotype c *S. mutans* TK 18 by ammonium sulfate precipitation and purified by chromatography on DEAE-cellulose (11, 26). rPAc fragments were prepared as fusion proteins with β-galactosidase by using expression plasmid vector pAX4a+ (Medac, Hamburg, Germany) (29). The fusion proteins were purified by chromatography on Sephacryl S-300 (Pharmacia) or *p*-aminophenyl-1-thio-β-D-galactopyranoside affinity chromatography as instructed by the manufacturer (Medac). After digestion with factor Xa (Boehringer, Mannheim, Germany), the rPAc fragments were further purified by chromatography on Sephadex G-100 (Pharmacia) and identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blotting (immunoblotting) analysis with a rabbit polyclonal antiserum to rPAc (28). The amino acid positions in the rPAc molecule of these rPAc fragments were as follows: rPAc-1, residues 39 to 217; rPAc-2, residues 200 to 481; and rPAc-3, residues 470 to 749.

Synthetic peptides. The peptides used in this study were designed in accordance with the amino acid sequence of PAc (28), synthesized by a stepwise solid-phase procedure using a model 350 multiple peptide synthesizer (Advanced Chemtech, Louisville, Ky.) (20), and purified by reverse-phase high-performance liquid chromatography (HPLC) on a TSK-GEL column (1 by 30 cm; Toso, Tokyo, Japan). To confirm the amino acid sequence of each of the synthetic peptides, several peptides were selected by random sampling and further ana-

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lyzed by using a System 7300 amino acid analyzer (Beckman) and model 477A protein sequencer (Applied Biosystems, Foster City, Calif.). All synthetic peptides used in the study were >95% pure as judged by analytical reverse-phase HPLC.

Purification of human salivary sIgA1. A sample of human salivary secretory immunoglobulin A1 (sIgA1) was obtained by affinity purification with a Sepharose 4B-jacalin (Pharmacia Fine Chemicals, Uppsala, Sweden) (12, 33). Briefly, the clarified human whole saliva (5 ml) was mixed with Sepharose 4B-jacalin (2.5 ml) and incubated with rotation for 2 h at 4°C. After removal of the nonabsorbed salivary components by centrifugation for 5 min at 2,000 × *g*, the sIgA1 binding to Sepharose 4B-jacalin was washed two times with 10 ml of phosphate-buffered saline (PBS). The bound sIgA1 was eluted by a 30-min rotating incubation at 37°C with 12.5 ml of 0.1 M melibiose in PBS and concentrated by ammonium sulfate precipitation. Since the antibodies against rPAc in the whole saliva might be classified as not only sIgA1 but also sIgA2 and some other immunoglobulins, the enzyme-linked immunoassay antibody (ELISA) antibody titer to rPAc in the affinity-purified sIgA1 preparation was adjusted to that (10^8) in the original whole saliva by dilution with PBS before use in the control experiments.

Immunization and collection of antisera. B10.D2 mice (groups of three) were immunized subcutaneously with 25 μg of the rPAc protein, the rPAc-1, -2, and -3 fragments, or various synthetic peptides, emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.). Two weeks later, the mice were boosted by subcutaneous injection with the same dose of the immunizing antigen emulsified in Freund's incomplete adjuvant (Difco). One week later, serum samples were obtained from clotted blood by centrifugation and used as antisera. The presence of specific antibodies in these antisera was tested by immunogen-specific ELISA. In the case of immunization with rPAc-2, spleen T cells were prepared from mice after collection of blood and then used for T-cell proliferation assays. The antisera or the spleen cells from the animals in each group were mixed and used for the experiments. The pooled serum from sham (PBS)-immunized B10.D2 mice was used as a control.

ELISA. For the ELISA, 96-well microtiter H-plates (Sumitomo Bakelite, Tokyo, Japan) were coated overnight at 4°C with 100 μl of rPAc or various synthetic peptides (each at 10 μg/ml) in 50 mM carbonate buffer (pH 9.6). The plates were washed with PBS containing 0.1% (vol/vol) Tween 20 (PBST) and blocked with 1% (wt/vol) skim milk in PBST for 1 h at 37°C. Excess skim milk was removed by three washes with PBST, 100-μl aliquots of twofold serial dilutions of murine antiserum were added to the wells, and the mixtures were incubated overnight at 4°C. The wells were then washed five times with PBST and further incubated for 1 h at 37°C with 100 μl of alkaline phosphatase-conjugated goat anti-mouse IgG (heavy and light chains) antiserum (Zymed Laboratories, South San Francisco, Calif.). After five washes with PBST, bound antibodies were detected by the addition of 100 μl of *p*-nitrophenyl phosphate (1 mg/ml) as the substrate and incubation for 1 h at 37°C. A_{405} was measured with a microplate reader (MPR A4; Tosoh, Tokyo, Japan). The ELISA antibody titer was expressed as the reciprocal ($\log_2$) of the highest dilution giving an A_{405} of 0.1 above that of the conjugate control (without coated antigen) after 1 h of incubation with the substrate. In the inhibition assay for binding of serum antibodies to the PAc(361-379) peptide, a 1/64 dilution of murine antiserum was allowed to react with various concentrations of a competitor, a truncated peptide of PAc(361-379), for 1 h at 37°C. After standing overnight at 4°C, the reaction mixtures were added to the PAc(361-379) peptide-coated ELISA plate (100 μl per well) and incubated for 1 h at 37°C. The bound antibodies were detected as described above. The percent inhibition of ELISA was calculated as $100 \times \{[A_{405}\ (\text{antiserum}) - A_{405}\ (\text{antiserum + competitor})]/[A_{405}\ (\text{antiserum})]\}$.

T-cell proliferation assay. B10.D2 mice (groups of three) were immunized subcutaneously with 25 μg of PAc(361-379) 19-mer peptide emulsified in Freund's incomplete adjuvant. One week later, the mice were boosted under the conditions used for primary immunization. Seven days later, spleen T cells were fractionated with a nylon fiber (Wako Pure Chemical Industries Ltd., Osaka, Japan) column (9) and adjusted to a concentration of 5×10^6 cells per ml in RPMI 1640 medium (GIBCO Laboratory, Grand Island, N.Y.) supplemented with 2 mM L-glutamine (GIBCO), 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, 20 mM sodium bicarbonate (Wako), 100 U of penicillin per ml, 100 μg of streptomycin per ml, 5×10^{-5} M 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum. One hundred microliters of the T-cell-enriched fraction (5×10^5 cells per well) was cultured with 100 μl of the above-described medium containing test peptide (0, 5, and 10 μg per well) and irradiated syngeneic spleen cells (3,000 rads, 5×10^5 per well) under a humidified atmosphere of 5% CO_2 in air for 3 days, and pulsed for a final 6-h cultivation with 1 μCi of [^{3}H]thymidine per well (ICN Radiochemicals, Irvine, Calif.); the cells were then harvested onto glass fibers by using a multiple-cell harvester. Radioactivity incorporated into proliferating cells was determined by liquid scintillation counter. All tests were carried out in triplicate. The proliferative response was expressed as the mean counts per minute incorporated ± standard deviation (SD) or stimulation index, which is the ratio of the mean counts per minute incorporated in the presence of antigen to the mean counts per minute obtained in the absence of antigen.

Determination of the interaction between the PAc molecule and human salivary components. The binding of fluid-phase salivary components to PAc protein was determined by using the BIAcore biosensor system (Pharmacia Biosensor AB, Uppsala, Sweden), which is a proven method to permit real-time interaction analysis of two interacting macromolecules (10, 38). In general, the use of this system offers some advantages over more conventional methods. The interaction is monitored directly as it proceeds. This allows semiquantitative comparison of different analyte-ligand interactions, which is often sufficient for many evaluation purposes. Concentration analysis may be performed on a kinetic rather than equilibrium basis, giving a shorter assay time. The biospecific surface can be regenerated so that a series of different analyses can easily be done under identical conditions. Thus, the results of different analyses are easily compared in overlay plots, directly visualizing relative interaction kinetics.

rPAc protein was immobilized to a carboxymethylated dextran-coated gold surface of a CM5 sensor chip via primary amino groups. The dextran surface was activated by injecting 30 μl of 400 mM *N*-ethyl-*N*′-(3-diethylaminopropyl)carbodiimide–100 mM *N*-hydroxysuccinimide solution at a flow rate of 5 μl/min. After activation, 30 μl of a 300-μg/ml solution of rPAc protein in 10 mM sodium acetate buffer (pH 4.5) was passed over the surface. Noncovalently bound rPAc molecule was washed off with HEPES-buffered saline (HBS; 10 mM HEPES [pH 7.4] containing 150 mM NaCl, 3.4 mM EDTA, and 0.05% Tween 20) for 5 min, and residual *N*-hydroxysuccinimide esters were inactivated with 1 M ethanolamine hydrochloride-NaOH (pH 8.5) for 6 min. A flow of HBS buffer was maintained at 5 μl/min throughout the immobilization procedure.

Twenty microliters of clarified human whole saliva was exposed to the rPAc protein-immobilized surface at a flow rate of 5 μl/min. The dissociation phase was followed during injection of HBS at 5 μl/min. All binding experiments were conducted at 25°C. At the end of each binding cycle, the biosensor surface was regenerated by exposure to 100 mM HCl for 1.2 min.

The BIAcore detection system uses surface plasmon resonance, a quantum mechanical phenomenon which monitors the change in optical properties at the surface of a sensor chip (13) and so eliminates the need to label interactants. The resonance angle depends on the refractive index in the vicinity of the surface, which changes as the concentration of molecules on the surface is modified. The signal is expressed in resonance units (RU). The surface plasmon resonance signal obtained in each binding cycle was recorded as a sensorgram, which is a real-time pattern with a sampling interval of 0.2 to 0.5 s plotted in RU versus time. The amounts of binding were represented as the increase of RU between the start and end of each binding cycle. A response of 1,000 RU corresponds to a shift of 0.1° in the resonance angle, which in turn represents a change in surface protein concentration of about 1 ng/mm^2 (17).

Inhibition of the binding of fluid-phase salivary components to immobilized rPAc protein by the murine antisera. Inhibition studies were carried out by injecting 20 μl of murine antisera to immobilized rPAc protein for 4 min at 25°C prior to injection with whole saliva. Percent inhibition of binding of salivary components to rPAc protein by the antiserum was calculated as $100 \times \{[\text{RU (rPAc + saliva)} - \text{RU (rPAc + antiserum + saliva)}]/[\text{RU (rPAc + saliva)}]\}$.

RESULTS

Distribution of T-cell and B-cell epitopes in the A region of the PAc molecule. The A region of the PAc molecule is a strong antigenic region in both the murine (29, 39) and human (19, 36) immune systems. We therefore synthesized a set of overlapping 19-mer peptides covering the entire sequence of the rPAc-2 fragment, including the A region (residues 219 to 464), and examined the distribution of T-helper-cell recognition sites (T-cell epitopes) and antigenic determinants (B-cell epitopes) in this region. The results are summarized in Table 1.

Many peptides enhanced the proliferation of T cells from B10.D2 mice immunized with the rPAc-2 fragment, and many peptides reacted to the serum antibodies of these mice. Some peptides, such as PAc(196-214), PAc(355-373), and PAc(361-379), significantly reacted to the serum antibodies but showed a very weak proliferation of rPAc-2-specific T cells. Some peptides, such as PAc(219-237), PAc(226-244), and PAc(241-259), significantly enhanced T-cell proliferation but reacted to the anti-rPAc-2 antibodies very weakly. Peptides reacting to both T-cell proliferation and the serum antibodies were also observed [e.g., PAc(331-349)]. Among these peptides, the dominant B-cell epitopes in the rPAc-2 fragment were found in the PAc(361-379) and PAc(407-425) peptides, while the dominant T-cell epitopes were found in the PAc(226-244) and PAc(219-237) peptides.

Immunogenicities of the synthetic 19-mer peptides for B10.D2 mice. Immunogenicities of the peptides were examined by immunization of B10.D2 mice with a test peptide. The ELISA antibody titers of the antisera were determined against

TABLE 1. Serum antibodies and T-cell responses to synthetic peptides in B10D2 mice immunized with the rPAc-2 fragment

Peptide[a]	Antibody response[b]	T-cell response[c]
PAc(196-214)	2.02 ± 0.14	1.70 ± 0.55
PAc(211-229)	0.04 ± 0.01	1.60 ± 0.53
PAc(226-244)	0.18 ± 0.02	3.90 ± 0.02
PAc(241-259)	0.06 ± 0.01	3.00 ± 0.07
PAc(256-274)	0.06 ± 0.01	1.80 ± 0.24
PAc(271-289)	0.04 ± 0.01	1.90 ± 0.12
PAc(286-304)	0.04 ± 0.01	1.21 ± 0.29
PAc(301-319)	0.16 ± 0.03	2.03 ± 0.02
PAc(316-334)	0.33 ± 0.03	1.90 ± 0.51
PAc(331-349)	2.86 ± 0.01	2.93 ± 0.37
PAc(346-364)	0.07 ± 0.01	2.20 ± 0.14
PAc(361-379)	>3.00	1.60 ± 0.11
PAc(376-394)	0.06 ± 0.01	1.60 ± 0.46
PAc(391-409)	0.08 ± 0.01	2.50 ± 0.04
PAc(407-425)	>3.00	2.30 ± 0.18
PAc(421-439)	0.19 ± 0.02	1.60 ± 0.24
PAc(436-454)	0.04 ± 0.01	2.50 ± 0.03
PAc(451-469)	0.45 ± 0.02	1.90 ± 0.20
PAc(466-484)	0.04 ± 0.01	1.60 ± 0.04
PAc(204-222)*	0.04 ± 0.01	0.87 ± 0.05
PAc(219-237)*	0.05 ± 0.01	2.95 ± 0.14
PAc(264-282)*	0.12 ± 0.01	2.40 ± 0.25
PAc(279-297)*	1.49 ± 0.06	1.89 ± 0.02
PAc(355-373)*	0.68 ± 0.09	1.10 ± 0.15
PAc(370-388)*	0.04 ± 0.01	1.20 ± 0.19
rPAc-2	>3.00	7.91 ± 0.40

[a] Nineteen sequential overlapping (four amino acid residues) 19-mer peptides covering the PAc(196-484) and six 19-mer peptides (*) containing the same four overlapping amino acid residues in the middle of the molecules were designed as described by Okahashi et al. (28).

[b] A 1/64 dilution of the antiserum from mice immunized with the rPAc-2 fragment was added in peptide-coated microtiter plates. Binding of the antibodies to the peptides was determined by ELISA. The A_{405} was measured after 1 h of incubation with the substrate for alkaline phosphatase at 37°C. The results are expressed as mean absorbance ± SD from triplicate assays.

[c] T cells (5×10^5 per well) from mice immunized with the rPAc-2 fragment to obtain the antiserum were cultured with a test peptide (10 μg per well) and irradiated syngenic spleen cells (3,000 rads, 5×10^5 cells per well). The background counts per minute when cells were cultured with medium alone was 1,115.3 ± 140.7. The proliferative responses were expressed as mean stimulation index ± SD from triplicate tests.

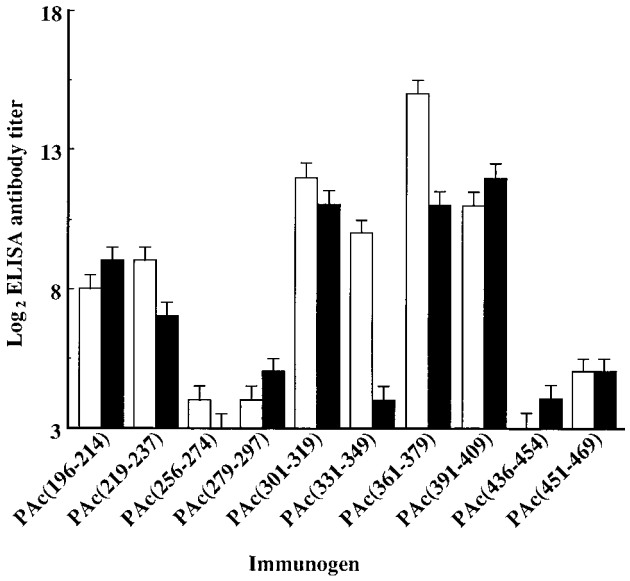

FIG. 1. Serum antibody responses to the immunogen (open bars) and rPAc (closed bars) in mice immunized with various synthetic peptides. Binding of antibodies to synthetic peptides was detected by ELISA. Results are expressed as means ± SD of the reciprocal titers ($\log_2$) of the lowest dilution that gives an absorbance of 0.1 above the conjugate control (no antigen coat) for triplicate assays.

the immunogen itself and against the whole rPAc protein. The results are shown in Fig. 1. Antisera from mice immunized with the PAc(301-319), PAc(361-379), and PAc(391-409) peptides showed very high ELISA antibody titers against both the immunogen and rPAc protein. The PAc(331-349) peptide also had strong antigenicity but very weakly induced cross-reacting antibodies to the rPAc molecule. On the other hand, the PAc(256-274), PAc(279-297), PAc(436-454), and PAc(451-469) peptides possessed very weak immunogenicity, at least in mice of this strain (Fig. 1). In these analyses, there was no significant correlation between the degree of immunogenicities of the peptides and the reactivities of the peptides to the T-cell and/or antisera from the mice immunized with the rPAc-2 fragment.

Analyses of T-cell and B-cell epitopes on the PAc(361-379) 19-mer peptide. Since the PAc(361-379) peptide possessed strong immunogenicity and could strongly induce cross-reacting antibodies to the rPAc molecule, we further investigated the precise T- and B-cell epitopes on this peptide, using 13 truncated synthetic peptides of the PAc(361-379) peptide listed in Table 2.

The T-cell-enriched fraction from the spleen cells of B10.D2 mice immunized with the PAc(361-379) peptide was used in the PAc(361-379)-specific T-cell proliferation assay for the truncated peptides. The results are shown in Fig. 2. The peptide, PAc(361-379), used as a positive control induced remarkable T-cell proliferation. Although the incorporated radioactivities found differed considerably, T-cell proliferation was significantly stimulated by the addition of the PAc(361-375), PAc(363-377), PAc(365-376), PAc(365-377), PAc(365-379),

TABLE 2. Analyses of T-cell and B-cell epitopes on the PAc(361-379) peptide

Peptide	Amino acid sequence[a]	% Inhibition of antibody response[b]	% of maximum T-cell proliferation[c]
PAc(361-379)	NAKATYEAALKQYEADLAA	100.0	100.0
PAc(361-370)	NAKATYEAAL	5.6	41.3
PAc(361-375)	NAKATYEAALKQYEA	13.0	61.9
PAc(363-370)	KATYEAAL		31.2
PAc(363-377)	KATYEAALKQYEADL	79.9	83.4
PAc(365-372)	TYEAALKQ		35.8
PAc(365-375)	TYEAALKQYEA	18.0	62.8
PAc(365-376)	TYEAALKQYEAD	19.0	65.0
PAc(365-377)	TYEAALKQYEADL	74.3	76.8
PAc(365-379)	TYEAALKQYEADLAA	100.0	87.1
PAc(366-373)	YEAALKQY		58.7
PAc(366-376)	YEAALKQYEAD	12.2	65.3
PAc(366-377)	YEAALKQYEADL	13.5	
PAc(367-377)	EAALKQYEADL		35.5
PAc(370-379)	LKQYEADLAA	4.9	37.5
None		0.0	28.7

[a] Given in one-letter code.

[b] Relative inhibitory effects of peptides on the binding of the anti-PAc(361-379) peptide. The mean percent inhibition of the positive control, PAc(361-379), 98.8%, was set at 100. The values were calculated from Fig. 3.

[c] T-cell proliferation with the peptides to relative maximum T-cell proliferation (^{3}H incorporation was 6,306.4 ± 433.7 cpm) with the PAc(361-379) peptide as a positive control. The values were calculated from Fig. 2.

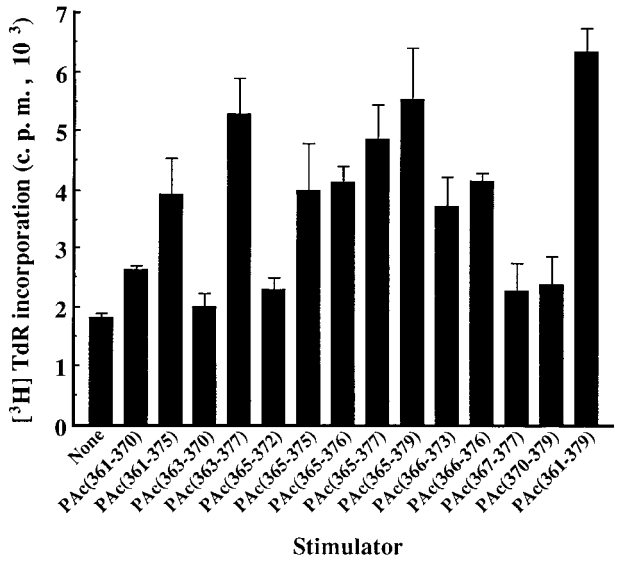

FIG. 2. The PAc(361-379)-specific T-cell response to truncated peptides of PAc(361-379). T cell (5×10^5 cells per well) from mice immunized with the PAc (361-379) peptide were cultured with the test peptide (10 μg per well) and irradiated syngenic spleen cells (3,000 rads, 5×10^5 cells per well). The proliferative responses were expressed as the mean counts per minute ± SD from triplicate tests.

PAc(366-373), or PAc(366-377) truncated peptide. The other truncated peptides exhibited almost no effect.

The precise B-cell epitopes on the PAc(361-379) peptide were also identified by using these truncated peptides. The inhibitory effects of the truncated peptides on the binding of serum antibodies to the PAc(361-379) peptide were determined by an ELISA inhibition assay with the antiserum from B10.D2 mice immunized with the PAc(361-379) peptide. The values for percent inhibition of ELISA with two different concentrations of each peptide are shown in Fig. 3. The mean percent inhibition observed in the positive control, in which the PAc(361-379) peptide was used not only as an ELISA antigen but also as a competitor, was about 98.8 under these experimental conditions. The truncated peptide PAc(365-379) inhibited the binding of serum antibodies to the PAc(361-379) peptide with almost the same magnitude as that of the positive control. In addition, although the inhibitory effects were not equal to that of the positive control, the PAc(363-377) and PAc(365-377) peptides were still found to strongly inhibit the binding of antibodies to the PAc(361-379) peptide. The other truncated peptides exhibited less than 20% inhibition.

The results obtained from these T-cell and B-cell epitope analyses using the truncated peptides are summarized in Table 2. The common amino acid sequence among the peptides that induced more than 55% of the maximum T-cell proliferation was YEAALKQY (residues 366 to 373). This sequence therefore seems to be a T-cell epitope core structure on the PAc(361-379) peptide in B10.D2 mice and probably includes the specific sites for binding to the antigen-presenting cells. Similarly, the common amino acid sequence among the peptides that induced more than 75% of the maximum proliferation was TYEAALKQYEADL (residues 365 to 377). On the other hand, the common amino acid sequence among the peptides exhibiting a significant inhibitory effect (≧70%) on the binding of serum antibodies to the PAc(361-379) peptide was TYEAALKQYEADL (residues 365 to 377) and must be a specific region for the B-cell epitopes recognized by the antibodies raised against the PAc(361-379) peptide. These results demonstrated that the T-cell epitope of the PAc(361-379) peptide seems to be involved in the region for the B-cell epitopes of this peptide, which raised the question of whether the shortened peptides, such as PAc(365-377), PAc(361-375), and PAc (361-373), could be immunogens for the induction of antibodies reacting not only to their own peptide but also to the PAc molecule.

We therefore immunized the B10.D2 mice with the shortened peptides and examined the antigenicities of the peptides by ELISA, in which the PAc(361-379) peptide was used as a coating antigen instead of the shortened peptides, which seemed to be inadequate for this purpose. In fact, we obtained apparently low ELISA antibody titers from the ELISA with PAc(365-377) peptide-coated plates (data not shown). The results are shown in Fig. 4. The PAc(365-377) peptide had enough antigenicity for the production of cross-reacting antibodies to rPAc protein as well as to the PAc(361-379) 19-mer peptide used as a positive control, although the ELISA antibody titer against its own peptide was decidedly less than that of the positive control. The PAc(361-375) 15-mer peptide and PAc(361-373) 13-mer peptide, which lacked two (Asp and Leu) and four (Glu, Ala, Asp, and Leu) residues at the C-terminal end of the PAc(365-377) peptide, respectively, but still had a part of the B-cell epitope and the T-cell epitope core structure, possessed very weak antigenicities and less production of such cross-reacting antibodies. Thus, as expected, the PAc(365-377) peptide induced mainly the production of cross-reacting antibodies to rPAc rather than non-cross-reacting anti-PAc(365-377) antibodies, in contrast with the PAc(361-379) peptide.

To confirm this finding, the inhibitory effects of rPAc protein on binding of the serum antibodies to a PAc(361-379) peptide were examined by the ELISA inhibition assay. The results are shown in Fig. 5. The binding of anti-PAc(365-377) antibodies to PAc(361-379) used as the ELISA antigen were almost com-

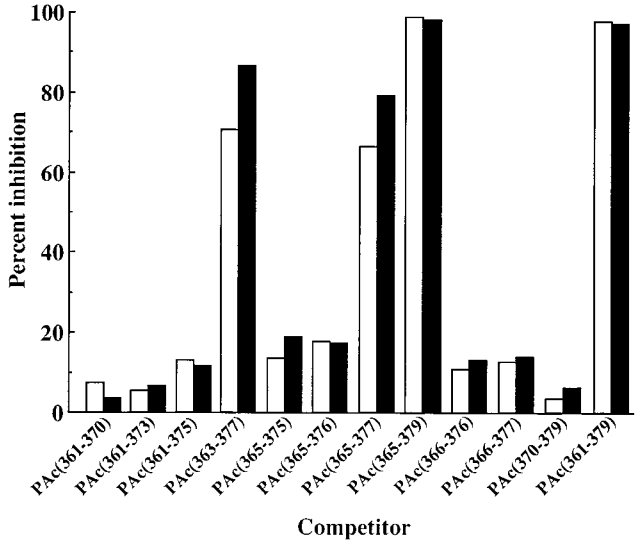

FIG. 3. Competitive inhibition of the binding of anti-PAc(361-379) antibodies to the PAc(361-379) peptide by various truncated peptides of PAc(361-379). Each well of an ELISA plate was coated with 100 μl of a 10-μg/ml solution of the PAc(361-379) peptide. A 1/64 dilution of the serum from mice immunized with PAc(361-379) was allowed to react with 300 μg (experiment 1; open bars) or 1 mg (experiment 2; closed bars) of the test peptides per ml for 1 h at 37°C. After standing overnight at 4°C, the reaction mixtures were added to the PAc(361-379)-coated wells. Binding of antibodies to the peptide was determined by ELISA.

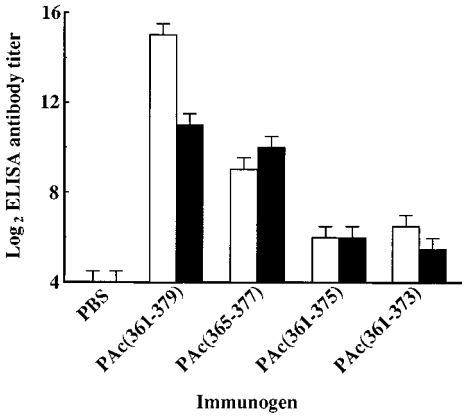

FIG. 4. Serum antibody responses to synthetic peptides PAc(361-379) (open bars) and rPAc (closed bars) in mice immunized with the indicated peptides. Binding of the antibodies to coated antigen was detected by ELISA. Results are expressed as means ± SD of the reciprocal titers (log_2) of the lowest dilution that gives an absorbance of 0.1 above the conjugate control (no antigen coat) for triplicate assays.

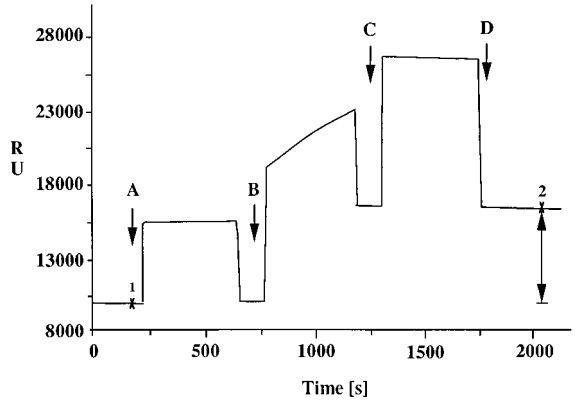

FIG. 6. Sensorgram showing immobilization of rPAc to the sensor surface. The flow rate was 5 μl/min. Arrows indicate the start of each injection. Arrow A, activation of the carboxymethylated dextran matrix by injection of 30 μl of *N*-hydroxysuccinimide–*N*-ethyl-*N'*-(3-diethylaminopropyl)carbodiimide mixture; arrow B, injection of 30 μl of rPAc (300 μg/ml) in 10 mM sodium acetate (pH 4.5); arrow C, inactivation of residual (unreacted) ester groups on the matrix with 30 μl of ethanolamine HCl (pH 8.5); arrow D, washing of excess reagents and unbound rPAc with HBS. The increase of 5,202 RU between points 1 and 2 corresponds to immobilized rPAc (5.2 μg/mm^2).

pletely inhibited with either the PAc(365-377) peptide or the rPAc molecule. In contrast, the rPAc molecule faintly inhibited the binding of anti-PAc(361-379) antibodies; nevertheless, the immunizing peptide, PAc(361-379), exhibited 100% inhibition. In addition, when the rPAc molecule was used as an ELISA antigen in a control experiment, either rPAc or the immunizing peptides inhibited the binding of both antibodies more than 90% (data not shown).

Binding of fluid-phase salivary components to immobilized rPAc protein. rPAc protein was used as a ligand for BIAcore analysis of the interaction between the PAc molecule and fluid-phase human salivary components. A sensorgram for immobilization of the rPAc protein to the sensor chip surface is shown in Fig. 6. The increase of 5,202 RU between the start (point 1) and end (point 2) of the immobilization procedure suggested that approximately 5.2 ng of rPAc protein per mm^2 was bound covalently to the activated carboxymethylated dextran of the biosensor surface.

The sensorgrams shown in Fig. 7 illustrate the binding of salivary components and salivary sIgA1 to the immobilized rPAc protein. The first time point (point 1) represents the baseline response of the immobilized rPAc sensor chip surface to the continuous flow of HBS. The second point indicates the response level of the immobilized rPAc surface after exposure to whole saliva (point 2) or salivary sIgA1 preparation (point 3) to the flow of HBS. The change in surface plasmon resonance between the first and second time points corresponds to the net binding of analytes to the immobilized rPAc on the sensor chip surface. Thus, about 0.7 ng (669 RU) of the salivary components per mm^2 or 0.06 ng (55 RU) of salivary sIgA1 per mm^2 bound to the surface rPAc protein in these experimental conditions. The ELISA antibody titer to rPAc in the affinity-purified salivary sIgA1 preparation used in this experiment was adjusted to that (10^8) in the original whole saliva before use. In addition, bovine serum albumin and α-amylase, also used as

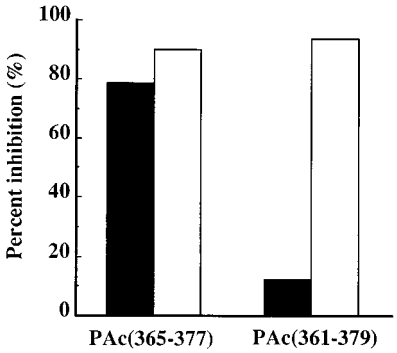

FIG. 5. Competitive inhibition of the binding of the anti-PAc(361-379) or anti-PAc(365-377) antibodies to the PAc(361-379) peptide by the rPAc molecule. Each well of an ELISA plate was coated with 100 μl of a 10-μg/ml solution of the PAc(361-379) peptide. A 1/32 [1/128 for the anti-PAc(361-379) antiserum] dilution of the antiserum raised against the PAc(365-377) peptide was allowed to react with 600 μg of the immunogen (open bars) or rPAc (closed bars) per ml for 1 h at 37°C. After standing overnight at 4°C, the reaction mixtures were added to the PAc(361-379)-coated wells. Binding of antibodies to the peptide was determined by ELISA.

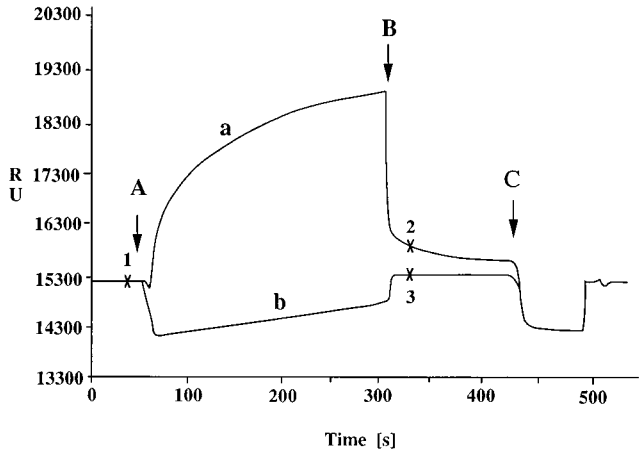

FIG. 7. Sensorgrams illustrating the binding of salivary components (a) and sIgA1 (b) to the immobilized rPAc protein. A total of 5,202 RU of rPAc is immobilized on the sensor surface. Arrows indicate the start of each injection. Injection of 20 μl of whole saliva (arrow A) was followed by injection of HBS (arrow B). Arrow C, washing of the unbound analytes with 100 mM HCl. The increase of 669 RU between points 1 and 2 corresponds to the binding of salivary components to rPAc, and the increase of 55 RU between points 1 and 3 corresponds to the binding of sIgA1 to rPAc.

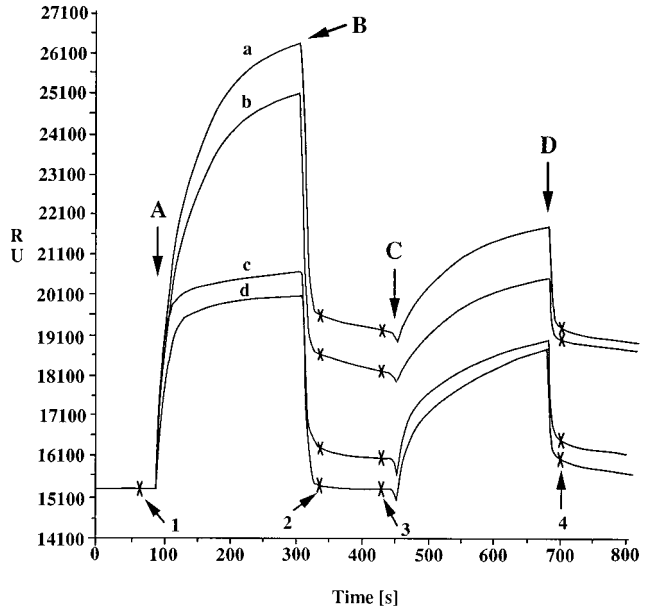

FIG. 8. Inhibitory effects of various antibodies against rPAc on the binding of salivary components to immobilized rPAc. The overlay plots of sensorgrams show the binding of antibodies raised against rPAc-2 (a), rPAc-1 (b), PAc(361-379) peptide (c), or PBS (d) to the immobilized rPAc (points 1 to 2) and the binding of salivary components to the antibodies bearing immobilized rPAc (points 3 to 4). Arrows indicate the start of each injection: 20 μl of an antiserum (arrow A) followed by HBS (arrow B), and 20 μl of whole saliva (arrow C) followed by HBS (arrow D).

control analytes in this experiment, gave increases of 50 to 70 RU (data not shown). These results strongly suggested that the salivary antibodies to rPAc were not the main contributors to the binding of the salivary components to rPAc, at least under the experimental conditions used.

Inhibition of the binding of fluid-phase salivary components to immobilized rPAc by murine antibodies recognizing the rPAc protein. B10.D2 mice were immunized with various antigens related to the PAc molecule, such as rPAc, rPAc fragments (rPAc-1, -2, and -3), and synthetic peptides [PAc(361-379) and PAc(365-377)]. Using the antisera from these immunizations, we examined the inhibitory effects of the serum antibodies against the interaction between the salivary components and rPAc protein in real time by BIAcore analysis. The results are compared in overlay plots in Fig. 8. For instance, the binding of anti-PAc(361-379) antibodies to the immobilized rPAc on the sensor chip surface is illustrated by sensorgram c. Twenty microliters of the antiserum from mice immunized with the peptide was injected at arrow A in Fig. 8. After a wash with HBS (arrow B), 20 μl of whole saliva was injected at arrow C, and then nonassociated components were washed off with HBS (arrow D). A baseline response of the immobilized rPAc (5.2 ng/mm^2) on the sensor chip surface was 15,196 RU, as shown at point 1 in Fig. 8. The difference between points 1 and 2 on sensorgram c was calculated as 819 RU, representing approximately 0.8 ng of antibodies against PAc(361-379) peptide per mm^2 bound to the immobilized rPAc protein. The further increase of 420 RU at point 4 on sensorgram c indicated that about 0.4 ng of the salivary components per mm^2 could bind to the antibodies bearing immobilized rPAc on the sensor chip surface. In control experiments using an antiserum from B10.D2 mice sham immunized with PBS (sensorgram d in Fig. 8), the increases of RU upon exposures to the serum and whole saliva were 61 and 670 RU, respectively. These data clearly show that about 0.7 ng of salivary components per mm^2 can bind to the control serum-treated rPAc on the sensor chip surface. Thus, the percent inhibition of the binding of salivary components to rPAc with the anti-PAc(361-379) antiserum was calculated as 37.2.

The binding amounts and the percent inhibition of various antibodies are summarized in Table 3. A large amount (9 ng/mm^2) of anti-rPAc antibodies in the antiserum from mice immunized with the entire rPAc molecule was able to bind to the immobilized rPAc on the sensor chip surface and strongly (89.3%) inhibited the binding of salivary components to the rPAc protein. A similar high percent inhibition (80.1%) was obtained with the anti-rPAc-2 antibodies in the antiserum from mice immunized with the rPAc-2 fragment, although the total binding capacity of these antibodies to rPAc was about 50% higher (4.6 ng/mm^2) than that of the anti-rPAc antibodies. On the other hand, the antibodies raised against the synthetic peptide PAc(361-379) or PAc(365-377) had only 10% of the total binding capacity of the anti-rPAc antibodies in the antiserum raised against the entire rPAc protein but still considerably inhibited the binding of salivary components to rPAc. In contrast, the antibodies produced by the immunization with rPAc-1 or -3 were able to bind to the immobilized rPAc protein but had almost no effect on the interaction between salivary components and rPAc.

DISCUSSION

For the development of peptide vaccines, specification of an appropriate peptide antigen for vaccination is essential. The peptide antigen must contain both T-cell and B-cell epitopes which are adequate to produce cross-protective antibodies against the target of vaccination (30, 34, 37, 41). Thus, scanning analyses of T-cell and B-cell epitopes in the target molecules by using synthetic peptides are necessary to find a clue to the development of such antigenic peptides, because the synthetic peptides are convenient tools with which to map cross-reactive epitopes within linear segments of immunogens. In this respect, recent scanning analyses of the antigenic (B-cell) epitopes in the PAc molecule carried out with mice (29, 39) and humans (19), using multiple pin-coupled peptide technology, revealed that the antigenic epitopes of PAc are multiple and scattered throughout the whole molecule, including the A region. However, the distribution of T-cell epitopes in the PAc molecule and the peptides that can induce the production of cross-reacting antibodies to PAc is still unclear. In this study, therefore, by using a complete set of purified sequential overlapping 19-mer peptides covering the entire sequence of the A region of PAc (Table 1) instead of the previous pin-coupled

TABLE 3. Inhibition of the binding of fluid-phase salivary components to immobilized rPAc on the sensor surface by various antisera

Immunization	Amt of binding antibodies (RU)	% Inhibition
PBS	61	0.0
Antiserum[a] against:		
rPAc	9,115	89.3
rPAc-1	3,336	0.0
rPAc-2	4,647	80.1
rPAc-3	1,188	1.3
PAc(361-379)	819	37.2
PAc(365-377)	918	47.2

[a] Obtained from mice immunized with the antigens.

peptides, we determined the distribution of T-cell and B-cell epitopes in the rPAc-2 fragment and examined the antigenicities of several peptides in B10.D2 mice (Fig. 1).

As a result, the wide distribution of both B-cell and T-cell epitopes in the rPAc fragment was suggested (Table 1), and so we could select several peptides for the immunization of mice on the basis of the anti-rPAc-2 antibody responses and rPAc-2-specific T-cell responses. The results (Fig. 1) suggest that the reactivities of the peptides against the anti-rPAc-2 antibodies and/or the rPAc-2 specific T cells do not directly reflect the immunological properties of these peptides. The PAc(331-349) peptide possesses both reactivities (Table 1) and shows strong antigenicity, but the anti-PAc(331-349) antibodies produced fail to cross-react against the rPAc molecule (Fig. 1). The PAc(391-409) peptide, which stimulates rPAc-2-specific T-cell proliferation but reacts weakly to anti-rPAc-2 antibodies, shows both strong antigenicity and cross-reactive antigenicity against the rPAc protein. In contrast, the PAc(436-454) peptide, which also induces primarily the T-cell response, exhibits weak antigenicity in B10.D2 mice. In addition, the PAc(301-319) peptide possesses weak reactivities in both cases but shows both strong antigenicity and cross-reactive antigenicity against the rPAc protein. Furthermore, PAc(196-214) and PAc(361-379), even if they do not significantly stimulate the rPAc-2-specific T-cell proliferation, can still be good antigens for production of cross-reacting antibodies against the rPAc molecule. This finding strongly suggests that many potentially immunogenic T-cell and B-cell epitopes may be hidden behind the limited numbers of the dominant T- and B-cell epitopes in the rPAc-2 fragment and possibly would display their own immunogenicities if it were not for those dominant epitopes.

Actually, the possibility of the presence of potentially immunogenic T-cell and B-cell epitopes has been demonstrated. Gammon et al. (7) demonstrated that in the case of hen egg white lysozyme, the T-cell responses induced often upon in vivo administration of the protein antigen are focused on a small number of epitopes, and the majority of potentially immunogenic T-cell epitopes are recognized only weakly or not at all. Nikcevich et al. (25) also pointed out that T cells elicited in response to an immunizing antigen usually recognize only one or a few immunodominant peptides; they also proposed the hypothesis that dominance is dependent on peptide binding affinity for the appropriate major histocompatibility complex class II molecule and the ability to compete with other peptides, derived from the same antigen, for class II binding. Similarly, Scheerlinck et al. (35) reported that an immunodominant B-cell epitope biases the immune response away from less immunogenic epitopes on the same molecule, although the mechanisms are still obscure. Thus, to obtain the appropriate peptide antigens for vaccination, a scanning analysis of T-cell and B-cell epitopes in the target molecules for vaccination was found to provide just a clue to the estimation of the test peptides, and so further immunological studies must be expanded to include various peptides containing not only both the T-cell and B-cell epitopes of the target molecule but also either of the two epitopes. In fact, the murine antibodies raised against the rPAc-2 fragment strongly recognized the PAc(361-379) 19-mer peptide, but the rPAc-2-specific T cells recognized the peptide very weakly (Table 1). However, this peptide possessed strong immunogenicity and could strongly induce cross-reacting antibodies to the rPAc molecule in B10.D2 mice (Fig. 1).

We therefore further investigated the immunological properties of the PAc(361-379) peptide, using 13 truncated synthetic peptides of this PAc(361-379) peptide, and determined the precise T- and B-cells epitopes of the peptide. As shown in Table 2, the results revealed that the amino acid sequence of the specific region for the B-cell epitopes, which are mainly recognized by the antibodies raised against the PAc(361-379) peptide, is TYEAALKQYEADL (residues 365 to 377), and a part of this sequence, YEAALKQY (residues 366 to 373), is probably the core structure of the T-cell epitope of the PAc (361-379) peptide in B10.D2 mice. These facts strongly suggested that the synthetic 13-mer peptide (TYEAALKQYEA DL), i.e., PAc(365-377), may be able to produce only cross-reacting antibodies against the PAc molecule. This thesis was substantiated by the immunization of B10.D2 mice with the peptide: in contrast with the PAc(361-379) 19-mer peptide, the ELISA antibody titer of the total antibodies raised against the PAc(365-377) 13-mer peptide was almost equal to that of the cross-reacting antibodies against the rPAc molecule (Fig. 4) and was almost completely inhibited with the rPAc molecule (Fig. 5). This sequence, therefore, must be composed of the cross-reactive B-cell epitopes of the PAc molecule and probably one of the shortest peptides which could still strongly induce only antibodies cross-reacting against PAc.

Recently, Partidos et al. (30) suggested that the orientations of epitopes and the amino acid compositions of chimeric peptides affect antigen processing and presentation to T cells, which govern both the specificity and affinity of the antibody produced, and they raised an antibody for the production of synthetic peptide immunogens with vaccine potential. Attention needs to be given to the number and orientation of the component epitopes required to produce antibodies of highest affinity. Subsequently, Shaw et al. (37) reported that the affinities of antibodies to the B-cell epitopes induced following immunization with chimeric T-B peptides were higher those that obtained following coimmunization. Sarobe et al. (34) also demonstrated that the insertion of two lysine residues (cleavage sites of cathepsin B) at the boundary of a B-cell epitope increased the antibody induction capacity of this type of peptide construct. Furthermore, it has been suggested as a general concept that a covalent bond between T-cell and B-cell epitopes is needed to induce anti-B-cell epitope antibodies cross-reactive with the native protein (41).

As shown in the present study, the presence of a unique peptide, such as PAc(365-377), seems to suggest the additional concept that if a minimum peptide corresponding to a B-cell epitope of the native protein has its own T-cell epitope within the molecule, the peptide probably induces only cross-reacting antibodies against the native protein. This new idea might be a very useful strategy for the development of absolutely safe peptide vaccines.

Since it had been reported that the PAc-defective mutants of *S. mutans* lacked the capability of saliva-mediated cell aggregation (11, 13), the PAc molecule, in particular the repeating regions, have been considered to play an important role in the cell adhesion and/or cell aggregation of *S. mutans* via an interaction with salivary components (3, 4, 8, 21, 23). In the present study, we focused on this interaction and further demonstrated the real-time binding of fluid-phase human salivary components to the immobilized rPAc molecule, using the BIAcore biosensor system (10, 38) (Fig. 7). In this system, whole cells of *S. mutans* are hard to use as the interactants, and so it is very difficult to analyze cell adhesion to immobilized salivary components. However, to determine the interaction of immobilized rPAc to fluid-phase salivary components, which probably represents PAc-mediated cell aggregation, this system seems to be adequate. There is no need to purify the analytes, i.e., salivary components and antibodies, in advance. Binding of the analytes to surface-immobilized rPAc is directly observed. The system permits one to record the real-time interaction of two

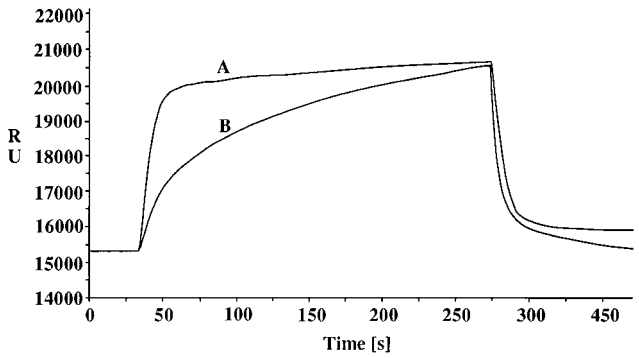

FIG. 9. Sensorgrams illustrating the association and dissociation phases of the binding of anti-PAc(361-379) antibodies (A) and salivary components (B) to the immobilized rPAc protein, redrawn from data in Fig. 8, sensorgram c, and Fig. 7, sensorgram a, respectively.

interacting macromolecules as a sensorgram, and the results of a series of different analysis are easily compared in overlay plots, directly visualizing relative interaction kinetics. Thus, association and dissociation phases of the sensogram for the binding of salivary components to rPAc protein (Fig. 7, sensorgram a) are redrawn in Fig. 9 together with those for the binding of antibodies to rPAc (Fig. 8, sensorgram c). As seen in Fig. 9, equilibrium of the association for the antibody rPAc interaction was reached within the initial 50-s period, while that for the salivary component-rPAc interaction took almost 240 s. This means that the affinities of salivary components to rPAc protein seem to be much weaker than those of antibodies, and so it seems possible to inhibit the interaction between salivary components and the PAc molecule with adequate antibodies against the PAc molecule. If the interaction between salivary components and PAc is involved somewhere in the initial attachment of *S. mutans* to the tooth surface, such antibodies may be useful to prevent dental caries. In fact, subcutaneous immunization of monkeys with purified PAc was found to be an effective measure against dental caries (15, 16, 40). Passive immunization with monoclonal antibodies against the protein antigen was also reported to prevent both dental caries in monkeys (14) and colonization of human teeth by *S. mutans* (18). Although Munro et al. (22) showed that the fragment (amino acid residues 816 to 1213) of the PAc molecule containing the P region mediates streptococcal adhesion to saliva-coated hydroxyapatite beads, the results shown in Table 3 further support the previous observation that the A region of the PAc molecule is a functional domain, interacting directly with salivary components (3, 4, 8, 21, 23); namely, the murine antibodies raised against rPAc protein strongly (89.3%) inhibited the binding of fluid-phase salivary components to the immobilized rPAc protein. In comparison, a similarly high inhibition (80.1%) was obtained with antibodies raised against the rPAc-2 fragment (residues 200 to 481), which contains the entire A region (residues 219 to 464), despite the fact that the total binding capacities of these antibodies to the rPAc seem to be almost 50% greater than those of the anti-rPAc antibodies (Table 3). Thus, the A region of the PAc molecule is now considered to be a main factor in the binding of fluid-phase salivary components to PAc and may be involved in the aggregation of this cariogenic bacterium.

In conclusion, a unique peptide, TYEAALKQYEADL, corresponding to the sequence of one of the B-cell epitopes of the rPAc molecule, contains its own T-cell epitope within the molecule and so strongly induces the production of only cross-reacting antibodies against the rPAc molecule in B10.D2 mice. Although the percent inhibition of the binding of salivary components to the rPAc protein by the antibodies raised against this unique peptide is less than 50%, this peptide must still be considered a candidate for a vaccinal immunogen for dental caries prevention. Because it is possible to develop other peptides of this kind by using the approach presented here, the antibodies raised against a mixture of these peptides may offer almost complete inhibition of binding of the salivary components to the PAc molecule at least at the same inhibition level as those raised against the rPAc-2 fragment.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in Aid for Developmental Scientific Research (05771537 to H.S.) from the Ministry of Education, Science, and Culture of Japan and by a grant from the Japan Health Science Foundation to T.N.

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Editor: J. R. McGhee