DEMONSTRATION OF ANTIBODY ACTIVITY TO CLIQUINOL IN SERA OF EXPERIMENTAL RABBITS AND PATIENTS WITH SUBACUTE MYELO-OPTICO-NEUROPATHY

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Abstract

Antibody to cliquinol (5-chloro-7-iodo-8-hydroxyquinoline: CIHQ) was detected by passive hemagglutinating reaction in rabbits receiving a prolonged administration of Emaform which was once a commercial preparation of CIHQ. The antibody was shown to be in the immunoglobulin fractions by separation with a specific immunoadsorbent, and it had relatively broad antigenic specificity.

The antibody was also demonstrated in sera from patients who suffered from Subacute Myelo-Optico-Neuropathy (SMON) and from normal healthy individuals. However, its titer and frequency of positive reactors in the former were higher than those in the latter, and the two groups could be differentiated each other in frequency distribution patterns of the antibody proprietors.

From these results, we discussed on desirable application of sero-epidemiological study to assessment of effects of chemical pollutants on living systems.

Key words: Cliquinol, Anti-hapten antibody, SMON

INTRODUCTION

Recently, increasing attention has been directed to the side effects of drugs and other chemicals contained in foods and materials of daily use. Some of them have been implicated in allergic reaction ranging from urticaria to fatal shock, as represented by allergy to penicillin (Manten 1972, Samson 1958), aspirin (Girard et al, 1969, Samter et al, 1967) or to a food dye such as tartrazine (Chafee et al. 1967, Juhlin et al. 1972).

From the early 1950s, a neurologic disorder “SMON” which was preceded by abdominal symptoms had increased year after year in Japan. A great deal of evidence had been accumulated supporting the view that the disease could have been caused by an excess administration of CIHQ (Kono 1975, Shigematsu 1975, Tateishi et al. 1975). The disease virtually disappeared in and after 1969 when the use of CIHQ was forbidden on recommendation of a SMON study group. However, despite extensive studies, the pathogenesis of SMON has remained imperfectly elucidated. Takeuchi et al (1971) demonstrated
cell infiltration which consisted mainly of plasma cells accompanying large mononuclear cells and lymphocytes, and amyloid deposition in the intestinal submucosa of patients with SMON, suggesting possible involvement of immunological mechanisms in the formation of the lesions. Based on this fact, we assumed that immune mechanisms might have taken part in the pathogenesis of disorders induced by the drug. This assumption included the possibility of CICHQ conjugating in vivo with body protein to display its antigenic activity. The present study was undertaken as a preliminary experiment to approach this problem.

MATERIALS AND METHODS

1. Antigens: Emaform (Tanabe Pharmaceutical Co.) was chosen as the form of CICHQ to be used for immunization. This preparation contains 0.9 g CICHQ, 0.095 g sodium carboxymethyl cellulose (CMC) and 0.005 g detergent in 1.0 g. 8-Aminoquinoline (8AQ) was conjugated with bovine serum albumin (BSA) with covalent bond by the method of Hoare and Kosherland (1966). 5-Chloro-8-hydroxy-quinoline (CHQ), tetracycline (TC), a-naphthol (aNAP), and p-nitrophenol (pNP) were conjugated with BSA or rabbit serum albumin (RSA) with bis-diazotized benzidine (BDB) coupling by the method of Johnson and Smith (1972). Their structural forms are indicated in Fig. 1. Hapten-protein ratios of these conjugates were estimated spectrophotometrically, and conjugates containing about 10 hapten groups per molecule were used as antigens for various immunological tests.

2. Immunization procedure: New Zealand albino rabbits weighing 2.5 to 3.0 kg were given 50 mg of Emaform in the form of a homogeneous mixture in 2.5 ml of isotonic saline.

![Fig. 1. Structural formula of antigens](image-url)
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for each injection. For the first group of immune rabbits (No. 1 to 8), the mixture was injected intraperitoneally every other day for six injections, and a week later for four injections. After five weeks of rest, each rabbit received five intravenous injections, and three weeks thereafter five intraperitoneal injections, on alternate days. For the second group of the rabbits (No. 9 to 16), three series of immunizations were carried out according to the schedule shown in Table 1, taking two and four weeks of rest periods after the first and the second series of immunizations, respectively. The third group of rabbits received only intravenous injections according to the same schedule as above. The rabbits were bled from an ear vein one week after the last injection in each series of immunizations. Control rabbits were injected with equal amounts of CMC and detergent to those in Emaform injected in the experimental rabbits, according to the immunization schedules for the first and the second groups of the immune rabbits.

3. Passive hemagglutination test: Tannic acid treatment of sheep red blood cells, their sensitization with an antigen, and other procedures were performed according to the method described by Stavitsky (1959). Test sera were heated at 56°C 30 min, and then absorbed for 1 hr with the tanned cells and BSA-sensitized cells in turn. The hemagglutinating reaction was carried out by a micromethod; 0.05 ml of test serum was serially diluted two-fold in wells on microplate holding 0.05 ml of 1% normal rabbit serum in pH 7.2 phosphate buffered saline as diluent, and the same amount of 1% suspension of sensitized cells in the same buffer was added. Final volume of the reaction mixture was 0.1 ml. After shaking, the mixture was kept at 37°C for 2 hr and then overnight at room temperature. Controls consisting of antigen-treated cells in the diluent and in the preimmunization serum, and tanned cells in dilutions of the test serum were provided in each test. Readings were made 2 hr after incubation and checked again after being left overnight. The hemagglutinating (HA) titer was expressed by the reciprocal of the highest serum dilution (by initial dilution) giving positive agglutination.

4. Hemagglutination inhibition (HI) test: 0.025 ml of each dilution of inhibitory antigen was added into 0.05 ml of each serial dilution of test serum. The mixtures were incubated at 37°C for 1 hr before adding 0.025 ml of 2% suspension of CHQ–BDB–BSA sensitized cells.

5. Fractionation of immunoglobulins: A specific immunoadsorbent was prepared by the method of Weetall and Weliky (1964) to apply to fractionation of serum immunoglobulin classes; carboxymethyl cellulose (CM cellulose) was coupled to benzidine at one side of its amino group in the presence of dicyclohexylcarbodiimide, and the resultant half benzidine amide of CM cellulose was diazotized and coupled to an anti-immunoglobulin. Fractionation of immunoglobulin was accomplished by the method of Avrameas and Ternynck (1969); an appropriate volume of test serum was mixed with the specific immunoadsorbent and stirred gently for 30 min at room temperature. After subsequent washing of the adsorbent to remove free proteins, immunoglobulin was eluted from the adsorbent with 0.1 M glycine–HCl buffer pH 2.8. The eluted protein was dialyzed against some changes of cold phosphate buffered saline and concentrated to the original volume.

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RESULTS

1. Hemagglutinating activity of immune rabbit sera: Twenty-four rabbits negative to CHQ-BDB-BSA in the HA test received intraperitoneal and intravenous injections of CIHQ according to the above-mentioned schedule. Sera collected during the immunizations were tested for HA activity using the CHQ-BDB-BSA antigen. Of the 24 rabbits, six (25.0%) reacted positive to the HA test (Table 1). The HA activity began to appear mostly after the progression of immunization, except in the case of rabbit No.12. The activity was reproducible even when it was tested using CHQ-BDB-RSA as an antigen, but was not observed when the positive sera were tested by BDB-BSA or TC-BDB-BSA sensitized cells. On the control rabbits, none of fourteen had a consistently positive HA test throughout the immunization process. Sera of twenty normal rabbits and the seven immunized with unrelated antigens were given the same test. Of these 27 sera, one of the normal (3.7%) was positive to CHQ-BDB-BSA antigen at dilution 1:64.

The specificity of HA activity in the immune and normal rabbit sera was examined by the HI test, using homologous and heterologous antigens as inhibitors (Fig. 2). HA activity of the immune serum (No. 12) was more effectively inhibited with CHQ-BDB-BSA and 8AQ-BSA than with αNAP-BDB-BSA, but not inhibited with pNP-BDB-BSA, TC-BDB-BSA, BDB-BSA and BSA. HA activity of normal rabbit serum was not only inhibited with CHQ-BDB-BSA and 8AQ-BSA but also with αNAP-BDB-BSA to the same degree. However, the inhibitory effects of both CHQ-BDB-BSA and 8AQ-BSA were lower compared to the case in which both antigens were tested for the CIHQ immune serum.

<table>
<thead>
<tr>
<th>Rabbit number</th>
<th>Pre-immunization</th>
<th>Immunization schedule</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
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<tr>
<td>CIHQ immune †</td>
<td></td>
<td>ip-6*</td>
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<tr>
<td>1</td>
<td>&lt;8+</td>
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<td>ip-6</td>
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<tr>
<td>C1</td>
<td>&lt;8</td>
<td>&lt;8</td>
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<td>C3</td>
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<td>C8</td>
<td>&lt;8</td>
<td>&lt;8</td>
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</table>

† Of 24 immune rabbits, data on positive responders were shown in the table.
* Six intraperitoneal injections.
** Five intravenous injections.
+ Hemagglutinating titer (reciprocal), detected with CHQ-BDB-BSA sensitized red cells.

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Fig. 2. Inhibition of the hemagglutinating activity of (A) CIHQ immune (No. 12) and (B) normal rabbits sera by various concentrations of inhibitors; ○ : CHQ-BDB-BSA, ● : 8AQ-BSA, △ : αNAP-BDB-BSA, ◊ : pNP-BDB-BSA, and ● : TC-BDB-BSA, BDB-BSA, BSA. The ordinate indicates the HA titer by the reciprocal of the highest serum dilution giving positive agglutination.

These results appear to suggest the possible participation of a quinoline nucleus for the specificity of the HA activity in the immune serum, although a naphthalene nucleus is able to cross-react to a much lesser extent. On the other hand, the specificity in the normal serum seems to be looser against the quinoline than in the immune serum.

2. HA activity of human sera: Sera were obtained from 128 patients with SMON, and 108 normal persons consisting of medical school students and the aged who were of almost the same age as the patients. The sera were tested for HA activity using CHQ-BDB-BSA as an antigen and the result is shown in Fig. 3. In this test, some sera which reacted to BDB-BSA were absorbed with BDB-BSA sensitized cells until reactivity to it was below 1:8. The proportions of positive reactors to the antigen with HA titer over 32 were distinctly larger in the SMON group (60.1%) compared with the control (36.1%) (Chi-square = 11.6, p < 0.01). As to the positive reactors to the antigen in the control
group, their HA activity was occasionally as high as the patients with SMON, although small in number. Due to the fact that CIHQ preparations had come into popular use in Japan, the possibility of adults and the aged having taken the drugs in the past could not be excluded. In this respect, the HA activity was determined on sera from infants who were born after the use of CIHQ was forbidden. Eight out of 28 infants (6 months to 3 years old) represented the HA titer of 32 to 512 (28.6%), and no significant difference in frequency of the positive reactors was found among the infants, the students or

Table 2. Distribution of the HA activity in immunoglobulin fractions.

<table>
<thead>
<tr>
<th></th>
<th>Whole serum</th>
<th>IgA</th>
<th>IgG</th>
<th>IgM</th>
<th>Residue</th>
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<tr>
<td>No. 2</td>
<td>512+</td>
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<tr>
<td>No.12</td>
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<td>256</td>
<td>N.D.</td>
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<td>SMON patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>S-S-217</td>
<td>512</td>
<td>0</td>
<td>32</td>
<td>16</td>
<td>0</td>
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<tr>
<td>-260</td>
<td>256</td>
<td>0</td>
<td>16</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>-286</td>
<td>1024</td>
<td>0</td>
<td>64</td>
<td>16</td>
<td>0</td>
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<tr>
<td>Control Students</td>
<td></td>
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<tr>
<td>WA</td>
<td>64</td>
<td>0</td>
<td>16</td>
<td>0</td>
<td>0</td>
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<tr>
<td>UG</td>
<td>256</td>
<td>2</td>
<td>0</td>
<td>4</td>
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<tr>
<td>The aged</td>
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<tr>
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<td>128</td>
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<tr>
<td>95</td>
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</table>

+ Hemagglutinating titer (reciprocal), detected with CHQ-BDB-BSA sensitized red cells.
N.D. Not determined.

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the aged groups.

The specificity of the HA activity in human sera was also studied by the HI test according to the same method as with rabbit sera. As shown in Fig. 4, there was the same tendency for specificity as seen in the rabbits; sera of the patients were more specific to quinoline than normal sera. Some variety was found on the specificity of the normal sera, that is, the serum D was more effectively inhibited by naphthalene than by quinolines, the serum C was not only inhibited by both but also by nitrophenol to some extent, and the rest were inhibited by naphthalene and quinolines at the same level (not shown in the figure) as seen in the normal rabbit serum.

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3. Fractionation of HA activity in serum: To examine the characteristics of HA activity, HA-positive sera were fractionated into immunoglobulin classes. Then each serum fraction was tested for activity to CHQ-BDB-BSA (Table 2). Most of the HA activity of immune rabbit sera was recovered in the IgG fraction, and lower activity in the residue. The activity of human sera was detected in both IgG and IgM fractions, regardless of whether the subject was a patient with SMON or not. These results possibly provide evidence that the HA activity of the sera is caused by antibody-like substance belonged to immunoglobulins.

DISCUSSION

There has been general acceptance of the concept that low molecular weight substances must react or conjugate in vivo with body protein to form a complete antigen. A similar reaction or conjugation is supposed to occur also in many cases of clinical allergy caused by drug and other chemicals of low molecular weight, but no substantial laboratory data providing the in vivo binding formula of these substances with body protein have been presented except concerning a few substances being represented by penicillin (Levine et al 1961).

In a past study using animal, antibody productions to penicillin (Wagelie et al 1963) and tetracycline derivatives (Queng et al 1965) have been demonstrated to not require the formation of in vitro conjugates, and recently this has been demonstrated in an antibody to morphine (Beranek et al 1976a, 1976b). The present study also demonstrated that antibody production to CIHQ could occur in animal given the drug alone, without the aid of artificial in vitro conjugation of the antigen to protein, although it must be recalled that CIHQ preparation used for immunization of rabbits might contain artificial conjugates as impurities of the manufacturing process which are sufficiently large in molecules to elicit antibody production.

The antibody specific to CIHQ was found in sera of patients with SMON, and the antibody reactive with CIHQ was also found in sera of normal individuals inclusive of non-recipients of CIHQ. The activity in sera of normal persons appears possibly to be caused by an antibody which may result from antigenic stimuli of some quinoline or naphthalene derivatives contained in drugs, antiseptics, fungicide and various kind of products of popular use, as explained by the antibody to dinitrophenol in normal human sera (Brandriss 1969).

As mentioned above, the antibody to CIHQ was found in sera from both normal persons and the patients. However, the group consisting of the patients who took significant amounts of CIHQ for a long time could be distinguished from the control group by the frequency distribution patterns of the antibody proprietors. As to correlation of the antibody to pathogenesis of SMON, further studies are now in progress, but at least, the difference in the two distribution patterns seems to reflect some biological conditions in SMON patients who have been distinctly exposed to CIHQ, and in normal individuals. This finding gives us a faint expectation that a sero-epidemiological study as described
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in this paper may play a useful role to survey circumstantial evidence of invasion in the living systems by various substances such as agricultural chemicals, environmental pollutants, and so forth, as we have already studied on infectious agents. To make it possible, of course, further accumulation of data concerning to immunogenicity of small molecules will be necessary.

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REFERENCES


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