

Acta Medica Okayama

Volume 24, Issue 3

1970

Article 6

JUNE 1970

Antigenicity of chlorpromazine and clozapine to rabbits

Ayako Ueki*

Hiroshi Tada[†]

Satimaru Seno[‡]

*Okayama University,

[†]Okayama University,

[‡]Okayama University,

Antigenicity of chlorpromazine and clozapine to rabbits*

Ayako Ueki, Hiroshi Tada, and Satimaru Seno

Abstract

Antigenicity of clozapine was studied in rabbits, comparing with that of chlorpromazine as control. The results indicate that chlorpromazine produces antibody in rabbit as revealed by passive hemagglutination test, giving the titer of 1 : 2, 000 or higher in all the five cases observed, though specific precipitin lines has not been obtained and PCA test proves to be negative. Clozapine failed to produce anti.clozapine antibody giving negative passive hemagglutination test, passive cutaneous anaphylaxis and precipitin reaction, in all forms tested. Some remarks were made on the possible close relation between the antigenicity of the drug and its affinity to protein.

*PMID: 4249889 [PubMed - indexed for MEDLINE] Copyright ©OKAYAMA UNIVERSITY
MEDICAL SCHOOL

Acta Med. Okayama **24**, 323—332 (1970)

ANTIGENICITY OF CHLORPROMAZINE AND CLOZAPINE TO RABBITS

Ayako UEKI, Hiroshi TADA and Satimaru SENO

Department of Pathology, Okayama University Medical School, Okayama, Japan
(Director: Prof. S. Seno)

Received for publication, May 2, 1970

A number of cases of agranulocytosis have been reported in patients treated with chlorpromazine, aminopyrine, chloramphenicol and others. In Japan we also encounter not infrequently the cases of agranulocytosis, aplastic anemia and panmyelopathy to which phenothiazine derivatives or some antibiotics like chloramphenicol seem to be responsible. About the induction mechanism of these blood dyscrasias, many investigations have been made and some authors (PISCIOTTA 1969, ROTSTEIN *et al.* 1955, HOLLISTER 1964) attributed the cause to some immune mechanism. LINDBERG and NORDEN (1961) demonstrated hemolysin for red cells coated with the medicaments, but others (FIRKIN and LINNANE 1969, AHTEE and PASSONEN 1965) disagree with them and PISCIOTTA *et al.* (1958) observed no hemolytic or granulolytic activity of the serum of patients. Apart from the immune mechanism, the opinions supporting the direct toxic effects of the drugs seem to be predominant in recent years, as the evidences demonstrating the special sensitivity of the cells from the patients to the drugs have been accumulated (PISCIOTTA and KALDAHL 1961, PISCIOTTA *et al.* 1965). In spite of these findings, histocytologic picture of the bone marrow gives a distinct evidence that many fatal cases of agranulocytosis suspected to be induced by some medicaments or of unknown origin should be an immune disease; in the bone marrow of the patients lymphocytes and plasma cells proliferate and reticulum cells and macrophages are swollen and degenerated (SENO 1966). The animals having disintegrated macrophages and reticulum cells, e. g. by soot (TOYAMA 1965, MATSUOKA 1965) and PVP (TOYAMA 1965) injection, develop aplastic anemia in which the maturation of erythroblasts to red cells is not arrested but the induction of stem cell to proerythroblasts is severely affected, the blood picture of which is very similar to that of human aplastic anemia. These facts seem to indicate the possibility that blood dyscrasia appearing after treatment with drugs, at least in some cases, are largely due to some immune mechanism that attacks mainly macrophages and reticulum cells.

Recently, a new tranquilizer, clozapine (8-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo (b, e)-(1, 4)-diazepine), has aroused an interest of psychopathists because of its powerful effectiveness to psychosis. Recently, however, a few cases of agranulocytosis were reported, which occurred during the psychotherapy with clozapine (TSUJI 1969). Besides clozapine the patients were also given other drugs like chloramphenicol or chlorpromazine, and it is uncertain whether clozapine is responsible to the induction of agranulocytosis in these patients. In these cases, severe anemia, the proliferation of plasma cells and lymphocytes and the degeneration of reticulum cells were observed in smears of bone marrow. Therefore, it may be important to make it clear whether clozapine has antigenicity or not.

This paper deals with the antigenicity of clozapine in comparison with chlorpromazine, the latter of which is known to have distinct antigenicity. Clozapine introduced into rabbits did not give rise to any anti-clozapine antibody in all the cases observed.

MATERIALS AND METHODS

Animals: Twenty-two adult rabbits, male and female, and 10 male young guinea pigs were used. The rabbits were injected with antigens to produce antibody, and guinea pigs were used for passive cutaneous anaphylaxis experiments.

Haptenic carrier: Bovine serum albumin (BSA) and human erythrocytes were used as haptenic carriers. BSA was obtained from Armour Pharmaceutical Company, New York. Human erythrocytes (group 0) were taken from a healthy adult at the beginning of experiments.

Adsorption of clozapine to human erythrocytes: Human erythrocytes were collected in Alsever's solution and washed three times with phosphate buffered saline (PBS, pH 7.2). They were treated with 0.005 % tannic acid at 37°C for 30 min and washed three times with PBS. After incubation with clozapine solution (0.006 % at final concentration) at 37°C for 30 min, human erythrocytes were washed twice with PBS.

Immunization: The antigens or drugs were injected subcutaneously by mixing with complete Freund's adjuvant in some animals, and by mixing with protein solution and then with complete Freund's adjuvant in others. The mixture of the drugs with protein solution was made expecting the formation of some weak static electrical bond between drug and protein.

Animals were divided into 5 groups, 3 to 6 animals per group, and immunized as follows:

Group I: Five rabbits. They received 4 injections of chlorpromazine emulsified in Freund's complete adjuvant without any carrier; forty milligrams of chlorpromazine at one time, and 3 injections with one week interval, and the 4th injection two weeks later, 40 mg in each.

Group II: Five rabbits. They received 4 injections of clozapine mixed with Freund's complete adjuvant without any carrier. The number and the intervals of injections of the antigens were the same as in group I, 40 mg in each and 160 mg in total.

Group III: Six rabbits. They received also 4 injections of clozapine as in group II but the drug adsorbed to human erythrocytes, which were preliminary treated with tannic acid, were used, 0.4 ml of packed cells at a time, and 1.6 ml in total.

Group IV: Three rabbits. They received 4 injections of clozapine as BSA conjugate by mixing with complete Freund's adjuvant with the same intervals as in group I. Clozapine-BSA conjugate was obtained by adding 10 ml of 1 % aqueous solution of BSA to an equal volume of 1 % clozapine solution in 0.1 N HCl. The mixture was kept at room temperature for 48 hours and then added with ammonium sulfate to 70 % saturation. The mixture was centrifuged and the precipitate was used as the antigen, i. e., for one injection 1/5 of the total precipitate was used.

Group V: Three rabbits. They also received 4 injections of clozapine-BSA conjugate in a mixing with complete Freund's adjuvant. But the conjugate was obtained in a different way from that just described. Clozapine dissolved in 1 N HCl, 50 mg in 100 ml, was mixed with an equal volume of 0.1 % BSA solution in NaOH. This mixture was shaken vigorously, centrifuged and 1/5 of the precipitate was used for one injection.

Passive hemagglutination test (PHA): Commercially available sheep erythrocytes (Nippon Biotest Institute, Tokyo) were used. The erythrocytes were incubated with 0.005 % tannic acid at 37°C for 30 min, and to these erythrocytes the drugs, clozapine and chlorpromazine, were adsorbed by the method of BORDEN (1951). The serial 2-fold dilutions of rabbits antisera were made with PBS (pH 7.2) containing inactivated normal rabbits serum (1 %), and to each diluted antiserum one drop of 0.5 % suspension of the erythrocytes having drugs was added. As control the same dose of sheep erythrocytes treated with tannic acid but without any drug was added to another series of diluted serum.

Agglutination reaction was recorded according to the standard described by STAVITSKY (1954). The reactions were taken as positive, in which the ratio of PHA titer of drug-adsorbed erythrocytes to the control was more than 1 : 16, as slight hemagglutination occurred often in the series of control.

Precipitin reactions: Micro-Ouchterlony technique was employed to detect the precipitating antibody. Chlorpromazine mixed with BSA as mentioned in the method of Group IV were used as antigens for the precipitin reactions.

Passive cutaneous anaphylaxis (PCA): One tenth ml of each antiserum was injected into the back of young guinea pigs intravenously. After 4 hours, 10 mg of chlorpromazine or clozapine dissolved in 1 % Evans blue saline solution was injected intravenously. And thirty minutes later, permeation of dye from blood vessels into the cutaneous tissue preliminary treated with the antiserum was observed according to the description of OVARY (1929).

RESULTS

Chlorpromazine gave rise to the distinct antibody formation in rabbits, i. e., the serum from the 5 animals treated with 4 injections of chlorpromazine by simply mixing with complete Freund's adjuvant gave positive PHA reaction in all cases. The titer of PHA exceeded 1 : 2,000 in all animals. The highest one gave the value of 1 : 327,680, next two animals around 1 : 8000, and one about 1 : 4,000 and the lowest one around 1 : 2,000. In spite of the high hemagglutination titer, none of these sera gave positive precipitin reaction, i. e. no precipitin line was observed when they were tested with chlorpromazine-BSA conjugate as the antigen by the method of OUCHTERLONY. PCA reactions were also tested on guinea pigs but none of these sera gave positive reaction to the cutaneous injection of chlorpromazine. The results are shown in Table 1 and Fig. 1.

TABLE 1 IMMUNIZATION TEST OF RABBITS WITH CHLORPROMAZINE. CHLORPROMAZINE WAS EMULSIFIED IN FREUND'S COMPLETE ADJUVANT AND INTRODUCED SUBCUTANEOUSLY WITHOUT ANY CARRIER PROTEIN.

No. of animals	PHA titer	precipitin reaction	PCA
9	1 : 4096	—	—
10	1 : 327680	—	—
1	1 : 8192	—	—
2	1 : 8192	—	—
5	1 : 2048	—	—

PHA : PASSIVE HEMAGGLUTINATION TEST

PCA : PASSIVE CUTANEOUS ANAPHYLAXIS

METHOD : SEE TEXT

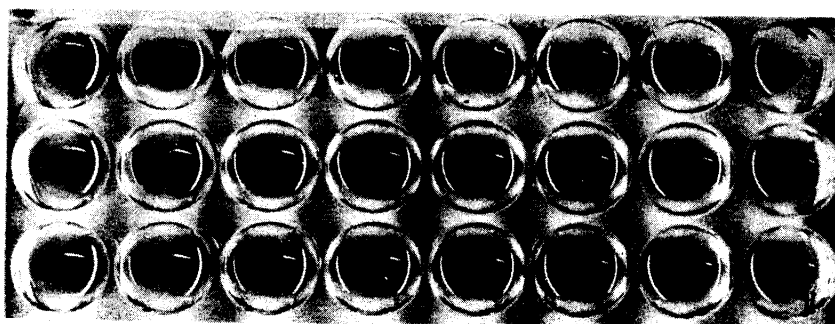


Fig. 1 Results of passive hemagglutination test. The upper line is control and added with erythrocytes treated with tannic acid alone. Antisera immunized with chlorpromazine mixed with Freund's complete adjuvant were diluted 4-fold. Lower two lines of series are added with erythrocytes treated with tannic acid and drugs. Antisera were diluted 2-fold.

In contrast to chlorpromazine it has been proven that the antigenicity of clozapine is very poor. In the 1st series of experiment (group II) 5 rabbits were treated with clozapine by injecting the drug with Freund's complete adjuvant. But all the sera obtained from these animals after 4 injections of clozapine, 160 mg as total gave negative PHA reactions. PCA test and precipitin reaction were also tested with the sera from these animals, but no serum showed positive reactions, indicating that the rabbits did not produce the antibody to clozapine by the subcutaneous introduction of the drug with Freund's complete adjuvant. The results are summarized in Table 2 and Fig. 2.

TABLE 2 IMMUNIZATION TEST OF 5 RABBITS WITH CLOZAPINE. CLOZAPINE WAS EMULSIFIED WITH FREUND'S COMPLETE ADJUVANT AND INJECTED SUBCUTANEOUSLY WITHOUT CARRIER PROTEIN.

No. of animals	PHA	precipitin reaction	PCA
3	—	—	—
4	—	—	—
6	—	—	—
7	—	—	—
8	—	—	—

PHA, PCA : SEE TABLE 1.

METHOD : SEE TEXT.

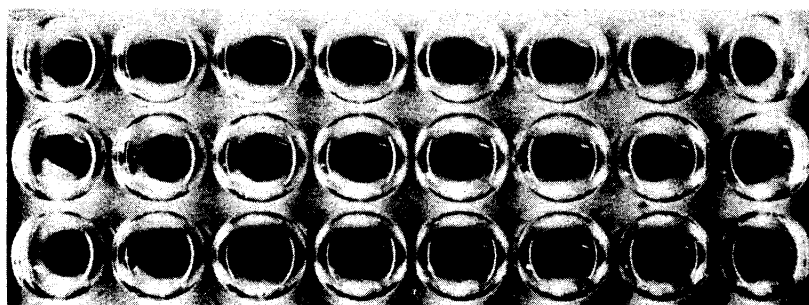


Fig. 2 Results of passive hemagglutination test.

The antigenicity of clozapine in rabbits was further tested by using clozapine adsorbed to human erythrocytes, which were previously treated with tennic acid. By this method clozapine was successfully adsorbed to erythrocytes that could be proved by treating the red cells with Nessler's reagent (containing no NaOH or KOH) followed by exposure to H_2S , as clozapine combines with the reagent forming insoluble conjugate which turned black being exposed to H_2S . By the injection of clozapine bound erythrocytes, rabbits were immunized four times as in the former experi-

ments. With the sera of the rabbits obtained after 4 injections of clozapine bound erythrocytes PHA test was made by using clozapine bound sheep erythrocytes. But all the sera gave negative reaction. Precipitin reaction was also made with the sera from these animals and BSA bound clozapine. This test also gave negative reaction. Finally PCA test against clozapine were carried out on guinea pigs as in the former experiments, but it gave negative reaction. Thus any one of these tests, PHA test, precipitin reaction and PCA test, did not give positive reaction showing again that clozapine has poor antigenicity in rabbits. These results are shown in Table 3 and Fig. 3.

TABLE 3 IMMUNIZATION TEST OF 6 RABBITS WITH CLOZAPINE ADSORBED TO HUMAN GROUP O ERYTHROCYTES MIXED WITH FREUND'S COMPLETE ADJUVANT. PASSIVE HEMAGGLUTINATION TEST, PRECIPITIN REACTIONS AND PCA WERE ALL NEGATIVE.

No. of animals	PHA	precipitin reaction	PCA
11	---	---	---
12	---	---	---
13	---	---	---
14	---	---	---
15	---	---	---
16	---	---	---

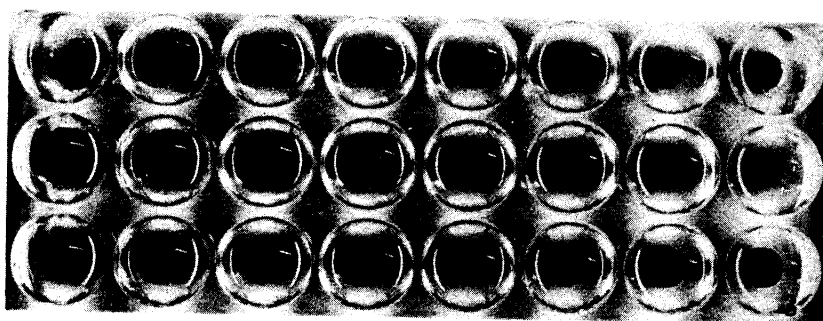


Fig. 3 Passive hemagglutination test. Antisera immunized with clozapine adsorbed to human group O erythrocytes mixed with Freund's complete adjuvant.

In the last experiment the antigenicity of clozapine was again tested by using clozapine-BSA conjugate precipitated by mixing the clozapine solution in 1 N HCl with an equal volume of BSA solution in 1 N NaOH. The clozapine-BSA conjugate thus precipitated was less soluble in water than BSA itself suggesting the electrostatic binding between the drug and BSA. With this precipitate the rabbits were sensitized by 4 injections as described (group V), and with the sera obtained after 4 injections the antigenicity of the clozapine was tested by PHA, PCA and precipitin

reaction. PHA and PCA tests were negative in all sera tested. Precipitin test carried out by using clozapine bound BSA gave one clear precipitin

TABLE 4 IMMUNIZATION OF 6 RABBITS WITH CLOZAPINE MIXED WITH BSA AND EMULSIFIED IN FREUND'S COMPLETE ADJUVANT. PASSIVE HEMAGGLUTINATION TESTS, PRECIPITIN REACTIONS AND PCA WERE ALL NEGATIVE.

No. of animals	PHA	precipitin reaction	PCA
17	—	—	—
18	—	—	—
19	—	—	—
20	—	—	—
21	—	—	—
22	—	—	—

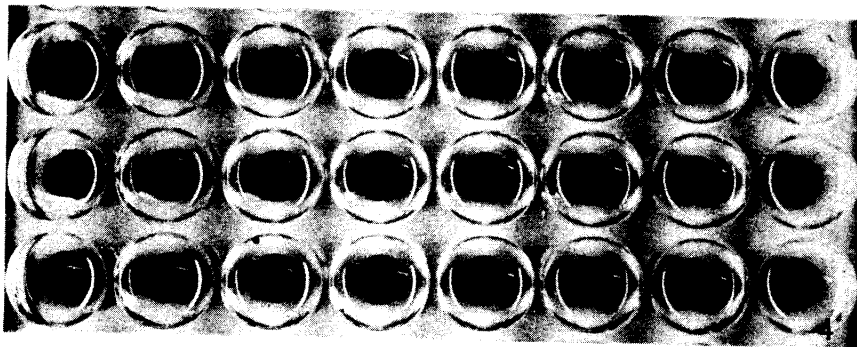


Fig. 4 Results of passive hemagglutination test.

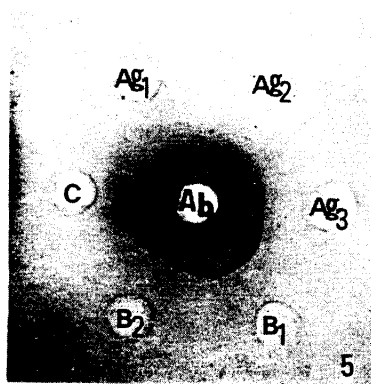


Fig. 5: The result of precipitin reactions for anti-clozapine antibody.

C: control (physiological saline)

Ag₁-Ag₃: 2-fold diluted antigen (the mixture of clozapine and BSA)

B₁-B₂: 2-fold diluted BSA

Ab: Antisera

Specific precipitin line for anti-clozapine antibody was not observed.

line. To clarify whether this precipitin line is specific to clozapine, clozapine bound BSA or to BSA itself as carrier protein, another precipitin test was performed, i. e., a small amount of BSA solution was poured into two basins of agar plate and antiserum into central one, and clozapine-BSA conjugate into three basins. Twenty-four hours later, one clear precipitin line was observed between antiserum and BSA, and between antiserum and clozapine-BSA conjugate. These lines fused completely with each other, indicating that the antibody formed was the one specific to BSA but not to clozapine. These results are shown in Table 4 and Figs. 4 and 5.

DISCUSSION

As has been demonstrated clearly by PHA test, the experiment has proved that chlorpromazine has a distinct antigenicity, which is inconsistent with the observations made by several authors in the past (PISCIOTTA 1969, PISCIOTTA *et al.* 1958), while clozapine showed no antigenicity to rabbits in all forms tested, as far as PHA test, precipitin reaction and PCA test were concerned.

It is generally accepted that to impart antigenicity to the low molecular compound less than 500 in molecular weight it requires some carrier protein. In this respect, chlorpromazine and clozapine, both of which are lower than 500 in their molecular weight, should combine with protein to be antigenic. In their structures chlorpromazine has a dimethyl amino group by which the drug is soluble in water and has a fairly good affinity to acidic protein or BSA, while clozapine has no charged group, hardly soluble in water and will have a poor affinity to protein. This difference between the two drugs may be correlated to their antigenicity. The other low molecular substances, which was proven to have antigenicity, such as aminopyrine, chloramphenicol will show a good affinity to protein as they have polar groups in their structure.

In view of this, we tried to conjugate clozapine with protein, BSA and erythrocytes *in vitro*, and to observe again the antigenicity of the drug by using such clozapine-BSA or clozapine-erythrocytes conjugates. Clozapine was readily soluble in N HCl probably being charged positive. When this solution was mixed with an equal volume of BSA solution in N NaOH, clozapine was bound with the negatively charged protein and precipitated losing its charge. Clozapine was also adsorbed to red cells at low pH. The binding should be the electrostatic bonding, similar to that of chlorpromazine to protein formed by mixing simply the drug and protein. As indicated in the experiment just presented, clozapine failed to produce

anti-clozapine antibody, as revealed by PHA test, precipitin reaction and PCA test.

Concerning the problem of sensitivity of the test, PHA test should be sensitive enough to reveal a weak antigen-antibody reaction which may be enhanced by the drugs. QUENG *et al.* (1965) reported the antigenicity of tetracyclines using the tanned-cell hemagglutination, and JUJI and MATSUHASHI (1969) observed the antigenicity of aminopyrine and chloramphenicol, demonstrating the antibody titer of over 1 : 2,000 in PHA test, but negative reactions in precipitin test and PCA test. The present observations also proved this is true in the cases of chlorpromazine. Negative precipitin reaction may be due to the failure in obtaining the stable drug protein conjugate *in vitro*, as the drug will bind with BSA only weakly in simple mixture.

SUMMARY

Antigenicity of clozapine was studied in rabbits, comparing with that of chlorpromazine as control. The results indicate that chlorpromazine produces antibody in rabbit as revealed by passive hemagglutination test, giving the titer of 1 : 2,000 or higher in all the five cases observed, though specific precipitin lines has not been obtained and PCA test proves to be negative. Clozapine failed to produce anti-clozapine antibody giving negative passive hemagglutination test, passive cutaneous anaphylaxis and precipitin reaction, in all forms tested.

Some remarks were made on the possible close relation between the antigenicity of the drug and its affinity to protein.

REFERENCES

1. AHTEE, L. and PASSONEN, M. K.: The hemolytic effect of some phenothiazine derivatives. *Ann. Med. exp. Fenn.* **43**, 101, 1965
2. BOYDEN, S. V.: The adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera. *J. Exp. Med.* **93**, 107, 1951
3. FIRKIN, F. C. and LINNANE, A. W.: Biogenesis of mitochondria, 8. The effect of chloramphenicol on regenerating rat liver. *Exp. Cell Res.* **55**, 68, 1969
4. HOLLISTER, L. E.: Complications from psychotherapeutic drugs. *Clinical Pharmacology and Therapeutics*. **5**, 322, 1964
5. JUJI, T. and MATSUHASHI, T.: Antibody production to low molecular drugs in experimental animals and in man. *Japan J. Exp. Med.* **39**, 21, 1969
6. LINDBERG, L. G. and NORDEN, A.: Severe hemolytic reaction to chlorpromazine. *Acta Med. Scandinav.* **170**, 195, 1961
7. MATSUOKA, K.: Studies on reticuloendothelial system and hematopoiesis. II. A study on the morphology and function of erythroblastic islet. *Acta Med. Okayama* **19**, 161, 1965
8. OVARY, Z.: Passive cutaneous anaphylaxis in the guinea pig: Degree of reaction as a

- function of the quantity of antigen and antibody. *Int. Arch. Allergy* **14**, 18, 1959
9. PISCIOTTA, A. V.: Agranulocytosis induced by certain phenothiazine derivatives. *JAMA* **208**, 1862, 1969
10. PISCIOTTA, A. V., EBBE, S., LENNON, E. J., METZGER, G. O., and MADISON, F. W.: Agranulocytosis following administration of phenothiazine derivatives. *Am. J. Med.* **25**, 210, 1958
11. PISCIOTTA, A. V. and KALDAHL, J.: Studies on agranulocytosis. IV. Effect of chlorpromazine on nucleic acid synthesis of bone marrow cells *in vitro*. *Blood*. **20**, 364, 1962
12. PISCIOTTA, A. V., SANTOS, A. S., KELLER, C., and HAYS, J.: Studies on agranulocytosis. VI. The effect of clinical treatment with chlorpromazine on nucleic-acid synthesis of granulocyte precursors in normal persons. *J. Lab. and Clin. Med.* **65**, 228, 1965
13. QUENG, J. T., DUKES, C. D., and MCGOVERN, J. P.: Antigenicity of the tetracyclines. *J. Allergy* **36**, 505, 1965
14. ROTSTEIN, J., FRICK, P. S. and SCHIELE, B. C.: Agranulocytosis associated with chlorpromazine therapy. *Arch. Int. Med.* **96**, 781, 1955
15. SENO, S.: Differentiation of erythroid cell. *Acta Pathol. Jap.* **16**, 457, 1966
16. STAVITSKY, A. B.: Micromethods for the study of proteins and antibodies. I. Procedure and general applications of hemagglutination and hemagglutination-inhibition reactions with tannic acid and protein-treated red blood cells. *J. Immun.* **72**, 360, 1954
17. TOYAMA, T.: Studies on the function of reticulo-endothelial system. I. Effects of the RES blocking with India ink on the hematopoiesis and the production of serum antibody. *Acta Med. Okayama* **19**, 255, 1965
18. TOYAMA, T.: Studies on the function of reticulo-endothelial system. II. Effects on the RES blocking with macromolecular PVP on the lymphoid cell reproduction and the production of serum antibody. *Acta Med. Okayama* **19**, 307, 1965
19. TSUJI, T.: Paper read at Japan Soc. of Hepatology, 1969