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HIGH PROSTAGLANDIN-E_1 BINDING TO SERUM PROTEIN IN ALLERGIC SUBJECTS

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Abstract—Protein binding to both salicylate and ^{3}H-labelled prostaglandin-E_1 ([^{3}H]PGE_1) was examined in the sera of 22 allergic and 16 normal individuals. Protein binding to salicylate ($P < 0.001$) and to [^{3}H]PGE_1 ($P < 0.01$) was significantly greater in the allergic than in the normal group. The nature of the binding sites of salicylate and PGE_1 was investigated with two fluorescent probes, dansylamide and dansylsarcosine as specific marker ligands for established Sites I and II, found to be specific for anionic drugs. The serum protein of 11 allergic subjects showed a higher binding at Site I ($P < 0.05$) and a lower binding at Site II ($P < 0.05$) than that of seven normal subjects. Salicylate and [^{3}H]PGE_1 bound competitively at the two sites. It was concluded, when comparing allergic to normal subjects, that the high protein binding of allergic individuals to salicylate and PGE_1 could be attributed to qualitative and/or quantitative differences in the lipophilic substances which are tightly bound to the albumin of normal sera, causing a reduction in binding ability at Site I.

Changes in the binding affinity of human serum albumin to small molecules have been reported in several disease states including penicillin hypersensitivity [1], rheumatoid arthritis [2], schizophrenia [3], uremia [4, 5] and certain liver diseases [6]. In these diseases, the alteration in the binding affinity of albumin may be related to a direct pathogenesis or may merely indicate metabolic changes modified by the disease states. In our recent study [7], an increase in binding affinity of serum protein, probably albumin, to salicylate was found in 82 allergic individuals with immediate types of allergy and 30 of their family members, in comparison to 24 normal subjects. Furthermore, in the binding study using serum proteins from both allergic and normal subjects fractionated with DEAE-Sephadex A-50 it was demonstrated that the salicylate molecule bound exclusively to albumin. It is known that salicylates inhibit the binding of prostaglandins (PGs) to serum albumin [8, 9] and that PGs act as important endogenous mediators in the inflammatory process [10]. Based on this evidence and our previous findings [7], it is speculated that the increased binding affinity of the albumin of allergic individuals to salicylate might reflect an increased affinity to the endogenous pro-inflammatory substances, PGs.

In the present study, serum protein binding to both salicylate and [^{3}H]PGE_1 was examined using the sera of allergic and normal subjects, in order to verify our speculation. As in the previous study [7], the sample collection from allergic subjects was done in the non-crisis state in order to find a certain underlying diathesis rather than an abnormal reaction to environmental stimuli. The nature of the sites on albumin to which salicylate and PGE_1 bound was also investigated by fluorescence titrations using two fluorescent probes (dansylamide, DNS-A and dansylsarcosine, DNS-S) as specific markers for two of the binding sites of anionic drugs on albumin [11, 12]. Changes in fluorescence characteristics associated with the binding of the probe to albumin depend on the binding environment. DNS-A, which has a hydrophobic nature without a negative charge, was used as the Site I probe and DNS-S, which has a hydrophilic nature with a negative charge in the carboxylic group, was used for Site II. The characteristics of albumin binding were determined by measuring the fluorescence produced by the binding of the probes to the two specific sites and studied to see whether there was any difference between allergic and normal subjects. Acid-treated albumin fractions which had been purified from the sera of allergic and normal subjects were also investigated at the two binding sites.

MATERIALS AND METHODS

Chemicals. The following chemicals were used in the study: sodium salicylate from Merk Japan Ltd (Tokyo, Japan); prostaglandin-E_1, human albumin (fatty acid-free, No. 110F-9350), dansylamide and dansylsarcosine from the Sigma Chemical Co. (St Louis, MO, U.S.A.); [7-^{14}C]salicylic acid (57 Ci/mmol) and [5,6-^{3}H(N)]prostaglandin-E_1 (89.5 Ci/mmol) from New England Nuclear (Boston, MA, U.S.A.). PGE_1 was dissolved in 70% ethanol to make a concentration suitable for use.

Subjects and sample collection. The criteria, described previously [7], for diagnosis in our study were met on the basis of careful clinical examinations and past histories. All allergic subjects used in the study were selected individuals with no other complications showing normal laboratory findings. The 22 allergic subjects consisting of six urticaria, four allergic rhinitis, five bronchial asthma, and seven drug allergy cases and the 16 normal subjects were investigated for protein binding to salicylate and [^{3}H]PGE_1. Another group consisting of 11 allergic cases and seven normal controls was examined in order to characterize the binding sites

on albumin using fluorescent probes and their competitive displacement with PGE_1. The allergic subjects here consisted of four urticaria, two allergic rhinitis, two bronchial asthma, two drug allergy cases and one case with seasonal eye irritation. In the binding experiment for the purified albumin after acid dialysis, the pooled sera from five normal controls and from two urticaria and two bronchial asthma cases and one drug allergy case were used. The age distribution for all categories was 22–60 years old.

Most blood samples were collected at the out-patient clinic in our hospital before drug administration. In the patients who had been under medication, samplings were made at least 7 days after withdrawal of all drugs. Normal subjects were drug-free, healthy adult volunteers. Serum specimens were kept in a frozen state at −20° until assayed.

Measurement of protein binding to salicylate. All experiments were performed at room temperature using an isotonic buffer, pH 7.4, which consisted of three parts 2.33% KH_2PO_4 and seven parts of 1.44% $NaHCO_3$. An aliquot of 0.5 mL of serum containing 1.25 mM salicylate, equivalent to the salicylate level in blood after a single oral administration of 1 g aspirin, was dialysed against 2 mL of isotonic buffer for 16 hr by shaking. After dialysis was completed, aliquots of 50 μL of the dialysed sera, dialysate buffer, standard solution of 1.25 mM sodium salicylate and serum without addition of salicylate as a blank were each added to 5 mL solutions of 0.15 M NaCl. The fluorescence produced by salicylate at an excitation wave length of 305 nm was measured at 405 nm [13] by a Hitachi MPF-2A fluorometer (Tokyo, Japan). The binding of serum protein to salicylate was calculated as follows: $S_b = Sin - Sout$; $S_f = Sout \times 5$; $\%\text{Bound} = S_b/(S_b + S_f) \times 100$; binding ability corrected for [Alb] $(M^{-1}) = (S_b/S_f) \times (1/[\text{Alb}])$, where S_b is the protein bound salicylate (mM); S_f is the free salicylate the volume of which was diluted from 0.5 to 2.5 mL (mM); *Sin* is the salicylate inside the dialysis tubing (mM); *Sout* is the salicylate outside the dialysis tubing (mM); and [Alb] is the albumin concentration (M). When an aliquot of 1.25 mM salicylate aqueous solution was dialysed by the method described above there was no detectable non-specific adsorption of salicylate to the cellulose tubing. In 10 replicate analyses using pooled serum with 1.22 mM sodium salicylate, the salicylate binding ability analysed by the semi-micro tubing dialysis method was $4.2 \pm 0.1 \times 10^3\ M^{-1}$ with a coefficient of variation of 2.4%.

Measurement of serum protein binding to PGE_1. The analytical method was basically identical to that for salicylate. An aliquot of 10 μL [^{3}H]PGE_1, 5 μCi/0.6×10^{-10} mol/mL in 70% ethanol with a specific activity of approximately 0.05 μCi/0.6 pmol of PGE_1, of which normal blood levels range from 0.8 to 1.1 nM, was added to 0.5 mL of serum inside the dialysis tubing. To study the displacement of PGE_1 from the serum protein by salicylate, the tubing was dialysed against 2 mL of various concentrations of salicylate in the isotonic buffer. After dialysis, the radioactivity of 0.05 mL of the dialysed serum and 0.2 mL of the dialysate buffer was measured using a scintillation counter. The serum protein binding affinity to PGE_1 was calculated in the same way as for salicylate except that only 1/4 of the radioactivity of PGE_1 in the dialysate was used because the sample volume of the dialysate was four times greater than that of the dialysed serum.

Determination of albumin. The commercial immunodiffusion plates Nor-Partigen-Albumin (Bohringer, Marburg, F.R.G.) were used for the quantitative determination of albumin in the serum.

Analyses of two specific binding sites on serum protein by fluorescence titration. Analyses of two specific binding sites on serum protein were performed by fluorescence titrations [11, 12] on the sera of allergic and normal subjects with or without addition of PGE_1 (10 μM). The serum was diluted with 1 μM probe solutions in the isotonic buffer to make the albumin concentration up to 10 μM. The fluorescence of solutions containing a probe (1 μM) and albumin (10 μM) was measured at 475 nm with an excitation at 350 nm. The fluorescence intensity was expressed relative to the fluorescence produced by fatty acid-free albumin (FAFA; 10 μM). In the preliminary experiment for this part of the study, serum from a normal subject was fractionated by DEAE-Cellulofine AM (Seikagaku Kogyo Co., Tokyo, Japan) chromatography into three peaks: all protein bands, except albumin, in peak 1; a single albumin band in peak 2; and a major albumin band with faintly stained α- and β-globulin bands in peak 3. The three peaks were diluted to give protein concentrations of approximately 10 μM with DNS-A (1 μM) and DNS-S (1 μM). Peak 1 did not show any measurable fluorescence relative to the fluorescence intensity produced by FAFA (10 μM) which was set as 100 %F for reference. This may indicate that either the two probes did not bind to serum proteins other than albumin or that the fluorescence produced by other serum proteins was so weak that it was undetectable. According to Bruderlein and Bernstein [14], the fluorescence intensity with albumin fraction V (20 μM) was similar to that with serum (albumin content, 20 μM) at Site I binding to DNS-A. The above evidence, combined with the results of our preliminary experiment, indicate that an analysis of two specific binding sites on albumin can be performed with sera.

Binding study at two specific sites on purified and acid-treated albumin. The protein-unbound substances in serum were separated from 10 mL each of sera pooled from five allergic and five normal subjects by Sephadex G-25 fine grade (Pharmacia Fine Chemicals, Uppsala, Sweden) gel filtration with 0.01 M NH_4HCO_3. After the protein fraction and the low molecular fraction had been lyophilized, the protein fraction was further chromatographed on DEAE-Cellulofine in order to obtain the albumin fraction, as described for fluorescence titration. The purified albumin after lyophilization was brought back to the original volume of serum and dialysed against the isotonic buffer and against the subsequent 500 mL solution of 1 N acetic acid, overnight. The acid dialysate was lyophilized whereas the acid-dialysed albumin was further dialysed against the isotonic buffer in order to return it to the physiological state. The protein concentration was determined by the Folin–Lowry method. Subsequently, the

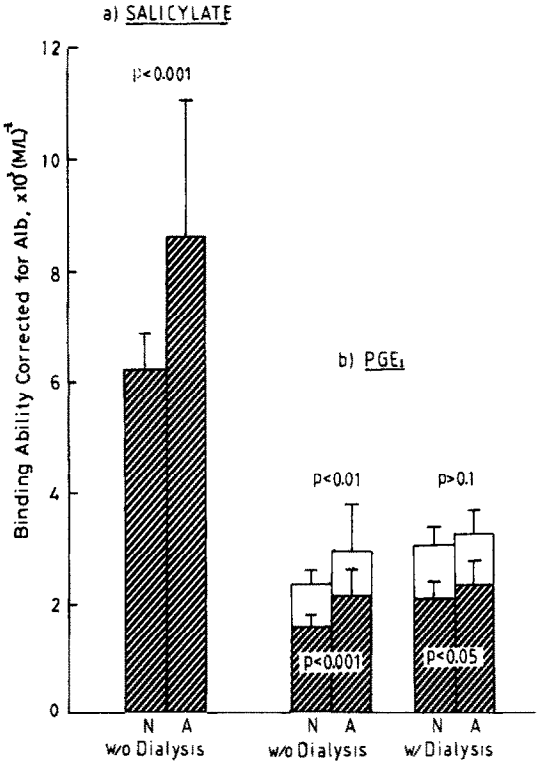

Fig. 1. Serum protein binding to both salicylate and [^{3}H]-PGE_1. (a) Binding to salicylate in the sera without dialysis. (b) Binding to [^{3}H]PGE_1 in the sera with and without dialysis. The hatched columns in (b) indicate the displacement of [^{3}H]PGE_1 from serum protein by salicylate, 0.313 mM. N, normal subjects (N = 16), A, allergic subjects (N = 22).

fluorescence titrations with DNS-A and DNS-S was performed before and after autologous and isologous cross-additions of serum-free substances to purified albumin in a concentration ratio of 1:1, with respect to albumin in the original serum. Acid dialysate was added in the same way to acid-dialysed albumin in a concentration of 0.5:1, with respect to albumin present in the albumin solution before acid dialysis, and fluorescence titration were similarly performed.

RESULTS

Binding capacity of serum protein to salicylate and PGE_1

The binding ability corrected for albumin was expressed by the formula ([bound ligand]/[free ligand]) × (1/[albumin]). The binding of serum protein to salicylate in the allergic and normal groups was $8.7 \pm 2.4 \times 10^3$ and $6.3 \pm 0.7 \times 10^3\ M^{-1}$, respectively, whereas the PGE_1 binding in the two groups was $2.98 \pm 0.84 \times 10^3$ and $2.39 \pm 0.26 \times 10^3\ M^{-1}$, respectively. As shown in Fig. 1, the binding in the allergic group of both salicylate ($P < 0.001$) and [^{3}H]PGE_1 ($P < 0.01$) was significantly greater than in the normal group. The same experiment using the serum dialysed against isotonic buffer overnight showed a similar result with slightly higher values for the PGE_1 binding. The values for the allergic and the normal groups were $3.26 \pm 0.42 \times 10^3$ and $3.10 \pm 0.34 \times 10^3\ M^{-1}$, respectively.

The sera of one normal subject and one subject allergic to antipyretics were examined for the displacement of [^{3}H]PGE_1 from serum protein in the presence of different concentrations of salicylate. As shown in Fig. 2, an increase in salicylate concentration of up to a molar ratio of 1:1 with respect to albumin caused displacement of PGE_1 in a more or less linear fashion. When salicylate was added to give more than a 1:1 molar ratio with respect to albumin, salicylate bound to its secondary non-specific sites and no further significant displacement of PGE_1 was observed.

This observation was confirmed in the study of PGE_1 displacement in the presence of salicylate (0.313 mM) in a 0.5:1 molar ratio with respect to albumin on the sera of 22 allergic and 16 normal subjects. The results are summarized in Fig. 1b. The fractions of PGE_1 displaced by salicylate in the allergic and normal groups which correspond to (b) in Fig. 2, as indicated by the hatched column, were $2.15 \pm 0.14 \times 10^3$ and $1.62 \pm 0.08 \times 10^3\ M^{-1}$ in sera without dialysis, respectively, whereas they were $2.38 \pm 0.40 \times 10^3$ and $2.11 \pm 0.32 \times 10^3\ M^{-1}$ in sera after dialysis, respectively. The degree of displacement of PGE_1 by salicylate was significantly greater ($P < 0.001$ for sera without dialysis and $P < 0.05$ for sera after dialysis) in the allergic group than in the normal group and this fraction of binding probably represents a primary binding site of salicylate. Accordingly, this portion of binding corresponds to the salicylate binding as presented in Fig. 1a. On the other hand, there was no significant difference between the two groups in the other portion of PGE_1 binding which was not displaced by salicylate, as indicated by the open column.

Two specific binding sites on albumin

The fluorescence titrations with two probes, DNS-A and DNS-S, revealed a difference in affinity of two assumed binding sites on albumin between the allergic and normal subjects (Fig. 3). When compared with the normal subjects, the serum protein of the allergic subjects showed a higher fluorescence intensity with DNS-A and a lower intensity with DNS-S. The average fluorescence values of the DNS-A binding at Site I for the allergic group and the normal group were 51.4 ± 13.6 and 37.0 ± 11.1 %F, respectively, whereas the values of the DNS-S binding at Site II for the two groups were 8.6 ± 5.2 and 13.9 ± 2.8 %F, respectively. The differences between the two groups at the two sites were statistically significant ($P < 0.05$). When present in a molar ratio of 1:1 with respect to albumin, PGE_1 (10 μM) binds to its primary binding site. Under this condition the displacement of probes from albumin by PGE_1 occurred to the same degree between the allergic and normal subjects at both sites as indicated by the stippled column in Fig. 3. This type of binding corresponds to the PGE_1 binding which was not displaced by salicylate, as shown by the open column in Fig. 1b. On the other hand, as indicated by the

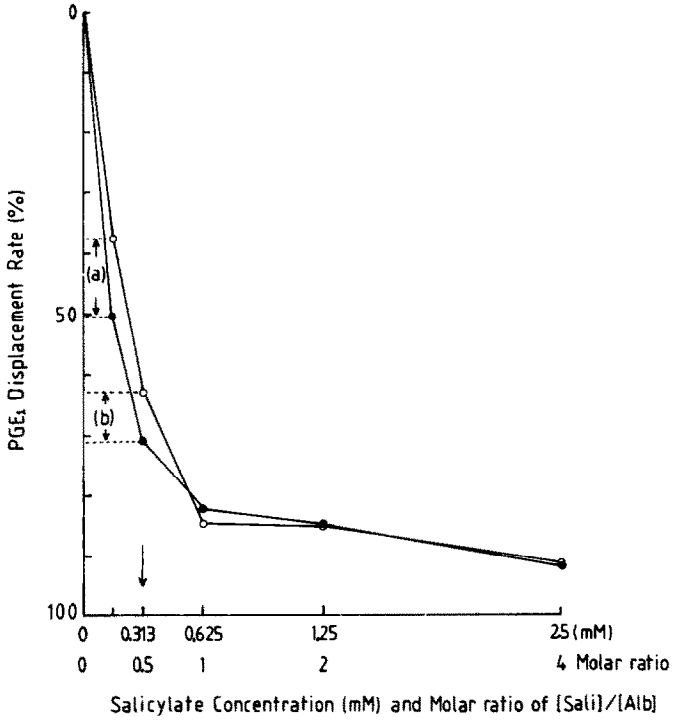

Fig. 2. Competitive displacement of [^{3}H]PGE$_1$ by salicylate. Displacement rate was expressed by [^{3}H]PGE$_1$ binding after displacement as a percentage of the initial binding before addition of salicylate. (○) Normal subject whose initial [^{3}H]PGE$_1$ binding ability corrected for albumin was $14.5 \times 10^3\ M^{-1}$ at 1.2 nM PGE$_1$. (●) Subject allergic to antipyretics whose initial [^{3}H]PGE$_1$ binding ability corrected for albumin was $17.8 \times 10^3\ M^{-1}$ at 1.3 nM PGE$_1$. The sera were tested after dialysis. Values shown are the means of triplicate analyses.

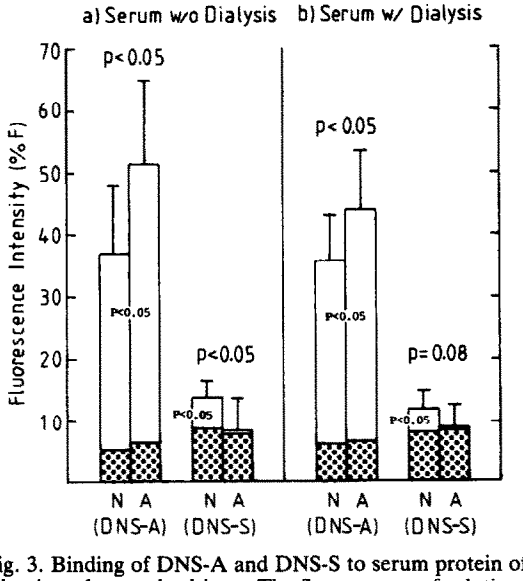

Fig. 3. Binding of DNS-A and DNS-S to serum protein of allergic and normal subjects. The fluorescence of solutions containing 10 μM albumin and 1 μM probes (DNS-A or DNS-S) was measured with and without addition of PGE$_1$ (10 μM). The fluorescence intensity was expressed relative to FAFA (10 μM). Stippled columns, a decrease of fluorescence in the presence of PGE$_1$ (10 μM); N, Normal subjects (N = 7); A, allergic subjects (N = 11).

open column in Fig. 3 the fluorescence intensities at Site I and II which were not displaced by PGE$_1$ at its primary binding site were 44.8 ± 13.4 and 0.5 ± 3.5 %F for the allergic group, whereas they were 31.5 ± 8.9 and 5.0 ± 2.7 %F for the normal group with DNS-A and DNS-S, respectively. The difference between the two groups at each site was statistically significant ($P < 0.05$). This type of binding is probably related to the site where salicylate binds at its primary binding site by displacing PGE$_1$ (the hatched column in Fig. 1b) and also corresponds to the secondary sites of PGE$_1$. Testing sera without dialysis is considered optimal for obtaining a correlation with *in vivo* biological states but gives little insight into the nature of the interaction of ligands and protein. Therefore, the sera were also tested after dialysis. The results showed a similar pattern although the difference in the values between the two groups were slightly less significant in the sera after dialysis. A similar phenomenon was also observed in the PGE$_1$ binding of dialysed sera, as presented in Fig. 1b.

Binding study at two specific sites for the purified albumin and the acid-dialysed albumin

The binding affinity of purified albumin to DNS-A at Site I was intensified in the presence of Cl^- ions (Fig. 4a) due to a decrease in the limiting fluorescence by a competitive displacement of Cl^- at the same site [15]. Albumin from the allergic

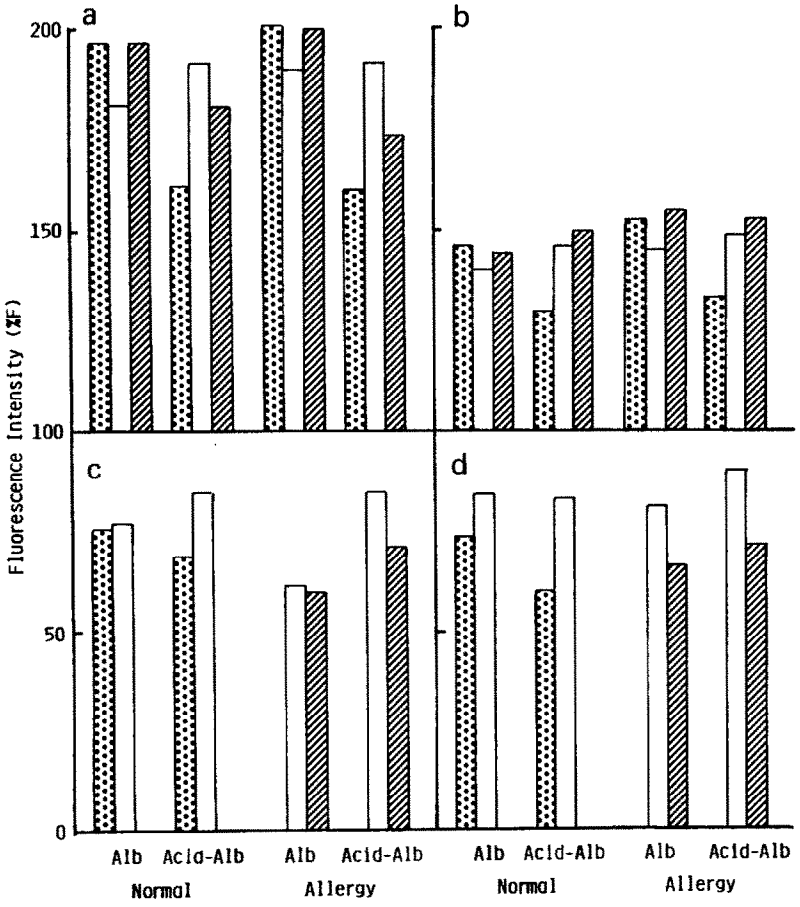

Fig. 4. Binding at two specific sites for the purified and acid-treated albumin. The fluorescence titrations with DNS-A (a and b) and DNS-S (c and d) were performed in the same way as those described in Fig. 3, before and after the autologous and isologous cross-additions of the serum protein-unbound substances to purified albumin and of the acid dialysate to the acid-treated albumin. The fluorescence of probes produced by FAFA (10 μM) was set as 100 %F for reference. Panels a and c, in the isotonic buffer with 0.15 M NaCl; panels b and d, in the isotonic buffer without NaCl; open columns, the purified albumin or the acid-treated albumin; stippled columns, after the addition of the normal-derived effectors; hatched columns, after the addition of the allergic-derived effectors. The values shown are the means of triplicate analyses.

subjects showed a slightly higher binding ability at Site I than that from the normal subjects in the presence and absence of Cl^- (the open columns in Fig. 4a and b). The cross-additions of serum-free substances, derived from both allergic and normal subjects, to autologous and isologous albumins induced a cooperative binding of DNS-A at Site I and either no change or an antagonistic binding with respect to DNS-S at Site II (Fig. 4c and d) where, as reported, Cl^- did not affect its binding [15]. The binding ability at Site I of the normal acid-dialysed albumin increased to the same degree as that of the allergic subjects. The cross-addition of the acid dialysate, which was derived from the albumin of both the normal and allergic subjects, in the same way as the serum-free substances resulted in decreased binding at the two sites except for a slight increase in binding at Site I by the addition of the acid dialysate from allergic subjects in the absence of Cl^- (Fig. 4b). The acid dialysate derived from normal albumin caused a greater decrease of the binding of the probes at the two sites of the acid-dialysed albumins than that derived from the allergic subjects.

DISCUSSION

As a parameter of the ligand binding ability of proteins, the product nK, where n is the number of binding sites and K is the apparent association constant, is commonly used [16]. Since albumin is the most probable serum protein responsible for binding to salicylate [7] and PGE_1 [17], the formula ([bound ligand]/[free ligand]) × (1/[albumin]) [18] was used in the present study, being a compromise for measuring n and K. Since any variation in the concentrations of ligand and protein in the samples to be compared may affect the parameter of the ligand binding to albumin, the total ligand levels in the samples were fixed at a constant concentration.

No effect of variation in the albumin concentration of the samples on the binding parameter was observed among the normal and allergic subjects, by testing a correlation between two variables.

In previous work [7], a high level of serum protein binding to salicylate was found in the sera of allergic individuals and the albumin fraction was considered to be responsible. We also speculated that this finding in the allergic subjects might reflect an increased binding ability of endogenous pro-inflammatory substances, possibly prostaglandins. Our speculation was verified in the present study. Serum protein binding to PGE_1 was high in the allergic subjects as was also the case with serum protein binding to salicylate (Fig. 1). The difference in the serum protein binding between the allergic and normal groups was more prominent with salicylate than with PGE_1. As suggested by Unger [19], there seemed to be two types of PGE_1 binding to albumin (Fig. 2). In one type, possibly at the PGE_1 secondary binding sites, the site was also the primary binding site of salicylate since an increase of salicylate concentration up to a molar ratio of 1:1 with respect to albumin caused displacement of PGE_1 in a linear fashion. In the other type of binding, possibly at the primary binding site of PGE_1, PGE_1 was not displaced by further increasing the concentration of salicylate. The significant difference in the binding of serum protein between allergic and normal subjects was caused by the former type of binding, which is indicated by the hatched column in Fig. 1b. Furthermore, binding to serum protein in the allergic group was higher at Site I and lower at Site II than in the normal group. The primary binding sites of salicylate and DNS-A are very likely to be related to each other at Site I [20–22]. Accordingly, the high salicylate binding in allergic subjects (Fig. 1a) clearly reflects a high binding at Site I, as shown in Fig. 3. Since the degree of displacement of probes by PGE_1 at its primary binding site was similar for the two groups at both sites (Fig. 3), the primary binding site of PGE_1 is probably not related to Site I. This may explain why the difference in the binding of albumin between allergic and normal groups was more prominent in the case of salicylate than that of PGE_1.

The increased binding of ligands on, presumably, albumin may be due to the presence of endogenous allosteric effectors. The endogenous effectors in serum were competitive for PGE_1 binding (Fig. 1b), whereas they were cooperative for DNS-A binding in the fluorescence titration (Figs 3 and 4a and b). It has been reported that fatty acids at a greater than one molar ratio to albumin enhanced the binding of DNS-A at Site I through allosteric cooperation but either decreased competitively or did not affect the binding of DNS-S at Site II [23–25]. From the results for sera (Fig. 3) and for purified albumin (Fig. 4), it can be presumed that the effective components of protein-unbound substances which originated from both allergic and normal subjects are probably fatty acids. Removal by dialysis of endogenous allosteric effectors such as fatty acids [23] and tryptophan [21] for Site II, and bilirubin [21] and chloride [15] for Site I resulted in an increase in PGE_1 binding for both allergic and normal sera and reduced the difference in binding between the two groups. This seemed to be due to the restoration of binding after dialysis in the normal sera at Site I, which was related to the primary binding site of salicylate (the hatched column in Fig. 1b), exceeding recovery in the allergic sera. This indicates the presence of endogenous competitive effectors for Site I in the normal sera. After eliminating those effectors by dialysis, there still remained a significant difference in binding at the primary binding site of salicylate (Fig. 1b) and at Site I (Fig. 3) between the two groups. This suggests that certain lipophilic substances which bound tightly to the albumin of either the normal or the allergic subjects and then caused either a decrease or an increase in binding, were not removed by dialysis at physiological pH. Under ordinary conditions, the molar ratio of fatty acids to albumin in human plasma varies from 0.5 to 1.5 [25]. Most of these acids are removed by dialysis at physiological pH but some are not [26].

A hydrophobic substance bound tightly to the albumin of normal subjects was released by acid dialysis [27] and this allowed the acid-treated normal albumin to gain the same degree of binding ability as the albumin of allergic subjects (Fig. 4a and b). Furthermore, the acid dialysate derived from normal albumin displaced DNS-A at Site I more efficiently than did that derived from allergic subjects (Fig. 4a). The displacement of the two probes by Site I- and Site II-specific compounds was altered by the presence of fatty acids [23, 24]. In these interactions, the structural differences of fatty acids together with their molar ratios to albumin were important determinants of the type of cooperativity (positive/negative) between the two probes and site-specific substances. Qualitative and/or quantitative differences in such hydrophobic substances, including fatty acids, tightly bound to albumin induced a modification of the tertiary structure of albumin at its two binding sites [23, 24]. This might cause less binding at Site I of normal albumin than of the albumin of allergic subjects. The pathophysiological significance of the high binding affinity of albumin for PGs in allergic subjects is that it may delay their metabolic degradation and clearance [8, 28], thus contributing to their prolonged inflammatory effect.

In this paper evidence has been presented that: (1) the sera of allergic individuals binds to a higher degree to both salicylate and PGE_1 than that of normal individuals and (2) this is attributable to qualitative and/or quantitative differences in the lipophilic substances bound tightly to, presumably, the albumin of normal sera, which causes a reduction in binding affinity to Site I of albumin.

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