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Facilitation of Penicillin Haptenation to Serum Proteins

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Traditionally, penicillin binding to serum proteins was believed to be a passive chemical process; however, it appears to be facilitated by serum factors. The objectives of this in vitro investigation were to examine facilitated penicillin haptenation, to study the kinetics of haptenation, and to determine the nature of haptenation-facilitating factors. The model involved addition of [³H]benzylpenicillin to serum or albumin solutions (at pH 7.3 to 7.4) and incubation at 37°C for up to 72 h. The extent of penicillin binding to proteins in serum was found to be four- to fivefold higher than with solutions having comparable concentrations of purified albumin, total protein, or total immunoglobulin. Ultrafiltration of serum reduced penicillin binding to serum proteins substantially. An ultrafiltrable haptenation-facilitating factor(s) was found to be less than 0.5 kDa but was not calcium or magnesium. Finally, the extent of penicillin binding was related to albumin purity, as binding substantially increased with albumin purity. These findings suggest that there is a factor(s) in serum that facilitates covalent binding of penicillin to serum proteins. The factor(s) can be removed and then restored to increase penicillin binding to albumin. It appears that at least one component of the facilitation factor is less than 0.5 kDa, which suggests that it is not a peptide and that it is some simple serum component other than calcium or magnesium.

For 50 years, since its introduction, clinical use of penicillin has been complicated by immediate hypersensitivity reactions. Allergic reactions to penicillin have been reported to occur in 0.7 to 8% of patients, depending on the study (3). We have previously reported that penicilloyl-specific immunoglobulin G (IgG) and IgE were detected in 38 and 18% of patients, respectively, who received ≥2 g of penicillin per day for at least 10 days (1), suggesting that major determinant-specific antibody responses are restricted to a minority of the general population. We postulated that heterogeneity of immune responsiveness to penicillin could be influenced by individual differences in immunogen formation and clearance, possibly involving differential rates of haptenation and dehaptenation to carrier serum proteins.

Lee and Sullivan (2) have reported that penicillin haptenation to serum proteins is facilitated and not passive. Haptenation of penicillin to serum proteins was 4.0- to 5.3-fold greater than that to purified human serum albumin. Sullivan (4) has also reported that penicillin may undergo dehaptenation from albumin and that this process may proceed at a lower rate in penicillin-allergic subjects.

A number of questions remain regarding the nature of facilitated penicillin haptenation to serum proteins. Specifically, the kinetics of facilitated haptenation and the nature of facilitating serum factors need to be addressed. The objectives of this in vitro investigation were to extend the findings of Lee and Sullivan regarding facilitated haptenation (2), to study the kinetics of haptenation further, and to determine the nature of facilitating factors.

MATERIALS AND METHODS

Materials. Serum was obtained from one healthy adult male volunteer with no history of allergy. The total protein and albumin in this serum were determined to be 7.2 and 4.6

g/dl, respectively. Where noted, sera from additional non-penicillin-allergic volunteers were tested. Three purity grades of human serum albumin were used. These included fraction V powder (A1653; Sigma Chemical Company, St. Louis, Mo.), crystallized and lyophilized fraction V powder (A9511; Sigma Chemical Company), and electrophoretically prepared, 100% pure powder (18.501.904, lot 4965F; Behring Diagnostics, Somerville, N.J.). Tritiated penicillin {phenyl-4(n)-[³H]benzylpenicillin} was obtained as a 93.1 to 98.6% pure solution with a specific activity of 10.5 to 25 Ci/mmol (TRK 779; Amersham, Arlington Heights, Ill.). The activities of experimental samples were determined on a beta counter (LS1701 Liquid Scintillation Counter; Beckman Instruments, Columbia, Md.). Unlabeled benzylpenicillin sodium was also obtained from Sigma Chemical Company. Trichloroacetic acid was obtained as a 100% solution (490-10; Sigma Chemical Company). Purified human IgG was obtained commercially (Sandoglobulin; Sandoz Pharmaceutical Corporation, East Hanover, N.J.). Phosphate-buffered saline (PBS), pH 7.3 to 7.4, was prepared fresh for each experiment. Ultrafiltrations were performed with an ultrafiltration cell (Amicon, Beverly, Mass.) with membranes having nominal molecular mass cutoffs of 0.5, 10, 30, and 50 kDa (YM membranes for 10, 30, and 50 kDa and YC membrane for 0.5 kDa). The membranes result in minimal retention of proteins (including albumin) at concentrations above 0.1 mg/ml (data on file at Amicon).

Procedures. Haptenation of penicillin to serum proteins was determined by adding 44,000 dpm of tritiated penicillin (in 50 μl of PBS) along with unlabeled benzylpenicillin (in 50 μl of PBS) to each milliliter of serum or 4.6% human albumin in PBS tested. The total reaction solution volume was 1 to 2 ml, depending on the number of time points at which samples were collected. The mixtures were incubated at 37°C for up to 72 h. At specified times, 110-μl aliquots were removed from each mixture for determination of amounts of penicillin bound to albumin. The concentration of unlabeled penicillin was varied from 1 to 1,000 μM and did not affect

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haptenation of labeled penicillin. The data reported below are for 1,000 μM penicillin G.

Each aliquot was treated as follows. To 110 μl of protein solution in which penicillin was incubated (serum or albumin), 1.5 ml of 3% trichloroacetic acid was added to precipitate proteins. The mixture was centrifuged (500 × g for 10 min at 20°C), and the supernatant was removed by vacuum. The precipitate was redissolved in 3 ml of PBS, and then protein was reprecipitated with 0.3 ml of 30% trichloroacetic acid. This redissolution-and-precipitation procedure was repeated a total of three times. The final protein pellet was dissolved in 150 μl of 0.5 N sodium hydroxide. The solution was placed in a scintillation vial, 4 ml of scintillation cocktail was added, and radioactivity was assayed with a beta counter and reported as counts per minute. All assays were performed in triplicate, and results were reported as means with standard deviations (SD). Background activity was determined from samples assayed as described above, except that precipitation and washing were initiated within 10 s of addition of labeled penicillin. For each test sample, the reported result in counts per minute is the net value of the counts per minute determined minus the background counts per minute.

A variety of protein-containing solutions were prepared and used in the experiments described below. These included the following: intact human serum; serum which had been concentrated by ultrafiltration (under nitrogen pressure) with a 30- or 10-kDa-cutoff membrane, washed with multiple volumes of PBS (diaflowed), and then reconstituted to the original volume with PBS; serum which had been concentrated by ultrafiltration with a 30- or 10-kDa-cutoff membrane, diaflowed, and then reconstituted to the original volume with serum ultrafiltrate (prepared with a 10- or 30-kDa-cutoff membrane); 4.6 or 7.2% human serum albumin (fraction V) diluted in PBS; 4.6% human serum albumin (fraction V) diluted in ultrafiltrate from serum (with a 10- or 30-kDa-cutoff membrane); 4.6% human serum albumin (crystallized and lyophilized fraction V) diluted in PBS; 4.6% human serum albumin (crystallized and lyophilized fraction V) diluted in ultrafiltrate from serum (with a 10- or 30 kDa-cutoff membrane); 4.6% human serum albumin (100% electrophoretically pure) diluted in PBS; 4.6% human serum albumin (100% electrophoretically pure) diluted in ultrafiltrate from serum (obtained with a 10- or 30-kDa-cutoff membrane); 2.6% IgG diluted in PBS, pH 7.4; and 2.6% IgG with human serum albumin (fraction V) in PBS, pH 7.4. For all albumin solutions, the pH was adjusted to 7.3 to 7.4. With the above-described protein solutions and procedures, a number of comparisons of serum, ultrafiltered serum, and albumin solutions were made.

Modification of penicillin binding to serum. An experiment was conducted to determine whether the putative factors responsible for increased penicillin binding could be removed from serum by ultrafiltration. The protein-containing solutions compared were intact serum, ultrafiltered serum diaflowed through a 30-kDa-cutoff membrane and reconstituted with PBS, and ultrafiltered serum diaflowed and reconstituted with serum ultrafiltrate (prepared with a 30-kDa-cutoff membrane). Labeled penicillin and unlabeled penicillin were added as described above, and the mixtures were incubated for 48 h.

The potency of the serum haptenation-facilitating factor(s) was assessed by reconstitution of serum that was ultrafiltered, diaflowed, and concentrated with serum ultrafiltrate (obtained with a 30-kDa-cutoff membrane) and PBS at various ratios (100, 75, 50, 25, and 0% ultrafiltrate) by using the incubation methods described above.

Characterization of haptenation facilitation factor(s). To characterize the haptenation-facilitating factor in serum, ultrafiltrate was prepared from intact serum by using membranes with molecular size cutoffs of 0.5, 10, 30, and 50 kDa. Serum that was ultrafiltered, diaflowed, and concentrated was then reconstituted by using each of these filtrates and compared with intact serum and 4.6% human serum albumin (fraction V) in PBS. The six test materials were studied by using the procedures described above and 48 h of incubation.

We then determined whether serum cations (calcium and magnesium) are the factors required for facilitated penicillin haptenation in 4.6% human serum albumin (fraction V) in PBS. The above-described procedures were followed for incubation and sample processing. Test materials included intact serum, 4.6% human serum albumin (fraction V) in PBS, 4.6% human serum albumin (fraction V) in PBS with 2.5 mM calcium (as calcium chloride), and 4.6% human serum albumin (fraction V) in PBS with 0.78 mM magnesium (as magnesium chloride). Serum that was ultrafiltered, diaflowed, and reconstituted with PBS was also tested by addition of 2.5 mM calcium or 0.78 mM magnesium.

Effect of albumin purity on penicillin binding. Since the fraction V human serum albumin used in the initial experiments was labeled as 96 to 99% pure, it was possible that non-albumin-haptenating factors from serum were present. Therefore, we determined whether albumin purity influences the extent to which penicillin binds. Three 4.6% human serum albumin products varying in purity were studied, i.e., fraction V, recrystallized and lyophilized fraction V, and 100% electrophoretically pure human serum albumin. Each was diluted in PBS and in serum ultrafiltrate (prepared from a 10-kDa-cutoff membrane), and the pH was adjusted to 7.3 to 7.4. The procedure described above was then followed, with the same amounts of labeled and unlabeled penicillin added, incubation time, wash and precipitation steps, and counting.

Haptenation kinetics. To investigate further the binding to intact serum and 100% pure human serum albumin reconstituted with serum ultrafiltrate, a kinetic study was done with seven determinations over the first 24 h.

RESULTS

Comparison of intact serum with 4.6% human serum albumin (fraction V in PBS). After incubation for 72 h with labeled penicillin, the mean binding of penicillin to intact serum was almost fivefold higher than with 4.6% human serum albumin (fraction V) (6,161 ± 1,272 versus 1,270 ± 141 cpm). Binding of penicillin appeared to plateau by 24 h for both protein solutions (Fig. 1). In a separate study (data not shown), when the human serum albumin concentration was increased to 7.2%, so that total protein concentrations were similar to that of intact serum, the extent of penicillin binding increased insignificantly, to 26% from 19.5% of the binding with intact serum. This suggests that there are factors in serum that enhance binding to albumin or that there are other substances in serum to which penicillin binds selectively.

In a related experiment, we determined whether immunoglobulin can account for the differences between penicillin binding to serum and penicillin binding to 4.6% human serum albumin. The protein solutions tested included intact serum, 4.6% human serum albumin (fraction V) in PBS, 4.6% human serum albumin (fraction V) with 2.6% IgG in PBS
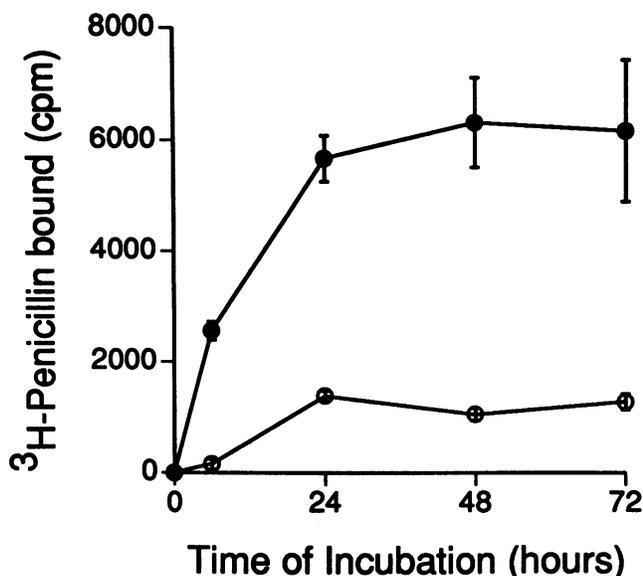
FIG. 1. Relative extent of penicillin binding to intact serum (●) or human serum albumin (○) over 72 h at 37°C (± SD).

with the pH adjusted to 7.4, and 2.6% IgG alone in PBS with the pH adjusted to 7.4. For intact serum and the solution of human serum albumin plus IgG, the total protein concentration was the same (7.2%). IgG at 2.6% added to 4.6% human serum albumin increased penicillin binding by about 25%, but the penicillin binding still did not approach that of intact serum. With intact serum, 4.6% human serum albumin in PBS, 4.6% human serum albumin plus 2.6% IgG, and 2.6% IgG as test materials, the mean ± SD counts per minute bound were 5,658 ± 160, 1,638 ± 206, 2,046 ± 176, and 740 ± 111, respectively. These data demonstrate that the presence of IgG does not account for the differences in penicillin binding between serum and human serum albumin.

Modulation of penicillin haptenation with ultrafiltration. Ultrafiltration of serum was found to reduce penicillin binding. Penicillin binding was 7,400 ± 288 cpm with intact serum, 2,113 ± 195 cpm with serum diaflowed and reconstituted with PBS, and 6,082 ± 306 cpm with serum diaflowed and reconstituted with serum ultrafiltrate. The binding of serum that had been ultrafiltered and reconstituted with PBS was 29% of that of intact serum. However, when the ultrafiltered serum was reconstituted with serum ultrafiltrate, the binding capacity was restored to 82% of that of intact serum. Serum ultrafiltrate alone showed minimal penicillin binding (96.4 ± 7 cpm). This experiment demonstrated that the haptenation-facilitating factors in serum are less than 30 kDa and can be removed to some extent and then restored to serum.

The serum factor(s) retained almost its full ability to facilitate penicillin haptenation at up to a dilution of 1 to 2 with PBS. The results are shown in Table 1. Beyond 1 to 2 dilutions, the facilitation activity was reduced in a concentration-dependent manner.

Characterization of haptenation factor(s). Reconstitution of serum with ultrafiltrate from 0.5-, 10-, 30-, and 50-kDa-cutoff membranes substantially increased penicillin binding compared with 4.6% albumin in PBS. The results are shown in Table 2. This demonstrates that a major component of the haptenation activity is below 0.5 kDa.

TABLE 1. Effect of dilution of serum concentrate reconstitution fluid on penicillin haptenation

% of serum reconstitution fluid that was:		Net cpm bound ± SD
Serum ultrafiltrate	PBS	
100	0	6,081 ± 306
75	25	6,072 ± 212
50	50	5,506 ± 69
25	75	4,214 ± 111
0	100	2,112 ± 195

Addition of calcium or magnesium did not increase penicillin haptenation of human serum albumin or ultrafiltered and diaflowed serum. Penicillin binding was 6,287 ± 341 cpm for intact serum, 2,188 ± 98 cpm for human serum albumin without calcium or magnesium, 1,811 ± 123 cpm for human serum albumin with calcium, and 1,772 ± 121 cpm for human serum albumin with magnesium. Binding of ultrafiltered and diaflowed serum was 3,791 ± 245 cpm with no added cations, 3,171 ± 65 cpm with calcium added, and 3,571 ± 58 cpm with magnesium added. Calcium and magnesium did not appear to be the facilitating factors.

Effect of albumin purity on penicillin binding. The extent to which penicillin bound to human serum albumin was directly related to albumin purity (Fig. 2). For each type of albumin, dilution in serum ultrafiltrate increased the extent of binding almost twofold. With the purest human serum albumin diluted in serum ultrafiltrate, the extent of binding was equivalent to that of intact serum (data not shown). When 4.6% electrophoretically pure human serum albumin was diluted with ultrafiltrate from human serum albumin fraction V (obtained with a 10-kDa-cutoff membrane), penicillin binding was decreased compared with dilution with PBS (3,375 ± 54 versus 3,834 ± 89 cpm, respectively), suggesting that a factor in the fraction V human serum albumin preparation inhibited haptenation.

Haptenation kinetics. The relationship of penicillin binding with time is shown in Fig. 3. By using linear regression to determine the slope of each line, we found average rates of binding to penicillin of 131 cpm/h for serum and 66 cpm/h for the human serum albumin solution. Therefore, the initial rate of binding to reconstituted purified human serum albumin was much less than the rate of binding to intact serum, suggesting that higher-molecular-weight factors in serum modulate penicillin haptenation.

Studies of sera from non-penicillin-allergic patients. Sera

TABLE 2. Effects of various-molecular-weight ultrafiltrates on restoration of penicillin-facilitated haptenation to diaflowed serum

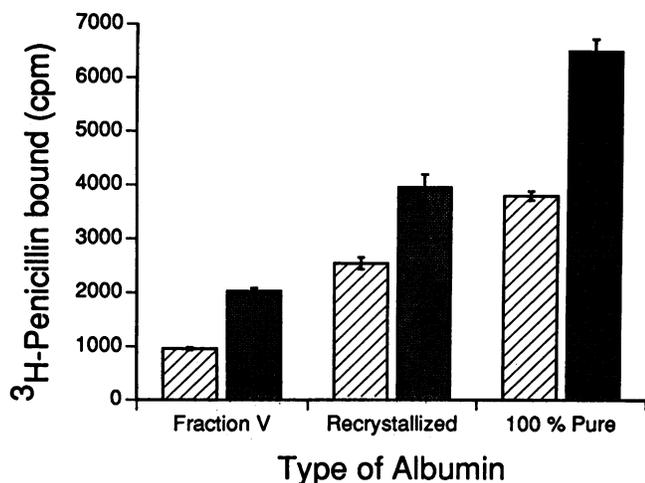| Test material	Mean cpm bound ± SD
Intact serum	7,611 ± 162
4.6% human serum albumin (fraction V) in PBS	2,078 ± 134
Ultrafiltered serum reconstituted with ultrafiltrate from:	
50-kDa membrane	6,869 ± 20
30-kDa membrane	5,946 ± 180
10-kDa membrane	5,680 ± 180
0.5-kDa membrane	6,564 ± 47

FIG. 2. Effect of albumin purity on binding of penicillin reconstituted with either PBS (▨) or serum ultrafiltrate (▩).

from seven subjects that were documented not to possess detectable anti-penicilloyl IgE were available. Penicillin binding averaged 4,914 ± 643 cpm (range, 3,550 to 5,388 cpm), while it was 2,780 ± 125 for 4.6% human serum albumin in PBS.

DISCUSSION

These studies confirm and extend the preliminary report of Lee and Sullivan (2) regarding facilitated haptenation of penicillin to serum proteins. We demonstrated that a factor(s) in serum indeed facilitates covalent binding of penicillin to serum proteins. The factor can be removed from serum by ultrafiltration, and when returned to the serum it restores penicillin binding. When serum ultrafiltrate is added to human serum albumin, penicillin binding is increased but at
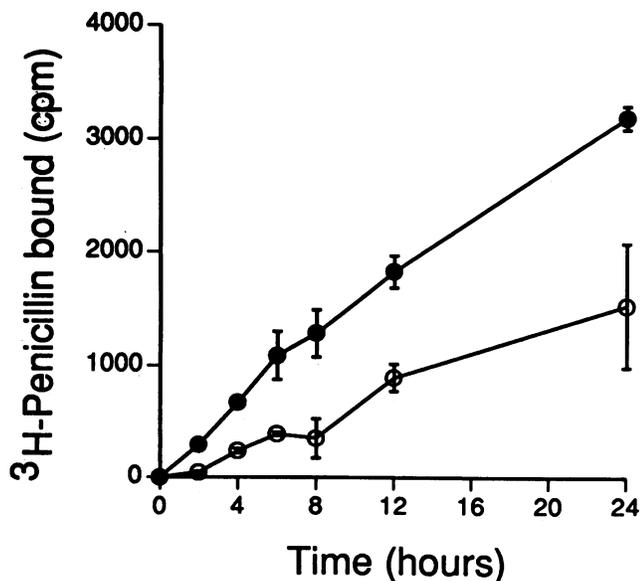

FIG. 3. Kinetics of penicillin binding to serum proteins (●) or to 100% electrophoretically pure human serum albumin reconstituted with serum ultrafiltrate (○).
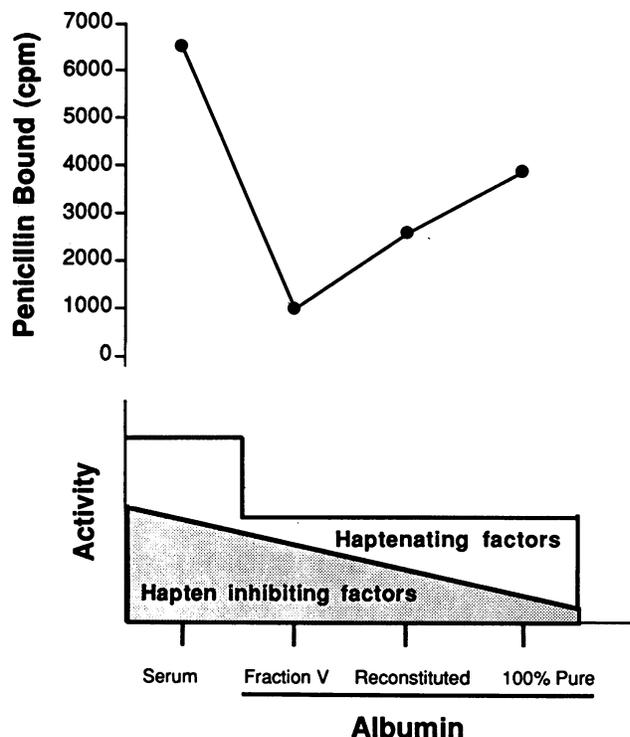

FIG. 4. Proposed model of penicillin haptenation and dehaptenation factors in serum and albumin fractions based on observed relative penicillin binding.

a rate less than that for intact serum. It appears that at least one component of the facilitation factor is less than 0.5 kDa, which suggests that it is not a peptide and that it is some simple serum component other than calcium or magnesium. We did not attempt to define the factor(s) further.

In the studies with whole serum, we did not determine the percentage of penicillin that is bound to proteins other than albumin. Studies with purified IgG demonstrated minimal binding to this fraction. Also, negligible binding to serum ultrafiltrate suggests that small peptides are not important as sites for penicillin binding. The study with 100% pure human serum albumin demonstrated an extent of binding similar to that obtained with intact serum and suggests that similar numbers of binding sites are available in serum and 4.6% albumin.

The demonstration that albumin purity influences penicillin haptenation suggests that there are also factors which inhibit penicillin haptenation. It appears that purification of albumin removes the inhibitory factors. With the presence of inhibitory and facilitating haptenation factors, the extent and rate of penicillin binding depend on the net effect. A proposed model compatible with the data is that with intact serum, facilitating factors predominate in effect (Fig. 4). With the preparation of human serum albumin fraction V, much of the facilitation factor is removed while some of the inhibitory factors are not. Thus, the net effect with fraction V human serum albumin is a relatively low rate of penicillin haptenation. With further purification of human serum albumin, the inhibitory factors are removed, allowing facilitating factors to predominate. This is in agreement with the lower rate of haptenation with 100% pure human serum albumin in serum ultrafiltrate compared with intact serum.

No direct relationship has been established between the extent of penicillin bound to serum proteins and the risk of penicillin allergy. Penicillin sensitivity also relates to mechanisms involved in antigen presentation and IgE production. However, even at some low level, production of penicilloyl-albumin conjugate should be associated with reactivity since current models of type I hypersensitivity assume that bivalent penicilloyl-albumin is required to cross-link IgE on basophils or mast cells.

These studies support the hypothesis that there are factors in serum which facilitate and inhibit penicillin haptenation to serum proteins. Perhaps these factors could partially explain the heterogeneity of immune responsiveness to penicillin in patients, although this possibility was not investigated in our study. Further studies are needed to determine the relationship between facilitated haptenation and the immune response.

REFERENCES

1. **Adkinson, N. F.** 1984. Risk factors for drug allergy. J. Allerg. Clin. Immunol. **74:**567–572.
2. **Lee, M., and T. J. Sullivan.** 1989. Facilitated haptenation of human proteins by penicillin. J. Allerg. Clin. Immunol. **85:**333.
3. **Sullivan, T. J.** 1982. Pathogenesis and management of allergic reactions to penicillin and other beta-lactam antibiotics. Pediatr. Infect. Dis. **1:**344–350.
4. **Sullivan, T. J.** 1989. Dehaptenization of albumin substituted with benzylpenicillin G determinants. J. Allerg. Clin. Immunol. **85:** 222.