Lymphocyte Stimulation Test with Tetrazolium-Based Colorimetric Assay for Diagnosis of Drug-Induced Allergic Hepatitis

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The lymphocyte stimulation test (LST) is useful for diagnosing drug-induced allergy and identifying the causative drug. In this study, we examined the usefulness of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as a marker for LST in diagnosing drug allergy. In a basic study using normal peripheral blood mononuclear cells, the normal range of stimulation index (SI) was 0.92—1.38, and the mean SI for all drugs tested was 1.134 ± 0.111 (mean ± S.D.). The cut-off value of SI for diagnosis of drug allergy was thus set at over mean + 2S.D. for possibly positive, and at over mean + 3S.D. as a definitely positive reaction. Forty-six cases of suspected drug-induced allergic hepatitis involving 85 drugs were diagnosed by this assay, and the possibly positive and definitely positive rates were 54.3% (SI ≥ 1.4) and 41.3% (SI ≥ 1.5), respectively.

A clinical study was made of 113 patients with diagnosed drug-induced allergic hepatitis. Forty-nine (43%) of the patients were male and 64 (57%) were female. In 85% of cases the allergic reaction occurred within one month of taking medication, but there were a number of cases in whom onset occurred after long-term incubation. The main clinical symptoms were jaundice, itching, eruption, fever, and general malaise. In about 75% of cases glutamic oxaloacetic transaminase (GOT) or glutamic pyruvic transaminase (GPT) returned to normal range within one month after medication was halted. Among the causative drugs, antimicrobial agents were the most numerous accounting for 33.9% of the total, followed by central nervous system agents 21.2%, and cardiovascular agents 16.9%.

These results indicate that LST with the MTT assay would be useful in diagnosing drug-induced allergic hepatitis, and that among the drugs examined, antimicrobial agents were responsible for the largest number of allergic reactions.

Keywords drug-induced hepatitis; lymphocyte stimulation test; 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT); drug allergy; tetrazolium

Drug-induced liver disease is broadly divided into two categories, one caused by allergic reactions and the other caused by toxic reactions. Recent conjecture, however, suggests that the greater part of such hepatic damage is actually caused by an immunological mechanism, especially delayed-type allergic reaction. This is supported by evidence of the existence of sensitized lymphocytes without anti-drug antibodies in the blood of patients with allergic hepatitis.

To identify these sensitized lymphocytes, the lymphocyte stimulation test (LST) is widely used.1–3 Test indicators generally rely on the incorporation of [3H]thymidine into DNA.4–7 We have already reported the usefulness of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as a marker in LST.8 The MTT assay relies on measuring enzymatic activity of succinic acid dehydrogenase in mitochondria. The benefit of this method is that the complete test procedure can be carried out in a general laboratory, and requires no special equipment or the need to deal with radioisotopes. However, the method has the disadvantage of needing large cell cultures from using a 24-well plate. We found that HCl—isopropanol as a solvent to dissolve the MTT formazan, when used according to Mosmann's method,9 was limited in its solubility. Therefore, in this study we used dimethylsulfoxide (DMSO) as a solvent according to Carmichael et al.10 and a 96-well plate, which allowed for simpler assay with fewer cell cultures. A cut-off value of the stimulation index (SI) for diagnosis of drug allergy was determined by means of analyzing the normal SI range, and 46 cases of suspected drug-induced allergic hepatitis involving 85 different drugs were diagnosed by this assay.

In addition to the laboratory study, we reviewed clinical features of 113 cases of drug-induced allergic hepatitis diagnosed in the gastroenterology department of Ogaki Municipal Hospital between January 1988 and March 1993.

MATERIALS AND METHODS

Materials RPMI 1640 culture medium and phosphate-buffered saline (PBS) (—) were obtained from Nissui Pharmaceuticals; fetal calf serum (FCS) was from Gibco; phytohemagglutinin (PHA) from Difco; MTT and DMSO from Dojin Chemicals; and LeucoPREP® and the 96-well plate from Falcon. A Fujiirebio MPR A4i microplater reader was also used for the assay.

Subjects Normal peripheral blood mononuclear cells taken from 3 healthy subjects were used as control components of the assay. Diagnosis was conducted for the 46 patients with suspected drug-induced allergic hepatitis.

The clinical study was carried out on the 113 patients with diagnosed drug-induced allergic hepatitis. Some of these patients had been diagnosed by the method used here and others by techniques, involving the MTT assay11 reported earlier and the [3H]thymidine assay.

Methods 1. Culture of Peripheral Blood Mononuclear Cell: The peripheral blood mononuclear cells
were separated from peripheral blood containing heparin using a lymphocyte separation tube LeucoPREP®. Peripheral blood was introduced into the LeucoPREP® tube and centrifuged for 15 min at 1800 g. The cells obtained were washed twice with PBS (−) and then adjusted to a concentration of 3–5 × 10² cells/ml in RPMI 1640 containing 10% FCS, penicillin (50 units/ml) and streptomycin (0.05 mg/ml). The cell suspension was seeded into 100 µl aliquots into the 96-well plate, and 50 µl of test drug solution was added to each well. The drugs tested were dissolved by sonication in RPMI 1640 containing 10% FCS, and then diluted to give six different concentrations (1/1000, 1/3000, 1/9000, 1/27000, 1/81000 and 1/243000 of standard single dose) by multiples of three on the basis of a standard single dose of the drug in the basic study and the clinical test. Cultures were incubated at 37 °C and 100% humidity in 5% CO₂ for 6 d; those with PHA added were incubated for 72 h.

2. MTT Assay: MTT was dissolved in PBS (−) to a concentration of 5 mg/ml, added to each well in aliquots of 25 µl, and then the mixture was incubated for another 4 h at 37 °C. The 96-well plate was centrifuged for 15 min at 2000 rpm and the supernatant was removed. Two hundred fifty microliters of DMSO was added to dissolve the MTT formazan produced, and when this was fully dissolved, the absorption was measured at a wavelength of 540 nm and a reference wavelength of 620 nm using a microplate reader.

3. Analysis of Data: The lymphocyte blastogenesis was expressed by the SI:

$$SI = \frac{A_2 - A_0}{A_1 - A_0}$$

where: $A_2$ is absorption of cultures with drugs added; $A_1$ is absorption of cultures without drugs; $A_0$ is absorption of the blank (culture medium only).

4. Determination of Drug Allergy by SI: In determining an SI cut-off value to diagnose drug allergy, we set the value of less than mean + 2S.D. as normal range, that between mean + 2S.D. and mean + 3S.D. as possibly positive and that of above mean + 3S.D. as positive.

5. Measurement of Liver Function Parameters: Glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), γ-glutamyl transpeptidase (γ-GTP) and total bilirubin (T-bil) were measured using a Hitachi 736-40 automatic analyzer.

RESULTS

1. Basic Study of LST with MTT Assay To determine the cut-off value of SI for diagnosis of drug allergy, the following basic study was conducted. As indicated in Fig. 1, the SI increased gradually to a peak value of 3.1 at a concentration of 10 µg/ml of PHA and decreased in accordance with PHA concentration. Next, the effects of various drugs on SI using normal peripheral blood mononuclear cells were studied. Figure 2 shows the SI distribution for 53 drugs tested in normal peripheral blood mononuclear cells; it ranged from 0.92 to 1.38 with mean value and standard deviation of 1.134 ± 0.111. Table I shows the 53 drugs categorized by clinical effect, numbers tested and SI of each drug. The SI values of antimicrobial agents were relatively close to 1, but central nervous system agents such as angesics and psychotropics were also comparatively high. Other categories contained digestive organ agents (SI: 1.01 ± 0.03), antiallergic agents (SI: 1.07 ± 0.10), respiratory organ agents (SI: 1.34 ± 0.15), urogenital agents (SI: 1.08 ± 0.03), peripheral nervous system agents (SI: 1.38 ± 0.12), hormones (SI: 1.20 ± 0.06) and enzyme preparations (1.05 ± 0.04). The reproducibility of this assay was tested by assaying peripheral blood mononuclear cells (2 × 10⁵ cells/ml) stimulated by 5 µg/ml PHA for 3 d. The coefficients of within-run assay and between-day assay were less than 13.3% (N = 10) and less
Table II. Category of 85 Drugs Tested and Distribution of the SI in Patients with Suspicious Drug-Induced Allergic Hepatitis

<table>
<thead>
<tr>
<th>Category</th>
<th>SI &lt; 1.3</th>
<th>1.3 ≤ SI &lt; 1.4</th>
<th>1.4 ≤ SI &lt; 1.5</th>
<th>1.5 ≤ SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotics (N=17)</td>
<td>12</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Vasodilators (N=9)</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Analgesics (N=7)</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Chemotherapeutics (N=5)</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Peptic ulcer agents (N=5)</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Antiallergics (N=5)</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Antitussives (N=4)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Antiepileptics (N=3)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Comprehensive common cold agents (N=3)</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Others (N=27)</td>
<td>11</td>
<td>5</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Total (N=85)</td>
<td>42</td>
<td>10</td>
<td>10</td>
<td>23</td>
</tr>
<tr>
<td>Case (N=46)</td>
<td>18</td>
<td>3</td>
<td>6</td>
<td>19</td>
</tr>
<tr>
<td>Incidence (%)a</td>
<td>39.2</td>
<td>6.5</td>
<td>13.0</td>
<td>41.3</td>
</tr>
</tbody>
</table>

a) Incidence (%) = number of cases/number of total cases (46) × 100.

Fig. 3. Distribution of the SI in 46 Cases (85 Drugs) of Suspicious Drug-Induced Allergic Hepatitis
Eighty five drugs were tested in peripheral blood mononuclear cells obtained from 46 suspected cases of drug-induced allergic hepatitis.

than 16.7% (N=10), respectively (data not shown). SI values of normal subjects showed 1.4 as mean ± 2S.D. and 1.5 as mean ± 3S.D., respectively. Therefore, we used the value 1.4 as possibly positive and the value 1.5 as positive for diagnosing drug allergy.

2. Test of Drug-Induced Allergic Hepatitis LST with the MTT assay was applied to study the allergic reaction to 85 drugs involved in the 46 cases of suspected drug-induced allergic hepatitis. Figure 3 shows a summary of the SI using the MTT assay for all 85 drugs. Table II shows SI distribution in various drugs. There were 10 drugs (6 cases) with SI of 1.4—1.5, and 23 drugs (19 cases) with SI of 1.5 or more. The possibly positive rate was thus 54.3% (SI ≥ 1.4) and the positive rate was 41.3% (SI ≥ 1.5).

3. Clinical Study of Drug-Induced Allergic Hepatitis In this study the following points were regarded as important in the diagnosis: onset of hepatic damage following medication, allergic symptoms, and lack of a hepatic virus. Determination of the time of onset of or recovery from drug-induced hepatitis was done on the basis of GOT or GPT, and was regarded as the time at which GOT or GPT exceeded or returned to normal range. Forty-nine (43%) of the patients were male and 64 (57%) were female; the distribution of age and sex is shown in Fig. 4. Many of the patients were in their 50's and 60's, about 65% being over 50 years of age. Figure 5 shows the length of period of exposure to a causative drug prior to the induction of allergic hepatitis. Onset was assumed as the time at which GOT or GPT rose to above 401 U/l. In about 85% of cases the allergic reaction occurred within one month of taking the medication, but there were a number of cases in whom onset occurred after a long period of incubation.

Fig. 4. Distribution of Age and Sex in Patients with Drug-Induced Allergic Hepatitis

Fig. 5. Length of Exposure to the Causative Drug Prior to Drug-Induced Allergic Hepatitis

Figure 6 shows the liver function parameters, the highest results obtained during clinical treatment of a patient were used as their scores. Means were GOT: 279.6 ± 246.2 IU/l, GPT: 322.9 ± 252.0 IU/l, γ-GTP: 241.6 ± 180.5 IU/l, and T-bil: 5.0 ± 5.1 mg/dl. The most frequent clinical symptoms
were jaundice, itching, eruption, fever, and general malaise (Table III); others included light-headedness, anorexia, nausea, and swelling of the limbs. Figure 7 shows the number of days required for recovery to normal ranges of GOT or GPT after withdrawal from the causative drug. Recovery was assumed as the time at which GOT or GPT decreased to less than 40 IU/l. Means in the remission state were GOT: 41.3 ± 19.1 (18.1-67.7) IU/l and GPT: 49.3 ± 27.4 (13.2-103) IU/l. In about 75% of cases GOT or GPT returned to normal within one month after medication was stopped, but in some cases recovery took longer. After withdrawal of the causative drug, patients were treated with the following medicines: liver protectives were given to 68.1% (77 cases), cholangoloue to 10.6% (12 cases), steroids to 10.6% (12 cases), antihistamines to 9.7% (11 cases), vitamins to 4.4% (5 cases) and Chinese medicines to 3.5% (4 cases). No treatment was made to 28.3% (32 cases). Table IV classifies 118 causative drugs by their clinical effect. The most numerous were the 40 antimicrobial agents (33.9%), 22 of them cephems; central nervous system agents were next numbering 25 (21.2%), cardiovascular agents numbered 20 (16.9%), digestive organ agents 7 (5.9%), and antiallergic agents or metabolic agents 5 each (4.2%).

Finally, the correlation between SI values and liver function parameters was studied for the 46 cases tested by the MTT assay. As shown in Table II, there were 18 cases with SI of less than 1.3, 3 with SI between 1.3 and 1.4, 6 with SI between 1.4 and 1.5, and 19 with SI of 1.5 or more. Means of GOT and GPT, which were the highest obtained during clinical treatment of the patient, were as
This resulted in 54.3% of the allergic patients tested with this assay being positive with $SI$ of 1.4 or more, and 41.3% having $SI$ of 1.5 or more. Therapeutic conjecture provides support to these results of positive incidence. Of the 25 cases with $SI$ of 1.4 or more shown in Table II, 10 were studied for one tested drug, 8 for two tested drugs, and 7 for three or more tested drugs. In 19 cases (10 with one tested drug, 6 with two and 3 with three or more), only drugs with $SI$ of 1.4 or more were withdrawn from the prescription and clinical symptoms were then reversed following cessation of medication. Thus, in these cases drugs with $SI$ of 1.4 or more are considered to be the true causative drugs. Accordingly, $SI$ of 1.5 or more may be viewed as a fairly reliable indicator of drug allergy. Any test performed in vitro, however, is subject to the following limitation. Firstly, there is a very wide variety of possible allergens involved. It is possible that allergenicity develops only after the drug enters the bloodstream and binds to plasma proteins, or metabolism of the drug in vivo may yield by-products that induce the allergic reaction. Secondly, there is the problem of the tested drug, such as cellular damage of the drug itself, its solubility and concentration. Finally, the timing of the test is also of importance. The reactivity of lymphocytes to the tested drug may differ at various stages of allergic symptoms.

In measuring absorption at 540 nm with the MTT assay, there is the possibility that drugs with absorbing properties in this range will affect measurements. We therefore studied the absorption spectra of all drugs tested, but found none with strong absorption in the 540 nm range that might affect assay results. Furthermore, most of the drug added was believed to be eliminated since the culture supernatant was removed before the addition of DMSO. Accordingly, measurements of spectra in this study were scarcely affected by residual drug traces.

The most common manifestation of drug-induced allergic reaction is eruption, and the second is hepatic damage. The eruption can usually be diagnosed by in vivo skin reaction tests, but diagnosis of hepatic damage relies on in vitro tests. Of these in vitro tests, LST, the leukocyte migration inhibition test, macrophage migration inhibition test, and the clonal assay are able to detect cell-mediated immune reaction, with LST being the most widely used. Since the majority of drugs are hepatotoxic with molecular weights of under 1000, they demonstrate little antigenicity, but they nevertheless can cause considerable damage to the liver. Several mechanisms have been proposed to explain this. One is that the drugs induce a subclinical cytotoxicity and combine with carrier proteins released from the liver, like hepatic microsome fractions or liver specific antigens, to create a complete antigen.

When this drug–protein complex is perceived as an antigen by Kupffer cells, cytotoxic T cells are introduced to the liver along with cytokines such as lymphokinin or cholestatic factor, all of which cause hepatic damage.

The disease is difficult to distinguish drug-induced reactions from viral hepatitis on the basis of the liver function test. However, when administration of a drug precedes hepatic damage, drug-induced hepatitis should be suspected. In such cases, diagnosis should rest on the following three points: (1) onset of hepatic damage following medication,
the presence of systemic allergic symptoms, and (3) proof of hypersensitivity to the drug. Of the 113 cases diagnosed at this hospital, the drug-induced symptoms occurred within 10 d of medication in about 50% and within one month in about 85%. In some cases, the patient was already sensitized to the drug, and this caused an immediate reaction when the drug was taken again. However, there were other cases where the symptoms developed after long-term medication. In these instances, diagnosis was more difficult and the patient would often continue to use the drug despite the allergy, which delayed recovery. In all cases, prescriptions of the causative drugs were within normal dosage, so the dose used in the present study seemed little apt to cause hepatic damage.

Antimicrobial agents ranked first among causative drugs in liver impairment and accounted for 33.9% of the total, followed by central nervous system agents for (21.2%), and cardiovascular agents (16.9%). Antibiotics such as cephems, penicillins, and macrolides often generate drug-induced hepatitis of a mixed type, which causes hepatocellular damage and intrahepatic cholestasis at the same time. Erythromycin estolate, propionate, and ethylsuccinate of macrolides have been reported to cause hepatic damage like acute cholecystitis. Acetaminophen and aspirin have been known to cause cytotoxic liver damage, but usually hepatic damage due to most of the analgesics is the allergic type. In hepatitis caused by benzodiazepines, there are some cases of intrahepatic cholestasis and cases of hepatocellular necrosis due to diazepam. In about 6% of cases antiepileptic agents such as phenytoin, phenobarbital and carbamazepine have been reported to cause allergic-type liver damage.

Because any drug could be the cause of liver damage, it is impossible to predict an allergic reaction in a medication. However, it is therapeutically essential in treatment both to diagnose drug allergy early and to halt administration of the causative drugs as soon as possible. For this reason, a physician should be familiar with the past drug allergy of a patient, and know the clinical manifestations and other features of the most common causative drugs. Accurate identification of the allergen is also necessary, though at present there is no means to do this other than a provocative test, which is accompanied by risk and is seldom practiced. Therefore, the development of a safe, accurate method of testing is eagerly awaited. LST with the MITT assay is one such method, the greatest attraction being that it uses no isotopes and thus can easily be performed in an ordinary laboratory. In these days of multiple drug therapies, the identification of drugs for use in diagnosis which may have allergy-inducing tendencies is a topic of increasing concern.

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