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IMMUNOLOGICAL MECHANISMS IN ASPIRIN HYPERSENSITIVITY. STUDIES ON THE IMMUNOGENICITY OF FREE ASPIRIN

By

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Some of the more alarming adverse reactions induced by aspirin have long been suspected to be allergic in nature (4, 5). Almost uniformly negative skin test results in aspirin-sensitive individuals have, however, led to opinions which contest the participation of immunologic mechanisms in these reactions (2, 18, 20, 23, 24). The familial occurrence of the triad consisting of asthma, aspirin intolerance and nasal polyps (15, 16, 24) has favoured the concept that aspirin intolerance is an inborn error of metabolism (22). However, some cases with aspirin reactions showing positive skin tests have been reported (7, 10, 20) and recent work by Phills & Perelmutter (19) demonstrates that immunologic mechanisms are responsible for aspirin reactions of anaphylactic type, including urticaria, angioedema and anaphylactic shock.

Conjugates of aspirin with various proteins have been prepared and specific antibodies directed against aspiryl and/or salicylyl hapten have been produced in experimental animals by inoculation with these conjugates (7, 9, 27-29). However, a demonstration of the immunogenicity of the *in vitro* prepared conjugates gives no indication concerning the ability of the drug to form immunogenic conjugates *in vivo*. In the latter case, the effective hapten could be different from that implanted on the carrier protein *in vitro*. A thorough examina-

tion of the pertaining literature shows that the immunogenic potency of free aspirin has not so far been explored. According to de Weck et al. (1, 7) pure aspirin cannot form aspiryl conjugates, but all commercial aspirin preparations contain aspirin anhydride that can easily aspirylate proteins.

These considerations led us to investigate whether aspirin as such is capable of (a) inducing specific antibody formation in experimental animals, (b) coating erythrocytes with aspirin-derived hapten(s) enabling them to detect anti-aspirin antibodies by passive haemagglutination and (c) of forming aspiryl conjugates with various proteins.

MATERIAL AND METHODS

Reagents. Acetylsalicylic acid (Merck, Darmstadt). Salicylic acid and lanoline (Carlo Erba, Milano). Paraffin oil (Loba-Chemie, Wien). Chromium chloride (Union Chimique Belge, Bruxelles). Evans blue, ovalbumin, bovine γ -globulin (Fluka, Basel). Tris (Serva, Heidelberg). Human γ -globulin and lyophilized BCG (Cantacuzino Institute, Bucharest). All other chemicals used for the preparation of various solutions were of reagent grade.

Aqueous solutions of aspirin and salicylic acid were prepared extemporaneously by adding 1 M NaOH drop by drop and warming in a water bath, the final pH being brought to near neutrality. When used for inoculation the solutions were Seitz-filtered.

Preparation of the adjuvant mixture. Incomplete Freund's adjuvant was prepared from one part lanoline (sterilized by tindallization) one part 10 per cent aqueous solution of aspirin or salicylic acid and two parts paraffin oil. Killed mycobacteria from lyophilized BCG were incorporated in the complete Freund's adjuvant, so that the final emulsion contained 0.5 mg bacteria/ml.

Animals. New Zealand rabbits weighing 2.5–3 kg and outbred guinea pigs weighing 400–500 g, of either sex were used.

Inoculation. Two millilitres of the drug emulsions were given intramuscularly to the rabbits and 0.4 ml to the guinea pigs, each dose being divided equally into the four extremities. The dose of aspirin and salicylate administered at one inoculation was about 20 mg/kg. The second inoculation was performed 2 weeks later, and then repeated at 1-week intervals in the same manner.

Serum samples. Serum samples were obtained from the blood taken from the marginal ear vein in rabbits and by cardiac puncture in guinea pigs 11 or 12 days after the first inoculation and 6 days after each of the other inoculations. Each serum sample was divided into two portions. One, stored at -10°C , was used for the final antibody assay after the elaboration of the passive haemagglutination test, while the other, kept at 4°C , was used within a week for

various trials to adjust the test. Two of the rabbits having higher titres of anti-aspiryl antibodies were finally exsanguinated under sterile conditions and their sera, divided in vials, were stored at -10°C and used as standards in various investigations.

Aspiryl-coated erythrocytes. The landmarks elaborated for erythrocyte penicilloylation (3, 6, 13, 14, 25) were closely followed in our trials. The finally adopted technique was as follows:

Rabbit erythrocytes, collected in Alsever's solution and kept at 4°C for not more than 2 weeks, were washed four times with at least 10 vol. of Tris-buffered saline pH 7.4. A 4 per cent aspirin solution was mixed with an equal volume of borate buffer 0.2 M, pH 9.5, and 0.1 ml of washed erythrocytes was added to make up 2 ml of this mixture. The suspension was incubated at 37°C for 50 min with gentle shaking at 10-min intervals. The erythrocyte suspension was then centrifuged 3-4 min at 1,000 g and the cells were washed four times with Tris-buffered saline. The sensitized erythrocytes were finally suspended at a concentration of 2 per cent in the diluent (Tris-buffered saline containing normal rabbit serum 1.5 per cent). Aspiryl-coated erythrocytes are prone to haemolysis and must, consequently, be freshly prepared. They can, however, be stored for 1 or 2 days in the storage solution recommended by Levine et al. (13) without appreciable decrease in their sensitivity, although partial haemolysis occurs.

Passive haemagglutination test. Serial dilutions with a 1/2 ratio were made in the diluent referred to above. When the sensitized erythrocytes were heterologous, the sera were inactivated and thoroughly absorbed with normal red cells from the same sample. Dilutions of normal serum, as well as a few tubes containing the diluent alone, were used as controls for possible non-specific agglutination. Readings were taken after 1-2 h at room temperature in three ways: (a) from settling patterns; (b) by lightly tapping the tubes after spontaneous sedimentation; (c) by resuspending the cells, centrifugating for 3-4 min at 1,000 g and gently tapping the tubes. Although a close parallel was noted between these kinds of readings, the last one seemed a little more sensitive than the others.

Aspiryl-protein conjugates. Conjugates of aspirin with human γ -globulin, bovine γ -globulin, ovalbumin and rabbit serum were prepared by a procedure similar to that described by Vickers & Assem (26) for the preparation of penicilloyl conjugates.

A 20 per cent solution of aspirin was mixed with equal volumes of sodium carbonate buffer 1 M pH 10.5, and a 10 per cent solution of protein. The reaction mixture was kept at room temperature and shaken at intervals. The pH, which tends to decrease, was readjusted around 10.5-11 after 2, 4, 8 and 18 h. After 24 h, the reaction mixture was dialysed against 100 vol. of phosphate-buffered saline pH 7.4, at 4°C with two daily changes, during 5 days. The final dialysate was brought to 2 per cent protein concentration and stored in vials at -10°C .

For comparative studies, aspiryl-protein conjugates of the same proteins were

also prepared from aspirin chloride following the technique described by Wicher et al. (28). The characteristic absorbance activity of these conjugates at 305 nm was examined with a Zeiss spectrophotometer.

Coupling of aspiryl-protein conjugates on erythrocytes. The procedure recently described by Dixon et al. (8) using chromium chloride was found suitable for this system. One volume of washed erythrocytes was suspended in 5 vol. of aspiryl conjugate diluted to 0.2 per cent (as carrier protein) in saline, and 0.025 ml of a freshly prepared 1 per cent aqueous chromium chloride solution was added for each ml of reaction mixture, drop by drop and with mixing. In order to avoid non-specific agglutination, the conjugates were previously fully absorbed with normal erythrocytes and the carrier protein (also absorbed with normal erythrocytes) was added to the serum dilutions in order to bind antibodies directed against it.

Passive cutaneous anaphylaxis (PCA) was performed following the procedure described by Ovary (17), the challenge being done either systemically or locally. The non-specific reactions of the conjugates and of the carrier proteins were carefully evaluated by appropriate control experiments.

Intradermal tests were performed following standard techniques, using concentrations of conjugates which elicited no reactions in normal animals. These concentrations were about 1/10,000.

RESULTS

Production of Specific Antibodies by Aspirin

Three groups of rabbits were inoculated as shown under 'Methods' with an equal dose of aspirin (20 mg/kg) given in aqueous solution (group I); in Freund's incomplete (group II); or complete adjuvant (group III). Sera obtained at various intervals during the sensitization period were examined for their ability to agglutinate aspirin-treated erythrocytes from various animal species.

Taking into account that aspirin, like penicillin, is an acidic drug, an alkaline pH was thought to be more suited for coupling aspiryl groups on erythrocyte membrane. Erythrocytes were therefore incubated at various time intervals in aspirin solutions of different concentrations (rendered isotonic with NaCl when necessary), mixed by vol. with various buffer solutions 0.2 M, pH 9-9.5. After various trials with sera collected during the sensitization period, the technique described under 'Methods' was adopted, and the frozen serum samples

from all the bleedings were finally titrated for haemagglutinating anti-aspiryl antibodies with the standardized technique. The results are summarized in Table 1.

As seen in this Table, no anti-aspiryl antibodies were detected in rabbits treated with aspirin in aqueous solution (group I). Specific haemagglutinating antibodies were, however, found in the sera of most rabbits given the drug incorporated in Freund's incomplete (group II) or complete adjuvant (group III). Although just detectable after the first inoculation, the titres of the aspiryl-specific antibodies increased in subsequent inoculations in both groups, but definitely higher titres were recorded in animals given the drug incorporated in complete adjuvant. The specificity of these antibodies was proven beyond any doubt, since, apart from the negative results in the group treated with aspirin in aqueous solution, none of the serum samples from 10 normal rabbits agglutinated aspirin-treated erythrocytes. Similar results were obtained in guinea pigs, but the antibody titres were smaller.

In sharp contrast to aspirin, the administration of sodium salicylate under absolutely identical conditions did not give rise to antibodies detectable by agglutination of either salicylate- or aspirin-treated erythrocytes. It is also worthwhile noting that none of the sera agglutinating aspirin-treated erythrocytes reacted with salicylic acid-treated cells.

Direct Binding of Aspiryl Groups to the Erythrocyte Membrane

Ability of various erythrocytes to bind aspiryl groups. A standard rabbit anti-aspiryl serum was inactivated and divided into four samples. Three of them were absorbed exhaustively with guinea pig, human and sheep erythrocytes, respectively, while one was kept unabsorbed. Meanwhile, rabbit, guinea pig, human and sheep erythrocytes were sensitized with aspirin under strictly identical conditions. Three dilution rows were made from each of the absorbed serum samples, as well as from the inactivated but unabsorbed serum and from non-

TABLE I
Mean Titres of Anti-Aspiryl Antibodies Detected by Passive Haemagglutination in Rabbits Inoculated with Aspirin in Aqueous Solution and Incorporated in Incomplete or Complete Freund's Adjuvant.

Time of blood withdrawal	Group I. Aqueous solution			Group II. Incomplete adjuvant			Group III. Complete adjuvant		
	No. of animals in group	No. of animals with antibodies	Mean antibody titre	No. of animals in group	No. of animals with antibodies	Mean antibody titre	No. of animals in group	No. of animals with antibodies	Mean antibody titre
12 days after 1st inoculation	8	0	0	8	3	2	7	5	2 ^{2.7}
6 days after 2nd inoculation	8	0	0	8	4	2 ^{1.5}	7	6	2 ^{3.8}
6 days after 3rd inoculation	8	0	0	8	6	2 ^{1.9}	7	6	2 ^{5.1}
6 days after 4th inoculation	8	0	0	8	7	2 ^{2.2}	7	6	2 ^{5.3}
6 days after 5th inoculation	8	0	0	8	7	2 ^{2.7}	7	6	2 ^{5.3}

inactivated serum. Haemagglutination assays were parallelly performed with sensitized rabbit erythrocytes and with sensitized erythrocytes from the respective species. Normal erythrocytes were added to one row of tubes in order to check the absorption completeness. The results demonstrated that rabbit erythrocytes gave haemagglutination titres four times higher than guinea pig and sheep erythrocytes and 16–32 times higher than human erythrocytes. The heat inactivation (30 min at 56°C) did not affect the haemagglutination titre, while the absorption caused a two-fold (one tube) decrease of the titre.

Influence of the buffer. Samples of a 4 per cent aspirin solution were mixed with an equal volume of one of five buffers 0.2 M, pH 9. Rabbit erythrocytes were incubated following the adopted procedure in each of these buffered aspirin solutions and then assayed for haemagglutinating ability with a standard anti-aspiryl serum. While somewhat lower titre was recorded with Tris-buffer, no important difference was noted in this respect among the other four buffers tested, namely: boric acid – NaOH; veronal – HCl; CO_3HNa – CO_3Na_2 ; glycine – NaOH.

Influence of the pH. Rabbit erythrocytes were incubated for 50 min in 4 per cent aspirin solution diluted by vol. in borate buffer 0.2 M of various pH values, namely 6, 7, 8, 9 and 9.5. The intensity of aspirylation was estimated by the intensity of the haemagglutination with a standard anti-aspiryl serum. The results showed that under these conditions no detectable erythrocyte aspirylation occurred at pH 6–8, while effective aspirylation took place at pH 9 and 9.5.

Influence of aspirin concentration. Rabbit erythrocytes were incubated for 50 min at 37°C in borate-buffered (pH 9.5) solutions of aspirin with concentrations ranging between 0.2 M (3.6 per cent) and 0.01 M (0.18 per cent) and the intensity of erythrocyte aspirylation was estimated as shown above. The results showed that under these conditions a detectable erythrocyte aspirylation occurred only at a concentration of 0.04 M, the intensity of erythrocyte sensitization improving with in-

creasing aspirin concentration. However, because of haemolysis occurring at higher concentrations, a 2 per cent (0.11 M) concentration was chosen for the standardized technique.

Influence of the source of aspirin preparations. Merck acetylsalicylic acid used throughout in this study and two samples of aspirin produced by two of the Rumanian drug factories were comparatively studied concerning their ability to sensitize rabbit erythrocytes. No significant difference was found between the three aspirin samples.

Influence of the temperature. The erythrocyte sensitization with 2 per cent aspirin in borate buffer was studied comparatively by performing the incubation at 37° C and at room temperature (20° C). The results showed that aspirylation was somewhat more efficient at 37° C than at 20° C.

Treatment with salicylic acid and acetic anhydride. As mentioned in a preceding paragraph, salicylic acid-treated erythrocytes were not agglutinated by aspiryl-specific anti-serum. In other experiments, erythrocytes incubated with acetic anhydride were not agglutinated by anti-aspiryl serum.

Formation of Aspiryl Conjugates by Direct Reaction with Aspirin

Aspiryl-protein conjugates, prepared by incubating proteins with aspirin at alkaline pH as shown under 'Methods' induced positive direct skin tests in three out of eight rabbits sensitized with aspirin incorporated in incomplete adjuvant. They also elicited positive PCA reactions in guinea pigs sensitized intradermally with serum from two rabbits of the same group, but no positive PCA test was recorded with sera from the rabbits inoculated with aspirin in complete Freund's adjuvant. Although both sera inducing PCA reactions contained haemagglutinating antibodies, they were not those with the highest haemagglutinating titre. When erythrocytes were coated with these conjugates by means of the chromium chloride technique, the erythrocytes could be agglutinated by the aspiryl-specific antiserum. The titres of passive haemagglutination tests performed with erythrocytes coated with these aspiryl-protein

conjugates generally equalled the titres of haemagglutination performed with either directly aspirylated erythrocytes or with erythrocytes coated with aspiryl conjugates prepared from aspirin chloride. Aspiryl conjugates prepared with either method were also equally effective in inhibiting the agglutination of aspirin-treated erythrocytes by the specific antiserum. These passive haemagglutination tests were significantly inhibited by aspiryl-protein conjugates at concentrations around 10^{-6} M, while aspirin as such produced no inhibition up to a concentration of 10^{-2} M.

In contrast to the similar behaviour of the conjugates in the immunological tests, striking differences were recorded concerning the molar absorbance at 305 nm between the conjugates prepared from aspirin chloride and those prepared by incubation with aspirin at alkaline pH. In accordance with previous data in the literature (7, 28), the conjugates prepared from aspirin chloride showed a molar absorbance at 305 nm of about 3,320. By contrast, the conjugates prepared by incubation of proteins with aspirin at alkaline pH were approximately 25 times less active in this respect. A parallel study of the absorbance of aspirin and salicylic acid at this wave length revealed that the former has a molar absorbance about eight times lower than the latter. The calculated number of haptenic groups coupled on a molecule of protein depends, therefore, on the supposed chemical nature of the hapten. Assuming that the haptenic groups in the aspiryl-ovalbumin conjugate prepared from aspirin chloride were exclusively aspiryl groups, approximately 200 of these groups would be coupled on a molecule of carrier protein. Supposing the haptenic groups in the conjugate to be exclusively salicylic groups, only 25 haptenic groups would be found on a molecule of ovalbumin. Similar calculations concerning the aspiryl-ovalbumin conjugate prepared by incubation of protein with aspirin at alkaline pH showed that it might contain eight haptenic groups if these were aspiryl groups, but only one haptenic group per carrier molecule if this group was a salicylyl group.

DISCUSSION

Although direct coupling of penicilloyl group to the erythrocyte membrane by simply incubating red blood cells with penicillin at alkaline pH has long become a routine procedure for preparing erythrocytes capable of detecting anti-penicilloyl antibodies in a passive haemagglutination test (6, 13, 14, 25), a similar approach for coupling aspiryl and/or salicylyl groups on the erythrocyte membrane has not yet been tried. The results reported in the present paper demonstrate that, as in the case of penicillin, at alkaline pH aspirin is capable of implanting its specific hapten(s) on the membrane constituents of the erythrocytes rendering them suitable for detecting anti-aspiryl antibodies in a passive haemagglutination assay. Since, even under these conditions, the acetyl group is lost in a percentage of aspirin, it seems likely that both aspiryl and salicylyl groups are bound to the erythrocyte membrane. However, since the reaction between aspirin and erythrocytes takes place under much milder conditions than those under which aspiryl conjugates have been prepared from aspirin chloride (7, 9, 27, 28, 29), we think that by directly reacting aspirin with erythrocytes, aspiryl rather than salicylyl groups are predominantly coupled on the erythrocyte membrane.

As in the case of penicillin, an amide bond is certainly established between the salicylic carboxyl of the aspirin molecule and the free amino groups of certain membrane constituents. A similar binding is currently envisaged to operate when aspiryl conjugates are formed by treating proteins with aspirin chloride or other highly reactive aspirin derivatives (1, 7, 21, 29). Recent work by de Weck et al. (1, 7) has shown that highly purified aspirin is not capable of forming conjugates with proteins or polylysine, while aspirin anhydride and acetylsalicylsalicylic acid, two common impurities in commercial aspirin preparations, are responsible for the binding of aspiryl and salicylyl haptens on the carrier amino groups. Since the Merck aspirin used in the present work has not, presumably, been without such impurities, it seems apparent

that erythrocyte aspirylation could have been due to these impurities rather than to aspirin itself. The finding that, among the three aspirin samples tested, no significant difference could be established concerning their power to aspirylate erythrocytes while the amount of impurities they contained were quite different, suggests that amino-reactive derivatives can be formed extemporaneously from aspirin itself when solubilized in water at alkaline pH.

Aspiryl-protein conjugates were successfully prepared in the present experiments following a procedure similar to that currently used for the preparation of penicilloyl-protein conjugates, namely by incubating proteins in sodium acetylsalicylate solutions at alkaline pH. Although in various immunological tests these conjugates behaved identically with the conjugates prepared from aspirin chloride, striking differences were noted concerning their molar absorbance at 305 nm, the former being about 25 times weaker than the latter. The finding that the molar absorbance of aspirin at this wave length is about eight times lower than the absorbance of salicylic acid strongly suggests that the differences between the molar absorbance of the two series of conjugates resides in the fact that the aspiryl/salicylyl ratio in the conjugates prepared by our method is much higher than in the conjugates prepared by reacting proteins with aspirin chloride. The almost identical conditions under which erythrocytes and protein aspirylation took place in our experiments suggest that the hapten spectrum on the erythrocyte membrane is very similar, if not identical, to the hapten spectrum in the aspiryl-protein conjugates. The high efficiency shown by these conjugates in inhibiting the passive haemagglutination performed with directly aspirylated erythrocytes also favours this conclusion. However, since the conjugates prepared from aspirin chloride were equally effective in this respect, the combined evidence seems to indicate that the differences in the aspiryl/salicylyl ratio in the conjugates do not essentially alter their antigenic specificity. Whether this fact is due to identical haptenic properties of aspiryl and salicylyl groups or to the presence of

a minimal amount of aspiryl groups in the predominantly salicylyl conjugates cannot be answered as yet.

Since a single aspirin sample was used for preparing aspiryl conjugates, and a comparative investigation on the ability of other aspirin samples to form such conjugates was not undertaken, the question, whether aspirin as such or its impurities supply the aspiryl groups on the carrier molecule, remains unanswered.

Aspirin has been shown to acetylate easily the protein amino groups, and the degree of acetylation increases with increasing pH (11). It follows, therefore, that under our experimental conditions, part of the amino groups of the erythrocyte membrane constituents, as well as of the proteins used to prepare aspiryl conjugates, were acetylated. Since acetylated homologous serum proteins have been shown to be antigenic (12), we investigated whether antibodies reacting with acetylated erythrocytes (obtained by incubating them with acetic anhydride) were found in the sera of aspirin-sensitized rabbits. The negative results suggest that under our experimental conditions, no *N*-acetyl specific antibodies were formed. In contrast to aspirin, salicylic acid did not give rise to antibodies capable of reacting with either salicylic acid- or aspirin-treated erythrocytes, and the sera showing positive haemagglutination tests with aspirin-treated erythrocytes did not react with salicylic acid-treated cells. There are at least three possible explanations for this discordance between the behaviour of aspirin and salicylic acid. Firstly, salicylic acid lacks the ability to generate conjugates *in vivo* and thereby become immunogenic. Secondly, although salicylyl-specific antibodies are formed, they are not detectable because salicylic acid does not bind to the erythrocyte membrane and/or because salicylyl-specific antibodies do not react with aspiryl-specific antibodies. Schwartz & Amidon, cited by Yurchak et al. (29), have already stated that salicylic acid is not able to form salicylamide derivatives with protein amino groups. Accordingly, our findings indicate that, contrary to aspirin, salicylic acid is not able to form either conjugates *in vitro* or to implant salicylyl groups on the erythrocyte membrane.

So far, aspiryl/salicylyl-specific antibodies have been obtained experimentally only by inoculation with *in vitro* prepared aspiryl-protein conjugates (7, 9, 27, 28, 29). The results reported here show that, as with penicillin (3), the inoculation of aspirin incorporated in Freund's adjuvant gives rise to aspiryl-specific antibodies as detected by passive haemagglutination or PCA tests. When given as such, in aqueous solution, aspirin does not induce antibody formation in either rabbits or guinea pigs; a finding which agrees with previous observations on penicillin immunogenicity (3), but disagrees with Suzuki's report cited by Wicher et al. (28), concerning the possibility of sensitizing guinea pigs by administration of aspirin without adjuvant.

SUMMARY

Anti-aspiryl antibodies were produced in rabbits and guinea pigs by inoculation of aspirin incorporated in complete or incomplete Freund's adjuvant. These antibodies were readily detected by passive haemagglutination using rabbit erythrocytes incubated with aspirin at alkaline pH. Aspiryl conjugates with ovalbumin, human γ -globulin, bovine γ -globulin and rabbit serum were also prepared by incubating the proteins with aspirin at alkaline pH. Aspiryl conjugates prepared by this technique behaved, immunologically, identically with the conjugates prepared from aspirin chloride. By contrast, the molar absorbance at 305 nm of the conjugates prepared from aspirin was about 25 times lower than the molar absorbance of the conjugates prepared from aspirin chloride. Since the absorbance of salicylic acid is about eight times greater than that of aspirin, the conclusion is drawn that the aspiryl/salicylyl ratio is significantly higher in the conjugates prepared by incubating proteins with aspirin at alkaline pH than in the conjugates prepared from aspirin chloride.

In parallel experiments, salicylic acid did not induce formation of specific antibodies capable of reacting with aspirin- or salicylic acid-treated red cells. Sera giving positive passive

haemagglutination with aspirin-treated erythrocytes did not react with erythrocytes treated with salicylic acid or acetic anhydride.

REFERENCES

1. Bundgaard, H. & de Weck, A. L. (1975): The role of amino-reactive impurities in acetylsalicylic acid allergy. *Int. Arch. Allergy* 49, 119-124.
2. Chafee, F. H. & Settiple, G. A. (1974): Aspirin intolerance. I. Frequency in an allergic population. *J. Allergy* 53, 193-199.
3. Cîrstea, M., Suhaciu, G. & Boros, I. (1975): Experimental studies on drug sensitization. I. Penicillin sensitization as an experimental model for drug allergy. *Rev. roum. Physiol.* 12, 243-247.
4. Cooke, R. A. (1919): Allergy in drug idiosyncrasies. *J. Amer. med. Ass.* 73, 759-761.
5. Cooke, R. A. (1947): Allergy in theory and practice. Saunders, Philadelphia and London.
6. de Weck, A. L. (1964): Penicillin allergy: its detection by an improved haemagglutination. *Nature (Lond.)* 202, 975-977.
7. de Weck, A. L. (1971): Immunological effects of aspirin anhydride, a contaminant of commercial acetylsalicylic acid preparations. *Int. Arch. Allergy* 41, 393-418.
8. Dixon, R., Rosse, W. & Ebbert, L. (1975): Quantitative determination of antibody in idiopathic thrombocytopenic purpura. *New Engl. J. Med.* 292, 230-236.
9. Giraldo, B., Blumenthal, M. N. & Spink, W. W. (1969): Aspirin intolerance and asthma. A clinical and immunological study. *Ann. intern. Med.* 71, 479-496.
10. Girard, J. P., Hildebrandt, F. & Favre, H. (1969/70): Hypersensitivity to aspirin: Clinical and immunological studies. *Helv. med. Acta* 35, 86-95.
11. Hawkins, D., Pinckard, R. N. & Farr, R. S. (1968): Acetylation of human serum albumin by acetylsalicylic acid. *Science* 160, 780-781.
12. Hoffman, D. R. & Campbell, D. H. (1970): Model systems for the study of drug hypersensitivity. II. Studies on the acetylation mechanism for aspirin intolerance. *J. Immunol.* 104, 582-584.
13. Levine, B. B., Fellner, M. J. & Levytska, V. (1966): Benzylpenicilloyl specific serum antibodies to penicillin in man. I. Development of a sensitive hemagglutination assay method and haptenic specificity of antibodies. *J. Immunol.* 96, 707-718.
14. Ley, A. B., Harris, J. P., Brinkley, M., Liles, B., Jack, J. A. & Cahan, A. (1958): Circulating antibodies directed against penicillin. *Science* 127, 1118-1120.
15. Lockey, R. F., Rucknagel, D. L. & Vanselow, N. A. (1973): Familial occurrence of asthma, nasal polyps, and aspirin intolerance. *Ann. intern. Med.* 78, 57-63.

16. Maur, K., Adkinson, N. F., Metre, T. E., Marsh, D. G. & Norman, P. S. (1974): Aspirin intolerance in a family. *J. Allergy* 54, 380-395.
17. Ovary, Z. (1960): Reverse passive cutaneous anaphylaxis in the guinea pig with horse, sheep or hen antibodies. *Immunology* 3, 19-27.
18. Parker, C. W. (1975): Drug therapy: Drug allergy. *New Engl. J. Med.* 292, 732-736.
19. Phills, J. A. & Perelmutter, L. (1974): IgE mediated and non-IgE mediated allergic type reactions to aspirin. *Acta allergol.* 29, 474-490.
20. Samter, M. & Beers, Jr., R. F. (1968): Intolerance to aspirin. Clinical studies and consideration of its pathogenesis. *Ann. intern. Med.* 68, 975-983.
21. Schlumberger, H. D. (1975): Immunogenicity of aryl esters of salicylic or acetylsalicylic acid in guinea pigs. *Int. Arch. Allergy* 48, 467-474.
22. Schlumberger, H. D., Löbbecke, E.-A. & Kallos, P. (1974): Acetylsalicylic acid intolerance. Lack of *N*-acetylsalicylic acid specific, skin-sensitizing antibodies in the serum of intolerant individuals. *Acta med. scand.* 196, 451-458.
23. Settupane, G. A., Chafee, F. H. & Klein, D. E. (1974): Aspirin intolerance. II. A prospective study in an atopic and normal population. *J. Allergy* 53, 200-204.
24. Settupane, G. A. & Padupakkam, R. K. (1975): Aspirin intolerance. III. Subtypes, familial occurrence, and cross-reactivity with tartrazine. *J. Allergy* 56, 215-221.
25. Spath, P., Garratty, G. & Petz, L. (1971): Studies on the immune response to penicillin and cephalothin in humans. I. Optimal conditions for titration of hemagglutinating penicillin and cephalothin antibodies. *J. Immunol.* 107, 854-859.
26. Vickers, M. R. & Assem, E. S. K. (1974): Tests for penicillin allergy in man. I. Carrier effect on response to penicilloyl conjugates. *Immunology* 26, 425-440.
27. Weiner, L. M., Rosenblatt, M. & Howes, H. A. (1963): The detection of humoral antibodies directed against salicylates in hypersensitive states. *J. Immunol.* 90, 788-792.
28. Wicher, K., Schwartz, M., Arbesman, C. E. & Milgrom, F. (1968): Immunologic studies with aspirin. I. Antibodies to aspiryl-protein conjugates. *J. Immunol.* 101, 342-348.
29. Yurchak, A. M., Wicher, K. & Arbesman, C. E. (1970): Immunologic studies on aspirin. Clinical studies with aspiryl-protein conjugates. *J. Allergy* 46, 245-253.

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