

Immunologic studies on aspirin

Clinical studies with aspiryl-protein conjugates

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Aspiryl-chloride was conjugated with human serum albumin, human gamma globulin, rabbit gamma globulin, and poly-L-lysine. These conjugates were evaluated in skin tests, indirect hemagglutination, lymphocyte culture, and in an immunofluorescent test on tissues and sera of patients sensitive to aspirin. No evidence of specific immunologic reactions was found. Possible explanations for these results are discussed, and three possible nonimmunologic mechanisms for adverse reactions to aspirin are offered.

The occurrence of asthma and hypotension following the ingestion of aspirin by certain patients has been documented by many authors.¹⁻⁵ Some persons develop troublesome urticaria after taking aspirin.³⁻⁵ Many of these patients have no other manifestations of an allergic diathesis.⁶ A safe and reliable diagnostic method for aspirin sensitivity is not available. In previous publications it has been shown that aspiryl-protein conjugates are immunogenic.^{7, 8} This report describes the use of aspiryl conjugates in testing of sera and tissues of patients sensitive to aspirin.

MATERIALS AND METHODS

Aspiryl conjugates

The aspiryl-protein conjugates were prepared as described previously.⁷ The conjugates used in this study were: aspiryl-human serum albumin (Asp-HSA), aspiryl-human gamma globulin (Asp-HGG), and aspiryl-rabbit gamma globulin (Asp-RGG). Aspiryl-poly-L-lysine (Asp-PLL) was prepared in a similar way: 250 mg. of poly-L-lysine, molecular weight 110,000 (Pilot Chemicals, Inc., Watertown, Conn.), was dissolved in 25 ml. of distilled water and partially succinylated by the addition of 100 mg. of succinic anhydride. Then 75 mg. of aspiryl chloride dissolved in ether was added and the mixture stirred until the reaction was completed. The conjugated Asp-PLL was exhaustively dialyzed against phosphate-buffered

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saline, 0.15M, pH 8.6. The protein concentration of the conjugates was estimated by the Kjeldahl method. The number of aspiryl groups conjugated with each protein was determined spectrophotometrically by measuring absorbance at 305 m μ . The ratio of aspiryl groups per carrier molecule was: 15 to 20 for Asp-HSA; 10 to 15 for Asp-HGG; 21 to 30 for Asp-RGG; and 5 to 10 for Asp-PLL. The activity of all conjugates was examined by gel precipitation and tanned cell hemagglutination tests against rabbit antisera specific for the aspiryl group. The tests were performed as described previously.⁷ All conjugates gave strongly positive reactions in both tests.

Skin test

Freshly prepared conjugates of Asp-HSA, Asp-HGG, and Asp-PLL diluted in buffered saline were used for direct skin tests. The concentrations of all conjugates used were 1 and 0.01 mg. per milliliter. Higher concentrations caused nonspecific skin reactions in some normal individuals. The skin test in the patients was performed by the intradermal injection of 20 μ g and 0.2 μ g of each conjugate in 0.02 ml. The results were read after 20 minutes, and 24 and 48 hours.

Immunofluorescent test

Tissue sections from nasal polyps obtained from 4 aspirin-sensitive patients were studied for the presence of antibodies by an indirect immunofluorescent technique. The preparation of tissue sections and the general technique of the fluorescent test were described previously.⁹ Two different aspiryl conjugates were used for this test. Tissue sections were incubated with Asp-HGG (10 mg. per milliliter) and, after washing, were covered with a fluorescent-labeled rabbit antiserum to human IgG (Hyland). Another set of tissue sections was incubated with Asp-RGG (10 mg. per milliliter) and after washing covered with a fluorescent-labeled goat antiserum to rabbit serum proteins. An additional set of sections was studied for the presence of complement with the use of a fluorescent-labeled rabbit antiserum to human β_{1c} (Hyland). All slides were examined immediately after preparation.

Lymphocyte culture

Lymphocytes of 6 patients sensitive to aspirin were examined in vitro with the use of different aspiryl conjugates. Heparinized blood of a patient was allowed to sediment and the supernatant containing the white cells was removed. The white cells were diluted in medium 199 (Grand Island Biochemical Company) to a concentration of one million mononuclear cells per milliliter. Four milliliters of the cell suspension was distributed in culture tubes (Falcon Plastics). The conjugates Asp-HSA, Asp-HGG, or Asp-PLL were diluted in medium 199. Each was added (0.1 ml.) to separate duplicate sets of tubes to final concentrations of 25 and 2.5 μ g per milliliter. Prior to each test, acetylsalicylic acid was dissolved and diluted in medium 199 at pH 7.4 and used in a final concentration of 2.5 μ g per milliliter. Control tubes without aspirin preparations and tubes containing 0.1 ml. of a 1:100 dilution of phytohemagglutinin-P (PHA-P, Difco) were included in each experiment. In addition, lymphocytes were incubated with a mixture of one of the aspirin compounds and PHA-P. The tubes containing PHA-P alone and PHA-P with one of the aspirin compounds were incubated for 3 days and the remaining tubes for 7 days at 37° C. Prior to harvest, 0.5 μ c of tritiated thymidine (New England Nuclear Corp., 6.7 c. per millimole) was added to each tube and the incubation at 37° C. continued for the final 16 hours. At harvest, the cells were centrifuged and washed twice in cold 5 per cent trichloroacetic acid (TCA). Two milliliters of 5 per cent TCA was then added and the nucleic acids extracted by heating the samples to 90° C. for 20 minutes. An aliquot of 0.5 ml. of each sample was then added into 15 ml. of Bray's¹⁰ solution and the radioactivity measured in a Packard Model 3000 scintillation counter.

Hemagglutination test

The sera of 17 patients were examined by a hemagglutination test with the use of tannic acid-treated erythrocytes coated with Asp-RGG and Asp-HGG as described previously.⁷ The

Table I. Description of patients and laboratory results

Patient	Age	Sex	Clinical disease*	Results of tests with aspiryl compounds		
				Skin tests	Hemagglutination titer	Lymphocyte culture
D. C.†	49	M	AR, P, S	Negative	Under 10	S.I. = 1‡
R. M.†	38	M	A, E, P, S	Negative	Under 10	S.I. = 1
W. M.	47	M	A, E, P, PR, S	Negative	Under 10	S.I. = 1
T. G.	57	F	A, E, P	Negative	Under 10	S.I. = 1
E. M.	59	F	A, P	Negative	Under 10	S.I. = 1
G. B.	63	F	A, E, P, PR	Negative	Under 10	S.I. = 1
Z. B.†	30	F	CS, P, PR, S	Negative	Under 10	ND§
J. D.	33	F	A, CS, P, PR, S	Negative	Under 10	ND
J. F.	33	F	A, CS, E, P, PR, S	Negative	Under 10	ND
Y. Y.	40	F	A, E, CS, P, PR, S	Negative	Under 10	ND
J. K.	43	M	A, P, PR, S	Negative	Under 10	ND
H. P.†	50	F	A, CS, E, P, PR	Negative	Under 10	ND
D. M.	57	M	A, CS, E	Negative	Under 10	ND
E. P.	69	F	A, CS, E, P, PR, S	Negative	Under 10	ND
J. W.	20	F	PR, U	Negative	Under 10	ND
F. K.	33	M	A, CS, E, PR, S, R	Negative	Under 10	ND
C. W.	34	M	U	Negative	Under 10	ND

*A, asthma; AR, seasonal allergic rhinitis; CS, corticosteroid therapy; E, eosinophilia over 6 per cent; P, nasal polyps; PR, perennial rhinitis; R, macular rash from aspirin; S, sinusitis; U, urticaria from aspirin.

†Polyps studied by immunofluorescent technique.

‡S.I.: specific incorporation of tritiated thymidine = counts with antigen/counts of control; a ratio of one (1) indicates no stimulation of thymidine incorporation.

§Not done.

sera prior to the test were absorbed with the carrier proteins. The coated erythrocytes were controlled by use of a rabbit antiserum to Asp-RGG absorbed with RGG and BGG.

RESULTS

A brief description of the 17 patients studied and the results of the immunologic tests performed are given in Table I. All but one patient was over 30 years of age. Ten of the patients were women. Only one patient had true seasonal allergic symptoms and was basically considered atopic. Fourteen of the patients had chronic perennial asthma and developed acute attacks of asthma upon ingestion of aspirin. Three nonasthmatic patients experienced repeated skin rashes after the ingestion of aspirin. Most patients had associated perennial rhinitis, nasal polyps, sinusitis, and peripheral blood eosinophilia. Eleven patients required a low dose (5 to 15 mg.) of prednisone to control nasal or bronchial symptoms.

The skin tests with the aspiryl preparations did not produce any definite immediate or delayed skin reactions, and no systemic symptoms were induced by the procedure.

The immunofluorescent examination of the nasal polyps did not show specific staining either with the aspiryl conjugates or the antiserum to β_{10} .

The lymphocyte cultures of the 6 patients studied showed no evidence of increased incorporation of tritiated thymidine over the control cultures. The lymphocytes of the same patients responded well to the action of PHA-P, indicating the potential for stimulation.

The tanned cell hemagglutination tests with the patients' sera showed no positive reactions, indicating lack of antibodies to the aspiryl conjugates in the sera. The rabbit antiserum to Asp-RGG reacted with the coated erythrocytes in a titer of 1:10,240.

DISCUSSION

Convincing evidence for an immunologic mechanism in adverse reactions to aspirin has not yet been found. Skin tests with aspirin performed in patients who are sensitive to aspirin are generally negative.¹¹ In addition, because hypotension and bronchospasm can be induced by such tests, they should be avoided.¹ Previous efforts to demonstrate skin reactions to protein conjugates of aspirin have been unsuccessful. Feinberg and Malkiel¹² used a preparation of whole human serum conjugated with aspiryl chloride in direct and passive transfer tests, with negative results. Mathews, Lovell, and Sheldon¹³ used sera for skin tests which were obtained from normal persons who had ingested aspirin. These sera contained from 60 to 240 μ g per milliliter of salicylates but were inactive in skin tests of aspirin-sensitive patients.

The immunofluorescent technique, to our knowledge, has not been utilized in tissue studies of patients sensitive to aspirin. Antibodies to the aspiryl conjugates were not detected in the patients' nasal polyps. In addition, the negative results with the use of the antiserum to β_{1C} suggest that complement is not involved in the pathogenesis of the polyps.

The negative results of the lymphocyte cultures agree with those of Caron¹⁴ and Giraldo and associates¹¹ but disagree with those of Halpern and associates.¹⁵ The latter authors reported uniformly positive results in lymphocyte cultures with the use of morphologic criteria to evaluate the results of the cultures. The studies of Caron and Giraldo and associates and our own employed a radioisotope technique to evaluate the response of the cells, which is more objective than counting lymphoblast-like cells as was done by Halpern.

Gantner and Zuckner¹⁶ reported a suppression of response to PHA when aspirin was added to lymphocyte cultures. However, in the concentrations of aspirin and aspiryl conjugates used by us, there was no suppression of PHA response. We could not culture lymphocytes from those patients who were receiving prednisone treatment since corticosteroids inhibit lymphocyte stimulation.¹⁷

The failure to find agglutinating antibodies to erythrocytes coated with the aspiryl conjugates contrasts with the positive results found by Weiner and associates¹⁸ in patients sensitive to aspirin or para-aminosalicylic acid (PAS). The antibodies found were peculiarly labile and disappeared after storage for 2 weeks at -10° C. Giraldo and associates¹¹ found hemagglutinating antibodies in the sera of normal and aspirin-sensitive patients, but it is unclear if their patients' sera were absorbed with the carrier proteins used for their aspiryl conjugates.

If adverse reactions to aspirin have an immunologic basis, 2 explanations can be offered for our negative results. First, a metabolic product different from that of an aspiryl conjugate might be involved in allergic reactions. The need

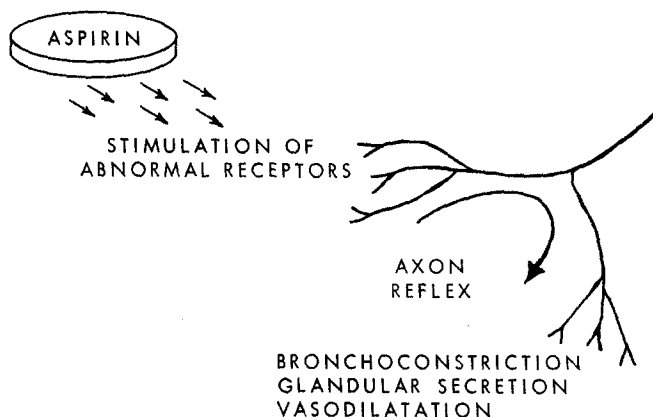


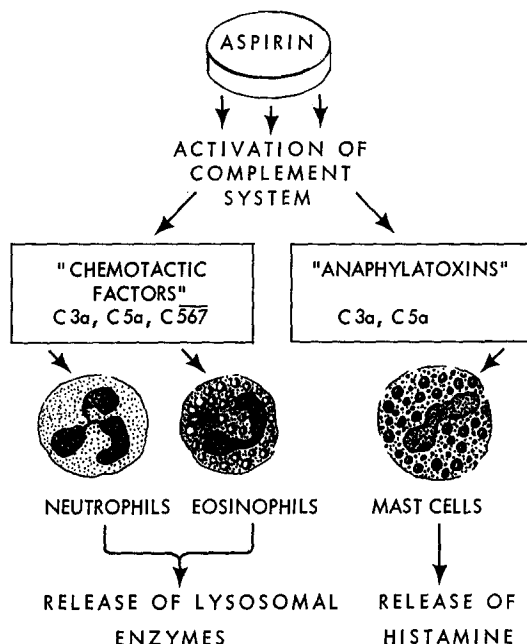
Fig. 1
Abnormal kinin receptor.

for testing patients sensitive to drugs with multiple metabolic products is well illustrated by the studies of Levine and associates¹⁹ on penicillin allergy. Aspirin has multiple degradation products and can react with serum proteins in a number of ways.²⁰ One of such reactions is the formation of a salicylamide derivative, a pathway that salicylic acid cannot follow.²¹ Another is the acetylation of proteins, such as albumin, as shown by Hawkins and associates.^{22, 23} Giraldo and associates²¹ found no positive responses with the use of aspirin-altered albumin in immunologic tests of aspirin-sensitive patients, but further studies of such products are awaited.

A second explanation for our negative results is the possibility that the conjugates used had lost their acetyl groups, since such antigens might not react with antibodies to acetylsalicylic acid. Acetylsalicylic acid can spontaneously hydrolyze to salicylic acid. In human beings, this process is accelerated by an enzymatic reaction so that only 25 to 50 per cent of ingested aspirin circulates after 30 minutes as the intact molecule.²⁴ We performed most of our tests within a few weeks of the preparation of the conjugates, but it is possible that the products used did not retain the acetyl group.

An alternative explanation of the negative results that could be offered is that nonimmunologic mechanisms are responsible for adverse reactions to aspirin. Samter and Beers²⁵ have proposed one intriguing hypothesis summarized in Fig. 1. These authors postulate that an underlying disease alters kinin receptors present in the lung, in capillaries, and in other tissues. Aspirin in sensitive patients stimulates these receptors and, in effect, causes symptoms similar to those produced by kinins. It is known that kinins can produce bronchospasm and vasodilation in man.^{26, 27} The induction of such symptoms in aspirin-sensitive patients by chemically unrelated compounds such as indomethacin, hydrazine yellow, and other drugs suggests that a nonimmunologic process may be involved.^{25, 28, 29}

Since no definite proof of an immunologic or nonimmunologic mechanism in aspirin sensitivity is available, we wish to offer two additional hypotheses.

**Fig. 2**

Direct activation of complement.

One mechanism for the action of aspirin in sensitive patients might be the direct activation of complement components. It is known³⁰⁻³³ that substances such as endotoxin, dextran, and plasmin can directly activate complement components, releasing "anaphylatoxins" which then release histamine. No antibodies are required in these reactions. Activation of $\overline{C3}$,* plasmin, or tissue proteases produces chemotactic factors that attract neutrophils and eosinophils.³⁴⁻³⁷ Thus the possible activation of the complement system by aspirin could produce the eosinophilia and local tissue edema seen clinically without requiring an immunologic mechanism. This sequence of events is outlined in Fig. 2. The release of anaphylatoxins and histamine would lead to well-known immediate effects. The attraction of neutrophils and eosinophils might result in the release of lysosomal enzymes causing chronic tissue damage.

We are not aware of studies of serum complement levels during acute reactions to aspirin. Our immunofluorescent studies were done on chronically inflamed polyps and might not indicate events occurring during acute reactions. Further studies of the complement system seem worthwhile.

A second hypothesis we wish to offer concerns the effect of aspirin on enzymes and the pathways leading to its ultimate pharmacologic effects. Aspirin in physiologic amounts affects a number of enzyme systems.³⁸ Indomethacin, a drug that provokes symptoms in aspirin-sensitive patients, can activate a proteolytic enzyme in rat skin.³⁹ Two nonimmunologic diseases that simulate allergic states are known to be associated with deficiencies of enzyme inhibitors. In hereditary angioedema there is deficiency of an inhibitor to multiple en-

*Proposed nomenclature of complement: Bull. W.H.O. 39: 935, 1968.

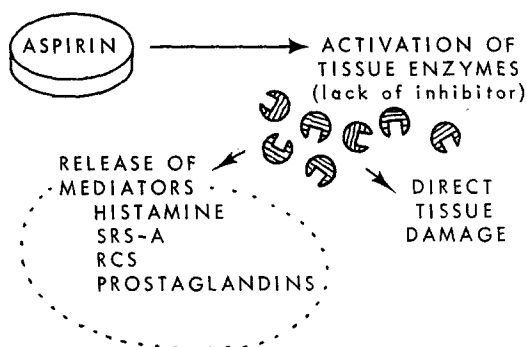


Fig. 3
Lack of enzyme inhibitor.

zymes.^{40, 41} This results in the development of life-threatening angioedema due to the activation of kinins and complement.⁴² Hereditary emphysema or alpha-1 antitrypsin deficiency is another example of a disease associated with the lack of an enzyme inhibitor.⁴³ This disease sometimes presents as "allergic bronchitis." Based on these two examples, it may be suggested that adverse reactions to aspirin occur because of a deficiency of an enzyme inhibitor as shown in Fig. 3. The activation of hypothetical tissue enzymes could lead to tissue damage and the further release of chemical mediators.

Piper and Vane⁴⁴ in a recent provocative article described effects of aspirin on a number of mediators released in anaphylaxis. Their studies show a potentiation by aspirin of slow-reacting substance of anaphylaxis (SRS-A) and an inhibition by aspirin of the release of a new mediator of anaphylaxis, rabbit aorta-contracting substance (RCS). Prostaglandins were also shown to be released subsequent to the action of SRS-A and the kinins. The role of all these mediators in adverse reactions to aspirin requires further study. Collier,⁴⁵ commenting on the work of Piper and Vane, notes that "aspirin and like-acting drugs block a route leading to or from the specific receptors for the agonists rather than blocking the receptors themselves."

We suggest that patients sensitive to aspirin may lack an inhibitor to an enzymatic reaction along this route. This lack might allow the release of kinins, the activation of complement, or the release of as yet unknown proteolytic enzymes. In future studies, it would be appropriate to search for nonimmunologic and enzymatic, as well as immunologic, mechanisms to explain adverse reactions to aspirin and other such chemical allergens.

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