

# Immunochemical analysis of sulfonamide drug allergy: Identification of sulfamethoxazole-substituted human serum proteins

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**Background:** Sulfonamides undergo oxidative metabolism to yield reactive metabolites that haptenate proteins readily. Although it has been shown that sulfonamide metabolites bind covalently to murine microsomes, sulfonamide-conjugated serum proteins have not been analyzed in the peripheral blood of treated individuals.

**Objective:** We hypothesized that during treatment with sulfamethoxazole, intracellular proteins are haptenated by drug metabolites, and some of these are destined for secretion into the serum.

**Methods:** Using antibodies specific for sulfamethoxazole and an alkaline phosphatase immunoblotting technique, we attempted to demonstrate the presence of sulfamethoxazole-substituted proteins in the serum of individuals during a course of treatment.

**Results:** Five days into therapy, serum protein haptenation by sulfamethoxazole was demonstrated in two of the three individuals studied. In addition, Western blot analysis revealed that haptenation is not indiscriminate, but highly selective. A single 30 kd protein is the target of haptenation in all instances. A kinetic analysis revealed that substituted proteins can be detected early, within hours of administration. Moreover, haptenated proteins remain detectable in the serum 48 hours after discontinuation of the drug.

**Conclusion:** The results presented here constitute the first direct evidence that sulfonamides, on being metabolized, covalently haptenate human serum proteins during a course of therapy. (J ALLERGY CLIN IMMUNOL 1994;94:1017-24.)

**Key words:** Sulfamethoxazole, haptenation, cytochrome P-450, immunoblotting, gel electrophoresis

Although Shear et al.<sup>1</sup> have shown that reactions to sulfonamides may be toxic in nature, evidence from several laboratories, including our own, supports the involvement of an immunologic mechanism in at least some of the reactions demonstrated. Specific antibodies of all isotypes,<sup>2-5</sup> as well as sensitized lymphocytes,<sup>6</sup> have been detected in individuals undergoing sulfonamide reactions. Moreover, the types of reactions demonstrated—

## Abbreviations used

BSA:	Bovine serum albumin
HSA:	Human serum albumin
KLH:	Keyhole-limpet hemocyanin
NRS:	Normal rabbit serum
PBS:	Phosphate-buffered saline
SDS-PAGE:	Sodium dodecylsulfate–polyacrylamide gel electrophoresis
SMX:	Sulfamethoxazole
TBS:	Tris-buffered saline

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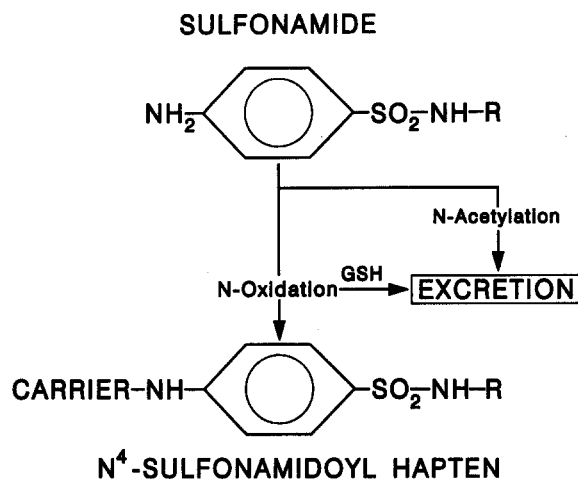
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anaphylaxis, urticaria, angioedema, maculopapular rashes, and contact sensitivity—further support the immunologic basis of sulfonamide reactions.

Sulfonamides are metabolized in the liver by N-acetylation, which leads to the formation of nontoxic metabolites, and by cytochrome P-450–catalyzed N-oxidation, the products of which are intermediates, possibly hydroxylamines, that are



**FIG. 1.** Sulfonamide metabolism. Sulfonamides are metabolized by N<sup>4</sup>-acetylation or by N<sup>4</sup>-oxidation by the cytochrome P-450 mixed function oxidase system. Acetylated sulfonamides and glutathionyl (GSH) compounds are excreted. Haptenation of protein carriers occurs if the capacity for glutathione conjugation is exceeded. The N<sup>4</sup>-sulfonamidoyl determinant is the major, and possibly the only, hapten formed during sulfonamide metabolism. Reproduced with permission from Sullivan TJ. Drug allergy. In: Middleton E, Reed CE, Ellis EF, Adkinson NF, Yunginger JW, Busse WW, eds. Allergy: principles and practice. 4th ed. St. Louis: Mosby, 1993:1726-46.

highly reactive with proteins. Rieder et al.<sup>7</sup> have demonstrated that both artificially synthesized hydroxylamines of sulfamethoxazole (SMX) and the reactive metabolites formed when SMX is incubated with a microsomal-activating system are toxic to human lymphocytes.

In addition to causing toxic reactions, the reactive intermediates formed during metabolism most likely play a critical role in immunologic reactions as well. We demonstrated previously the presence of SMX-specific IgE in patients with histories of immediate hypersensitivity reactions to SMX.<sup>2, 4</sup> In addition, we showed that the major antigenic determinant to which the antibodies are directed is the N<sup>4</sup>-sulfonamidoyl group, the structure of which is very similar to both the free drug and the hydroxylamine intermediate (Fig. 1).

The purpose of this study was to determine more clearly the underlying immunopathology of sulfonamide allergy. In light of our knowledge of sulfonamide metabolism, we hypothesize that during treatment with SMX the reactive intermediates that are formed subsequently bind covalently to serum proteins, forming potentially immunogenic complexes. To test this hypothesis, serum

samples from several subjects were analyzed for the presence of SMX-haptenated serum proteins before and during a course of SMX therapy.

## METHODS

### Materials

**Rabbit immunizations/antibody purification.** Hunter's Titermax adjuvant was obtained from CytRx Corporation (Norcross, Ga.). SMX-bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.) was prepared according to the method described previously<sup>4</sup> and was used as the immunizing antigen. An Econo-Pak Protein A kit from Bio-Rad (Richmond, Calif.) was used for the purification of the rabbit IgG.

**Enhanced ELISA.** Borate-buffered saline, phosphate buffered saline (PBS), blocking solution/standard diluent (ovalbumin, 5 gm/L in PBS/Tween), PBS/Tween (Tween 20, 0.5 ml/L in PBS), Tris/NaCl, and substrate/amplifier solutions were all obtained from Gibco BRL (Grand Island, N.Y.). Alkaline-phosphatase-coupled affinity-purified goat F(ab')<sub>2</sub> anti-rabbit IgG was obtained from Tago, Inc. (Burlingame, Calif.).

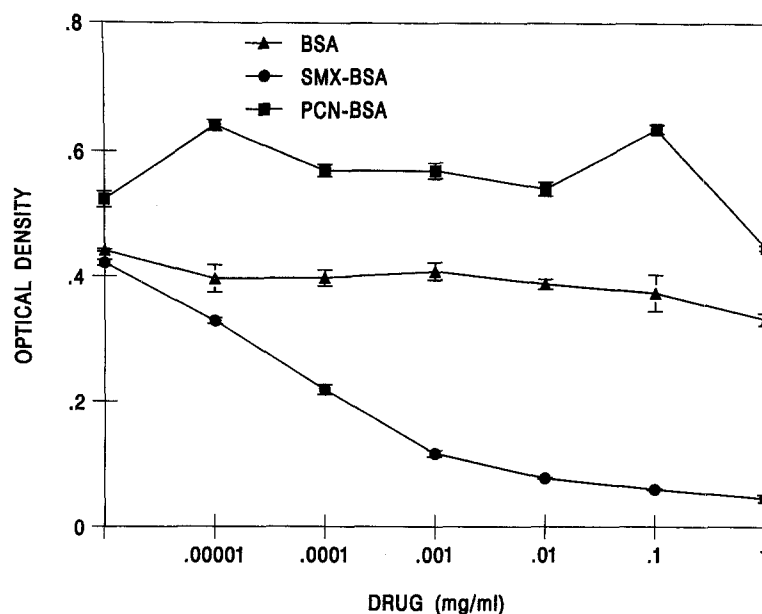
**Amplified alkaline phosphatase goat anti-rabbit immunoblotting assay.** Tris-buffered saline (TBS), TBS with 0.05% Tween-20, blocking solution (5% nonfat dry milk in TBS), second antibody solution (biotinylated goat anti-rabbit antibody, 1:3000 in TBS with 0.05% Tween-20), and streptavidin-biotinylated alkaline phosphatase complex (1:3000 in TBS) were obtained from Bio-Rad Laboratories.

**Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).** Nitrocellulose, 12% mini-gels, electrophoresis chemicals, and molecular weight markers were obtained from Bio-Rad Laboratories.

### Procedures

**Rabbit immunizations and antibody purification.** Two female New Zealand white rabbits (Myrtle's Rabbitry, Thompson Station, Tenn.) were injected intramuscularly with 25 µg SMX-BSA (emulsified in Hunter's TiterMax) in each hind flank and boosted 6 weeks later. One month after boosting, the animals were bled and killed, and serum samples were assayed for the presence of SMX-specific IgG antibodies by ELISA inhibition.

**Enhanced ELISA.** An enhanced inhibition ELISA was performed according to that described by Macy et al.<sup>8</sup> with slight modifications. Sixteen microliters of either normal rabbit serum (NRS) or serum from SMX-BSA-immunized rabbits (final dilution 1:10,000 to 1:20,000) was incubated with 300 µl BSA, SMX-BSA, or penicillin-BSA (each at 0.25 mg/ml) and 284 µl ovalbumin diluent in polystyrene tubes overnight at 4° C. Microtiter plates were coated with 100 µl SMX-keyhole-limpet hemocyanin (KLH) (50 µg/ml in borate-buffered saline) and incubated overnight at room temperature in a humidified atmosphere. The next day, the plate was washed and blocked, and 100 µl of the antibody-



**FIG. 2.** Specificity of rabbit anti-SMX-BSA antibodies. Diluted serum from a rabbit hyperimmunized with SMX-BSA was preincubated with varying concentrations of BSA, SMX-BSA, or penicillin (PCN)-BSA and then used in ELISA inhibition experiments. Microtiter plates were coated with SMX-KLH as described in the Methods section. These data are presented as the mean ( $\pm$  SEM) of triplicate determinations.

inhibitor mixture was added to each well. Subsequently, the alkaline phosphatase-coupled developing antibody (1:24,000) was added, followed by the substrate-amplifier solution. The plate was watched carefully for color development, and absorbance was measured at 490 nm with an automated microtiter plate reader. Triplicate samples were assayed.

**Amplified alkaline phosphatase goat anti-rabbit immunoblotting assay.** The Bio-Rad Bio-Dot Microfiltration Apparatus was used for the dot blot assays. One hundred microliters of diluted human serum (which had been extensively dialyzed) from individuals before and during treatment with SMX was added to the Bio-Dot apparatus, which contained a moistened nitrocellulose membrane. After the samples were allowed to filter through the membrane by gravity flow, the membrane was washed and removed for the blocking step. The membrane was blocked overnight in 5% nonfat dry milk in PBS and washed, and BSA-absorbed rabbit anti-SMX antibodies (or NRS) were added. After 2 hours of agitation, the first antibody solution was decanted, the membrane was washed twice, and the biotinylated goat anti-rabbit antibody solution (1:3000) was added. The final steps included incubation with the streptavidin-biotinylated alkaline phosphatase complex (1:3000), followed by color development.

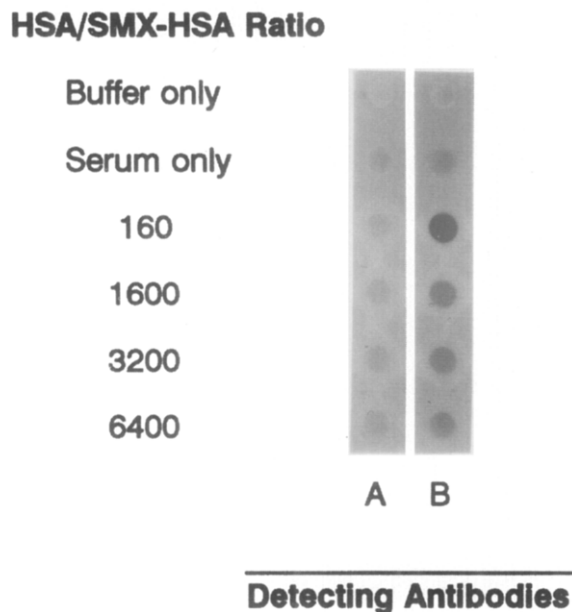
**SDS-PAGE.** Human serum samples taken from individuals before and during a course of SMX were immunochemically analyzed for SMX-conjugated proteins with partially purified rabbit anti-SMX antibodies (or NRS). Proteins were separated according

to molecular weight (25  $\mu$ g/lane) by using the procedure of Laemmli<sup>9</sup> on a discontinuous 12% SDS-PAGE system with a 4% stacking gel layer. Electrophoretically separated proteins were electroblotted onto nitrocellulose membranes, and SMX-conjugated proteins were detected with the amplified alkaline phosphatase goat anti-rabbit immunoblotting assay described above.

**Experimental subjects.** A 5-day course of SMX (500 mg twice a day) was administered to three volunteers. Serum was taken before and at varying times after the initiation of therapy for use in the studies described. Volunteer 1 was a 70-year-old woman with a remote history of urticaria in response to SMX and angioedema in response to penicillin. Before oral administration of SMX, the patient was shown to have a negative skin test response to a multivalent SMX conjugate, sulfamethoxazole poly-L-tyrosine. The skin testing procedure was performed as described previously.<sup>4</sup> Volunteer 2 was a 38-year-old woman with no history of antibiotic drug allergies. She did have urticaria when a second course of SMX was administered, however. Volunteer 3 was a 52-year-old man with no history of antibiotic drug allergies.

These experiments were approved by the University of Texas Southwestern Institutional Review Board, and informed consent was obtained from each subject before phlebotomy and drug administration.

**Experimental animals.** The guidelines that were followed for the care and use of the animals used in these studies are described in the "Guide for the Care and



**FIG. 3.** Sensitivity analysis of the detection of SMX-substituted human serum proteins. Normal human serum (with and without added SMX-substituted HSA at varying ratios) diluted 1:25 was applied to nitrocellulose. The first two rows are controls containing buffer alone (no serum or SMX-HSA) and diluted human serum without added antigen, respectively. Rows 3 to 6 contain diluted human serum with added SMX conjugate (SMX-HSA) at concentrations that yielded varying ratios of HSA to SMX-HSA (160, 1600, 3200, 6400). After extensive blocking, strips were incubated with either preimmune rabbit serum (*A*) or partially purified rabbit anti-SMX-BSA antibodies (*B*) which had been preabsorbed with BSA to remove any BSA-specific antibodies. After extensive washing, the strips were then incubated sequentially with biotinylated goat anti-rabbit IgG, streptavidin-biotinylated alkaline phosphatase complex, and color development reagent.

Use of Laboratory Animals" (NIH publication no. 86-23, as revised).

## RESULTS

### Characterization of rabbit anti-SMX antibodies

SMX-specific polyclonal antibodies were generated in New Zealand white rabbits. After animals were immunized and boosted with SMX-BSA (prepared by covalent linkage of the diazonium salt of SMX to BSA), serum was obtained, partially purified, and used in inhibition ELISAs. Prebleed serum was also obtained and partially purified for use as a negative control. Before addition to microtiter plates containing antigen, antibodies diluted 1:24,000 were incubated with one of three inhibitors: BSA, SMX-BSA, or penicillin-BSA.

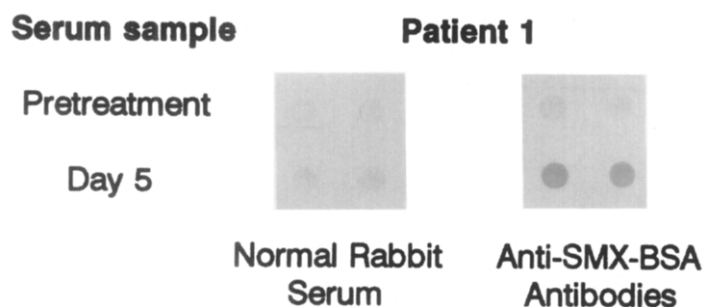
SMX-KLH was the antigen chosen for microtiter plate coating. After extensive washing, the plate was then incubated sequentially with alkaline phosphatase-conjugated goat anti-rabbit IgG, substrate, and color development reagent. Optical density values from one representative experiment are presented in Fig. 2.

As shown, SMX-BSA inhibited antibody binding in a dose-dependent manner, with as little as 10 µg/ml causing almost complete inhibition. BSA and penicillin-BSA were not inhibitory at any concentration tested. NRS demonstrated little binding, regardless of the presence or absence of inhibitor (data not shown). These results indicate that rabbit antibodies, like those of humans, recognize the sulfamethoxazolyl hapten and that on immunization with a large molecular weight SMX conjugate, high-titer anti-SMX-specific antibodies are generated.

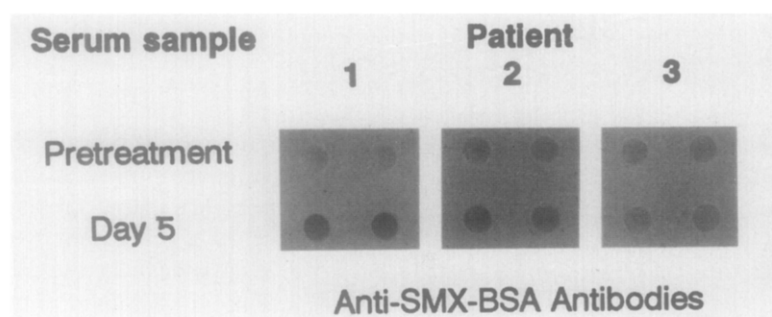
### Sensitivity of immunoblotting assay system

Before determining whether haptenated serum proteins are present in the peripheral blood of individuals treated with SMX, it is critical to first determine whether, and to what extent, the SMX-specific antibodies generated are able to detect known concentrations of SMX-haptenated proteins artificially added to normal human serum. An amplified alkaline phosphatase goat anti-rabbit immunoblotting assay was used in these studies, and a representative experiment is presented in Fig. 3. Normal human serum (with and without added SMX-substituted human serum albumin [HSA] at varying ratios to endogenous HSA) diluted 1:25 was applied to nitrocellulose. The first two rows are controls containing buffer alone (no serum or SMX-HSA) or diluted human serum without added antigen, respectively. Rows 3 to 6 contain diluted human serum with added SMX conjugate (SMX-HSA) at concentrations that yielded varying ratios of HSA to SMX-HSA (160, 1600, 3200, 6400). After extensive blocking, strips were incubated with either preimmune rabbit serum (Fig. 3, *lane A*) or partially purified rabbit anti-SMX-BSA antibodies (Fig. 3, *lane B*), which had been preabsorbed with BSA to remove any BSA-specific antibodies. After extensive washing, the strips were then incubated sequentially with biotinylated goat anti-rabbit IgG, streptavidin-biotinylated alkaline phosphatase complex, and color development reagent.

As shown in Fig. 3, no color development occurred when the preimmune rabbit serum was used as the primary detecting antibody. In con-



**FIG. 4.** Demonstration of SMX-substituted human serum proteins in a patient undergoing SMX therapy. Serum was obtained from one individual before and 5 days after daily administration of SMX (500 mg twice a day). After extensive dialysis, serum samples were applied to nitrocellulose in duplicate, and the immunoblotting assay was performed as described in the Methods section.



**FIG. 5.** Demonstration of the variability of SMX serum protein haptenation by different individuals. Serum samples were obtained from three different individuals before and 5 days after daily administration of SMX. After extensive dialysis, samples were applied to nitrocellulose in duplicate, and the immunoblotting assay was performed as described in the Methods section.

trast, positive reactions were demonstrated when the rabbit anti-SMX-BSA antibodies were reacted with diluted human serum containing varying concentrations of SMX-HSA. SMX-HSA added to diluted human serum to yield ratios (with endogenous HSA) of 1 to 160 and 1 to 1600 HSA molecules was easily detectable. Although less intense, positive reactions were observed with as little as 1 molecule of SMX-HSA per 6400 endogenous HSA molecules.

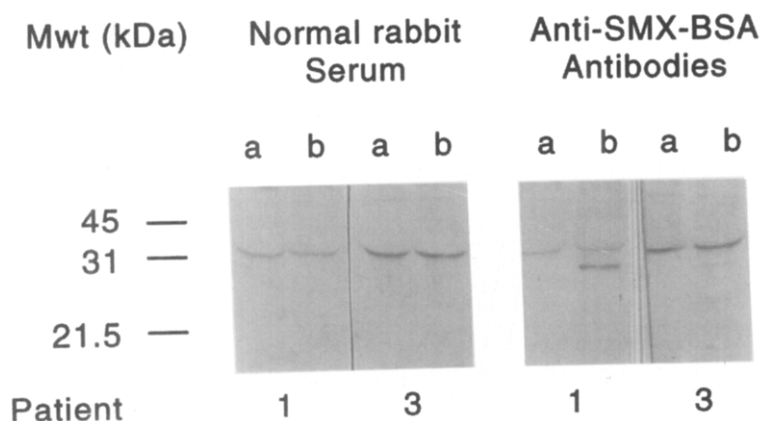
#### Detection of SMX-substituted human serum proteins

Because of the sensitivity of the immunoblotting assay it was used to determine whether SMX-haptenated serum proteins could be detected in the peripheral blood of individuals undergoing treatment with SMX. Serum was obtained from one individual before treatment and on the fifth day of treatment with SMX (500 mg twice a day). After extensive dialysis, samples were applied to nitrocellulose in duplicate. Results of a representa-

tive experiment from one individual are presented in Fig. 4. Although no color development was noted when pretreatment human serum was used as antigen, detectable SMX substitution of proteins was demonstrated 5 days into therapy. No color development was seen when preimmune rabbit serum was used in this assay system. These findings indicate that by using a sensitive immunoblotting technique, SMX-conjugated serum proteins can be found in the peripheral blood of individuals treated with SMX.

#### Comparison of haptenating ability by different individuals

To determine whether treated individuals haptenate serum proteins equally, serum was taken from three individuals immediately before therapy and again 5 days into therapy with 500 mg SMX twice a day. Patients 2 and 3 had no history of antibiotic allergies. Patient 1, who had a history of urticaria after administration of both penicillin and SMX, was given a course of SMX after the



**FIG. 6.** Western blot analysis of SMX-substituted human serum proteins. Human serum samples taken from individuals before and during a course of SMX were immunochemically analyzed for SMX-conjugated proteins with SDS-PAGE and immunoblotting. *a*, Pretreatment serum; *b*, serum taken 5 days into therapy with SMX.

absence of SMX-specific IgE had been ensured. Both prick and intradermal skin tests with SMX-poly-L-tyrosine were performed as described previously,<sup>4</sup> and results were negative. The patient tolerated the course of SMX without complications.

After extensive dialysis, the serum samples were applied to nitrocellulose in duplicate, and the immunoblotting assay was performed as described previously. Results of a representative experiment are presented in Fig. 5. Although little color development occurred when pretreatment human serum from each of the individuals was used as antigen, detectable color was demonstrated in serum samples, taken 5 days into therapy, from patients 1 and 2 but not from patient 3. These results suggest that although substitution of serum proteins may occur during a course of therapy with SMX, the degree to which haptenation occurs is variable. Robust haptenation occurs in some, but not all, treated individuals.

#### Western blot analysis of SMX-substituted serum proteins

Although the immunoblotting technique can be used to determine the existence of SMX-substituted proteins in human serum, the particular proteins haptenated cannot be identified by this method. To determine whether SMX haptenates proteins indiscriminately or selectively, serum proteins were separated according to molecular weight with polyacrylamide gel electrophoresis before immunoblotting. Western blot analysis was performed on serum samples taken before treat-

ment and 5 days into therapy from patients 1 and 3. The results are presented in Fig. 6.

In the presence of the rabbit anti-SMX-detecting antibodies a prominent band, approximately 30 kD in size, is seen in the 5-day serum sample from patient 1. Although not prominent, this band is also faintly visible in the 5-day sample from patient 3. This band does not appear in the pretreatment samples from either of the patients, nor is it visible when preimmune rabbit serum is used as the detecting antibody. A kinetic analysis was performed, and it demonstrated that haptenation is detectable, albeit weakly, at 5 hours after the administration of SMX (Fig. 7). The band then becomes more prominent 2 and 3 days into therapy and remains detectable 48 hours after the drug is stopped. These data indicate that SMX protein substitution is highly selective and that haptenation occurs within 5 hours after drug administration.

#### DISCUSSION

A significant number of adverse drug reactions appear to be immune-mediated, and as for any immune-mediated event involving simple haptens, induction and elicitation of the reaction requires the formation of immunogenic conjugates. Although it has been assumed that these conjugates are formed through covalent binding of the drug hapten itself or a reactive intermediate to tissue macromolecules, knowledge of the metabolic processes involved in the generation of these immunogenic conjugates is limited. The results presented here constitute the first direct evidence

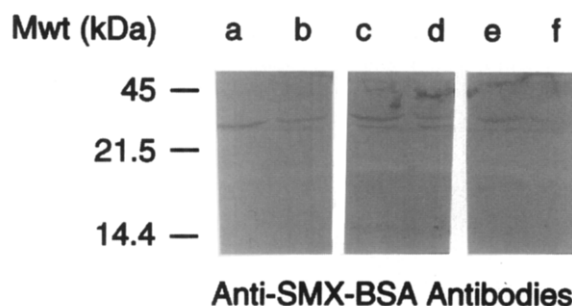
that sulfonamides, on being metabolized, covalently haptenate human serum proteins during a routine course of therapy.

In 1942 Rich<sup>10</sup> suggested that sulfonamides may covalently attach to plasma proteins and that, on doing so, specific sensitization may occur. Subsequently, Leftwich<sup>11</sup> reasoned that circulating hapten-carrier conjugates might be present in the blood of sulfonamide-treated individuals and found that serum samples from nonallergic SMX-treated patients (used as a skin test reagent) caused positive skin test responses in 28 of 30 patients who had previously experienced allergic reactions to sulfonamides. These data provide indirect evidence that multivalent drug-protein conjugates are routinely present in the peripheral blood during a course of sulfonamide therapy.

Shear and Spielberg<sup>12</sup> have demonstrated covalent binding of a metabolite of sulfadiazine to murine hepatic microsomes. However, we now demonstrate, in a human system, the covalent substitution of serum proteins. Although sulfamethoxazole hydroxylamine metabolites have been found in human urine,<sup>13</sup> to our knowledge this is the first demonstration of *in vivo* haptenation of human proteins by sulfonamides.

The mechanism by which serum proteins are haptenated has not yet been elucidated. Intracellular haptenation may occur, leading to the generation of substituted proteins that are subsequently secreted into the serum. Alternatively, haptenation of serum proteins by reactive intermediates may occur directly. In light of the short reactive half-life of most intermediates, it would not be expected that significant concentrations of the intermediate would accumulate at sites distant from the site of origin. Evidence supporting the intracellular formation of hapten-protein adducts has been previously demonstrated for acetaminophen, a drug that like SMX is rendered bioactive after oxidation by the hepatic cytochrome P-450 mixed function oxidase system. Bartolone et al.,<sup>14</sup> using acetaminophen-specific antibodies, demonstrated the presence of acetaminophen-protein adducts in microsomal and cytosolic extracts from liver, lung, and kidney, organs that sustain cellular damage after drug administration. Similar to our findings, these drug-protein conjugates were also found in the plasma; and, as we postulate occurs for SMX, the conjugates were not of plasma origin but had been released after hepatic metabolism.

Since their introduction in the 1930s, apparently toxic or immunopathologic adverse reactions



**FIG. 7.** Kinetic analysis of the appearance of SMX-substituted human serum proteins. Human serum samples, taken from an individual before and at varying times during SMX therapy, were immunochemically analyzed for SMX-conjugated proteins with SDS-PAGE and immunoblotting. *a*, Pretreatment serum; serum taken 5 hours (*b*), 24 hours (*c*), 48 hours (*d*), and 120 hours (*e*) after the initiation of SMX therapy; *f*, serum taken 48 hours after the last dose of SMX.

have been attributed to sulfonamides. However, the mechanisms responsible for these reactions have not been clearly elucidated. Although it has been proposed that covalent binding of sulfonamide metabolites to cell macromolecules could lead to hypersensitivity reactions, little evidence exists to support this hypothesis. We now demonstrate that drug-protein conjugates do exist in the sera of some SMX-treated individuals. Although we have yet to demonstrate that haptenation leads to sensitization or provokes clinical reactions, in one of our subjects who demonstrated marked SMX haptenation during an uneventful course of therapy urticaria later developed when the drug was reintroduced (patient 2). It is possible that individuals who demonstrate robust haptenation are at greater risk of developing allergic reactions because multivalent conjugates may promote immunologic sensitization. Studies are in progress to test this hypothesis.

Interestingly, Western blots (Figs. 6 and 7) demonstrate that a single 30 kd protein becomes haptenated. Thus it appears that SMX does not haptenate proteins indiscriminately. This was not an unexpected finding, however, because halothane and acetaminophen, other therapeutic agents that undergo oxidative metabolism, also have been found to bind covalently to a limited number of proteins<sup>14-16</sup> only. The mechanism underlying this high degree of protein specificity is most likely due to the reactivity of the intermediates formed and the population of proteins in the subcellular "vicinity" of newly formed reactive intermediates. In the case of halothane it has been proposed that most of the intermediates are

probably trapped by the cytochrome P-450 molecules that produce them before they have a chance to diffuse away to react with other proteins.<sup>17</sup> In favor of this hypothesis is the finding that the major haptenated protein found in human microsomes and in the plasma membrane after halothane treatment is a 54 kd cytochrome P-450 enzyme. It remains to be seen whether the target of SMX haptentation is the enzyme responsible for its metabolism.

Although we have demonstrated that protein substitution by sulfonamides occurs during therapy, further studies will be required to determine the relationship between haptentation and sensitization. The mechanism responsible for sulfonamide haptentation, its variable presence in treated individuals, and its necessity for development of hypersensitivity reactions are all important and unresolved issues that are the subject of current studies.

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