

## DETECTION OF SERUM ANTIBODIES TO AMINO- PYRINE OR ITS DERIVATIVES BY A PASSIVE HEMAGGLUTINATION ASSAY METHOD\*

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### ABSTRACT

An attempt has been made to detect antibodies to aminopyrine or its derivatives (pyrazolone derivatives) with a passive hemagglutination assay system. Formalinized red blood cells were sensitized by coupling diazotized 4-aminoantipyrine to the surface membrane. With this method, circulating antibodies were detected in a patient with aminopyrine-induced agranulocytosis. Serial determinations revealed that the concentration of antibody increased rapidly after the onset of clinical manifestations, reaching a maximum in about a week, and thereafter began to fall off gradually. The presence of antibody was found in 32 sera of 75 patients with a history of skin reaction due to aminopyrine or its analogues and in 15 sera of 62 control subjects having no such history. The highest hemagglutination titer was 1:16.

Adverse reactions to drugs occur commonly in clinical practice and constitute a problem of considerable importance. Some of these reactions are judged to be allergic in mechanism<sup>1)-3)</sup>. For example, the administration of aminopyrine or its derivatives (pyrazolone derivatives) has been known to induce agranulocytosis or skin reactions<sup>4)</sup>. Moeschlin and Wagner<sup>5)</sup> demonstrated that agranulocytosis was elicited in volunteers to whom blood from a patient with aminopyrine-induced agranulocytosis had been given intravenously. They also found a substance in the plasma of the patient at the height of disease which produced *in vitro* agglutination of homologous and heterologous leukocytes. In addition to this finding, Thierfelder *et al.*<sup>6)</sup> succeeded in demonstrating in the serum of a patient suffering from agranulocytosis due to aminopyrine an antibody-like principle able to agglutinate donor leukocytes in the presence of aminopyrine. The detection and quantitative measurement of such

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a specific serum component would be of practical value, not only for diagnostic purposes, but also for insight into the etiology of drug hypersensitivity. However, no specific tests have as yet been established for the measurement of antibody to aminopyrine. Recently, antipenicillin antibodies have been detected in human sera by a specific and highly sensitive passive hemagglutination system<sup>7)8)</sup>. In this study it has been attempted to detect serum antibodies to aminopyrine with a passive hemagglutination assay method.

#### MATERIALS AND METHODS

In the experiments reported here, human sera were investigated by the following technique.

Human type O red cells (from whole blood kept no longer than 10 days in acid-citrate-dextrose solution) were formalinized and stored in formalin-saline in the cold (3-5°C) by the method of Ingraham<sup>9)</sup>. Cells stored in formalin-saline were washed 4 times with at least 30 volumes of 0.005 M phosphate-saline pH 7.4 and used as a 50 per cent suspension. 1 ml of the cell suspension was incubated with 0.2 ml of N sodium hydroxide and 0.7 ml of diazotized 4-aminoantipyrine (4 AA) solution for 1 hour at 0°C with occasional shaking. This diazo solution was prepared by adding 1 mmole of sodium nitrite to 1 mmole of 4 AA dissolved in 13 ml of a solution containing 3 mmoles of hydrochloric acid<sup>10)</sup>. The sodium nitrite (7 g/dl) was added over a period of 10 minutes with continuous stirring to the 4 AA solution kept in an ice-cold bath. The completion of diazotization was checked by the color reaction of zinc iodide-starch paper.

Settling pattern titrations were performed according to the method of Ingraham<sup>9)</sup>. Cells coupled with antigen (4 AA) were washed 7 times with at least 30 volumes of cold phosphate-saline and adjusted to give a concentration of  $8 \times 10^7$  cells/ml. One-tenth ml of the cell suspension was added to 0.5 ml of serial two-fold dilution of serum in phosphate-saline containing 1:100 normal rabbit serum, which had been inactivated at 56°C for 30 minutes and then absorbed with an equal volume of formalinized cells. After allowing test tubes to stand for 18 to 24 hours at room temperature, the patterns were observed. Titers were reported as the reciprocal of the highest dilution of serum in 0.5 ml which caused a pattern clearly distinguishable from the controls. Non-specific agglutination by test sera was controlled by running a parallel titration with cells which had not been coupled with antigen.

Specificity of agglutination was examined by inhibition experiment in the presence of antigen. Solutions of 4 AA were made up in phosphate-saline in concentrations from 0.001 M to 0.1 M. 0.05 ml of each 4 AA solution was added to 0.5 ml of serial two-fold dilution of serum. These were incubated at room temperature for 30 minutes, after which 0.1 ml of the suspension of 4 AA-coated cells was added.

Leukocyte agglutination tests were performed by the procedure of Thierfelder *et al.*<sup>6)</sup> with a slight modification. Leukocyte suspension was prepared from a healthy donor of the same blood group as the patient. 9.0 ml of fresh blood were added to a solution of 10 mg EDTA dissolved in 1.0 ml of saline in a siliconed test tube and then mixed with 2.5 ml of 6% Dextran\* in saline. After incubation at 37°C for 60 minutes, the middle layer of plasma was removed with a fine pipette and was used without further treatment. One drop of the leukocyte suspension was mixed with 2 drops of the patient's serum to which 1 drop of saline or aminopyrine solutions varying in concentration from 0.01 to 0.1 M had been added. As a control, preparations were made using 1 drop of the leukocyte suspension and 2 drops of normal serum to which the drug had been added as described above. Each preparation was placed in a shallow glass chamber devised by Moeschlin and Schmid<sup>11)</sup>, incubated at 37°C in a moist container and observed under a microscope at intervals for 2 hours.

#### RESULTS

*Hemagglutination titer in a patient with aminopyrine-induced agranulocytosis:* A 35-year-old taxi driver complained of a dull headache, cough and sputa on February 24, 1967. A diagnosis of common cold was made, and he was treated with a drug mixture containing aminopyrine. On March 1 he developed a temperature of 39.7°C and was admitted to the hospital on the next day. Initial blood examination: R.B.C. 4.98 million; W.B.C. 2,000 per cu.mm; differential count (per cent), lymphocytes 77, monocytes 22, basophils 1; platelet count normal. Examination of bone marrow obtained by aspiration revealed that the cells of the granulocytic series were decreased, and the inhibition of maturation was remarkable. Agranulocytosis induced by aminopyrine was suspected. Fig. 1 shows the clinical, hematologic, and serologic changes over the course of seven months.

On the day of admission the leukocyte agglutination test was negative both in the presence and absence of aminopyrine in the reaction mixture. At this time, the hemagglutination titer was only 1:2. Five days after admission, however, the titer had risen to 1:8, and it reached the level of 1:16 on March 10 (eight days after admission). The leukocyte agglutination test carried out at this time clearly demonstrated that only preparations containing patient's serum showed significant agglutination. The increase in agglutination of leukocytes was found with the reaction system to which aminopyrine was added. Agglutination of the patient's own leukocytes was not investigated. The patient was treated with sigmamyacin and betamethasone. His temperature returned to normal by the fourth day and remained normal thereafter. The leukocyte count rose rapidly; on the third day it was 3,150 and on the seventh day 6,300.

\* Supplied by Meitō Sangyo Co., Ltd., Nagoya. Mean Molecular Weight was 180,000.

## AGRANULOCYTOSIS

35 yrs. ♂

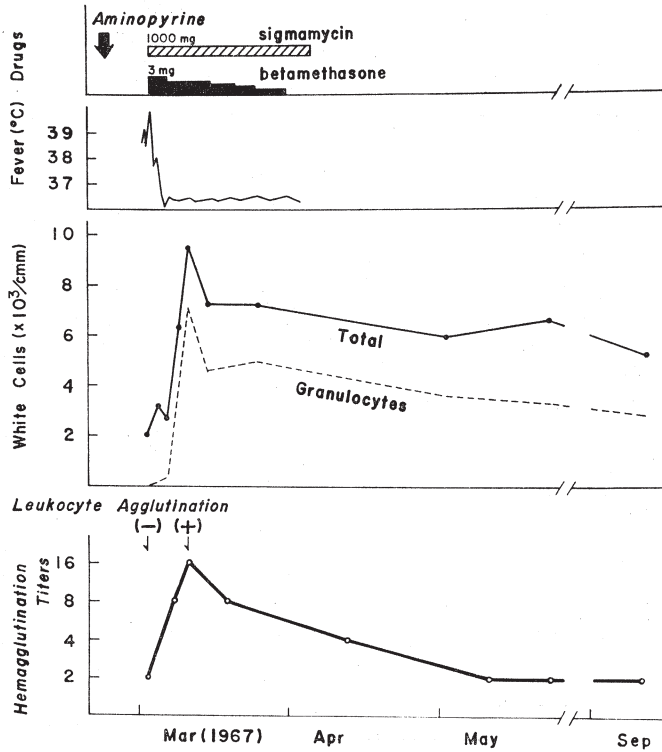


FIG. 1. Clinical, hematologic, and serologic course of a patient with aminopyrine-induced agranulocytosis.

The antibody titer fell progressively from its peak of 1:16 until on May 10, 10 weeks after onset of symptoms of agranulocytosis, it was only 1:2; the titer determined in September was also 1:2.

Reproducibility of the hemagglutination titers was examined in duplicate determinations and in tests performed on different days. No significant vari-

TABLE 1. Inhibition of Hemagglutination by Prior Addition of 4 AA to Reactive Serum

Concentration of 4 AA (M)*	Reciprocal Titer
0	16
0.001	8
0.01	8
0.1	4

\* 0.05 ml was added to 0.5 ml of each dilution of serum.

ations in titers were found in these tests. The antibody was stable for six months at refrigeration temperature ( $-20^{\circ}\text{C}$ ). Table 1 is a summary of data obtained from the inhibition experiment. By adding 0.05 ml of  $0.1\text{M}$  4 AA the hemagglutination titer of 1:16 was lowered to the level of 1:4. In the concentrations used here, 4 AA had no effect on the agglutination of human type A RBC by anti-A sera and type B RBC by anti-B sera.

*Antibody assays in patients with a history of skin reaction due to aminopyrine or its derivatives:* Sera of 75 patients from several medical clinics in Nagoya City were investigated. The reactions in these patients were manifested mainly by various kinds of skin rashes (erythema, maculopapular, maculovesicular, etc.), occasionally accompanied by fever, itching and other signs. The hemagglutination reaction was positive in 32 cases (42.6%). The distribution of hemagglutination titers of these patients is shown in Fig. 2 A. Twenty-seven of the 32 cases had titers ranging from 1:4 to 1:8. The highest antibody level was 1:16, which was found only in one patient. Only four cases showed the titer of 1:2. Volunteers from the laboratory or hospital staff and other apparently healthy subjects without such a history provided sera for the control series. The hemagglutination titers of these sera are presented in Fig. 2 B. In marked contrast to the foregoing group, the highest titer was 1:4. Of the 62 sera tested, six had a titer of 1:4, and nine a titer of 1:2. No antibody

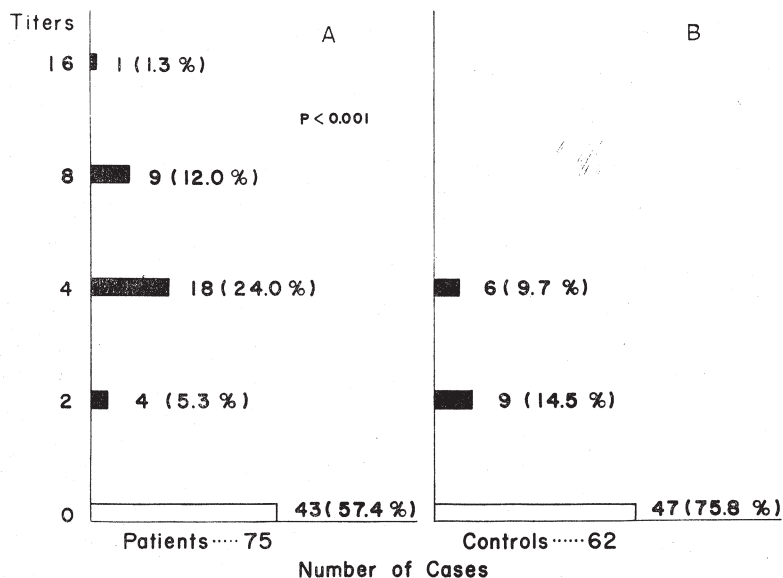


FIG. 2. Distribution of hemagglutination titers determined in cases with and without a history of skin reaction due to aminopyrine or its derivatives. The difference between these two groups is statistically significant,  $P < 0.001$ .

was detected in the remaining 47 sera (75.8%). Chi square analysis indicates a significant difference ( $P < 0.001$ ) between these two groups. An analysis of the relationship between the antibody levels and the intervals after the last clinical manifestation was done in 56 patients, whose histories about the last onset of allergic reaction were clearly noted. This is shown in Fig. 3. The hemagglutination reaction was positive in 25 of the 35 when the sera were investigated less than one year and a half after the onset of the allergic manifestations. On the other hand, the reaction was negative in most cases (16 out of 21) when the sera were investigated more than one year and a half after the allergic reactions. However, no definite relationship was demonstrated between the antibody titers and the frequency of clinical manifestations.

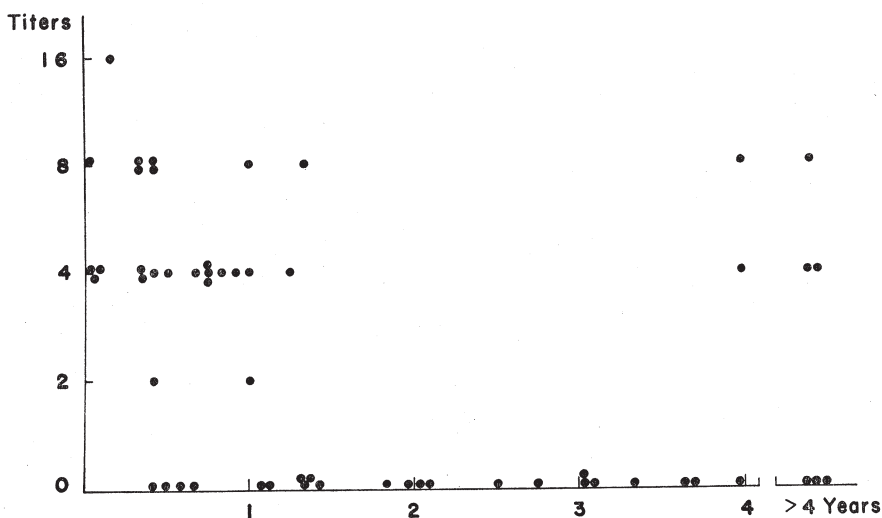


FIG. 3. Antibody levels of sera taken at various intervals after onset of clinical allergic manifestations.

#### DISCUSSION

The results indicate that the administration of aminopyrine or its derivatives to man gives rise to the synthesis of antibodies specific for these substances. In the hemagglutination technique presented here diazotized 4 AA was employed as the hapten to be attached to red blood cells. Though incompletely, hemagglutination reactions were inhibited in sera tested after prior incubation with 4 AA. According to previous reports<sup>(9,12,13)</sup>, the serologic or allergic specificity of drugs related to aminopyrine is determined by the substituents in position 1-3 of the antipyrene molecule, while substitution in position 4 is obviously less important or irrelevant in this regard. It seems possible, therefore, that circulating antibodies to pyrazolone derivatives of several sorts might

be detected by this method.

In the present study, serial observations performed in a patient with aminopyrine-induced agranulocytosis have revealed that the concentration of antibody increased rapidly, reaching a maximum in about a week, and thereafter began to fall off gradually. Furthermore, a certain correlation was found between the antibody levels and the intervals after the last clinical manifestation in patients having had skin reactions: the hemagglutination reaction was frequently positive (71.4%) when the sera were examined less than one year and a half after the onset of the allergic manifestations; on the contrary, the reaction was negative most of the time (76.2%) when the sera were investigated more than one year and a half after the allergic reactions. It can be seen from the above that the antibody content in the circulation falls gradually in the course of time.

Levine *et al.*<sup>14,15</sup> found that 97% of patients selected at random and all of patients with recent penicillin therapy had detectable hemagglutinating antibody specific for the benzylpenicilloyl group. On the other hand, de Weck<sup>2</sup> had previously shown a statistically significant difference in hemagglutination titers between patients with and without allergic reactions after administration of penicillin. The data in this investigation reveal that the incidence of positive hemagglutination reaction was higher in the group of patients with a history of skin reaction due to pyrazolone derivatives than in the group of subjects having no such history. Hemagglutinating antibodies could be found occasionally in the sera of the latter group. In such cases, the occurrence of hemagglutinating antibodies seems to indicate a specific immunological response to these drugs but is not obligatorily correlated with clinical allergic symptoms. Since aminopyrine or its derivatives are remedies frequently used in Japan, it is quite plausible that the sensitization by these drugs might be relatively prevalent among the people. Though the presence of circulating antibodies to aminopyrine or its analogues has been made clear in this investigation, their role in the development or in the clinical manifestation of allergic reactions remains obscure. The *in vitro* characteristics of circulating antipenicillin antibody have been studied in detail<sup>14,16,17</sup>. Of particular interest is the finding that two classes of antipenicillin antibodies, IgM and IgG, were defined, and the latter might be more related to the clinical manifestations than the former.

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