Inhibition of Leukocyte Migration by Tumor-associated Antigen in Soluble Extracts of Human Hepatoma

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SUMMARY

The leukocyte migration inhibition assay was used to detect cell-mediated immune reaction of hepatoma patients. Extraction of 3 pairs of human hepatoma tissue and normal liver tissue was made by 3 M KCl solution, and soluble hepatoma antigens were used to test against leukocytes of 18 hepatoma patients by the leukocyte migration inhibition assay. In all, 25 tests were performed; most of them were allogenic, and only 3 tests were autologous. Migration inhibition was observed in 13 of 25 tests, and there were only a few cases of migration inhibition in the control groups. It was concluded that tumor-associated antigen of human hepatoma was contained in the 3 M KCl extract of hepatoma tissue and that tumor-associated antigen of human hepatoma was shared by some hepatoma patients.

INTRODUCTION

Among the various tests to evaluate CMI of cancer patients, the LMI assay has been widely used because of its simplicity and high reproducibility (2, 4, 6, 16) and because it correlates well with the delayed cutaneous hypersensitivity test (1, 7, 17—20).

Since crude insoluble TAA of solid tumor is insufficiently purified for determining its chemical structure, tumor antigen in soluble form is urgently needed for studying tumor immunology.

Since Kahn (15) successfully used hypertonc potassium chloride to isolate the soluble transplantation antigen from cultured human lymphoid cells, the same method has been used to isolate soluble tumor antigens in experimental animals and even in humans (5, 14). Soluble antigens derived from tumor tissue have been proved to contain TAA, as demonstrated in human breast cancer patients by the LMI assay (13) and in human melanoma patients by the delayed cutaneous hypersensitivity test (8).

Although the CMI reactions against TAA have been demonstrated both in vitro and in vivo in chemically induced hepatoma of experimental animals (3, 11, 12), little had been known about the immunogenicity of soluble antigen of human hepatoma. In this study, the LMI assay was used to attempt to identify the TAA contained in soluble extracts of human hepatoma prepared by 3 M KCl solution.

MATERIALS AND METHODS

Patients. Whole blood and liver tissue were obtained at the Department of Surgery, National Taiwan University Hospital, during the period from January 1974 to December 1975, from patients aged 21 to 72 years whose hepatoma had been histologically confirmed or operatively proved. Normal male and female hospital employees (aged 20 to 40 years) and patients with gastric or colon cancer (aged 40 to 56 years) voluntarily donated blood to serve as a source of control leukocytes. Among the patients and controls, only 1 married woman with colon cancer had previously been pregnant, and 3 hepatoma patients had received previous blood transfusions. Blood was taken from these 3 hepatoma patients 2 weeks after the operation and was tested with autologous soluble antigen of hepatoma. All other blood samples of hepatoma patients were obtained before the operation. No chemotherapy or irradiation had been applied to the patients before the operation. Among the 18 hepatoma patients, 2 had ruptured and 3 were resectable; the other cases were unresectable due to metastasis to the opposite lobe.

Antigen Preparation. Hepatoma or normal liver tissue was minced in Eagle’s MEM, (pH 7.3 to 7.4) and passed through a wire screen at room temperature to obtain a single cell suspension. The cell suspension was centrifuged at 200 x g for 5 min and was washed 3 times with Eagle’s MEM. The cell pellet was resuspended in a minimal volume (16 to 25 ml) of the medium. 3 M KCl solution in phosphate buffer at pH 7.4 was added to the cell suspension (0.5 x 10^6 cells/ml). This mixture was stirred with an electromagnetic bar in a 4—10° room for 16 to 24 hr. The mixture was then centrifuged at 100,000 g for 60 min at 4°. The supernatant was dialyzed against 0.9% NaCl solution, with 3 exchanges within 24 to 36 hr at the same temperature. The depotsodium chloride supernatant was centrifuged at 200 x g for another 15 min at 4°. The supernatant was concentrated 3- or 4-fold with an Amicon ultrafiltration unit (UM 10 membrane and 60 psi pressure). The concentrated KCl extract was then sterilized by Millipore membrane (pore size, 0.22 μm) filtration, and the final protein concentration of the extract was determined by the biuret method. The extract was stored at −70°.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: CMI, cell-mediated immune; LMI, leukocyte migration inhibition; TAA, tumor-associated antigen; MEM, minimal essential medium; MI, migration index.

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A total of 3 hepatoma extracts and 3 extracts of normal liver tissue that had accompanied each resected hepatoma tissue were used.

Preparation of Leukocytes. Blood specimens (20 to 30 ml) from patients with hepatoma, or gastric or colonic cancer, and from normal individuals, were drawn into each syringe containing 0.5 ml (500 units) of preservative-free heparin. After staying at 37° for 1 hr, the buffy coat layer was collected and was centrifuged at 200 × g for 5 min at room temperature. The plasma layer was discarded. The leukocytes (mostly contaminated by RBC) were resuspended in Eagle's MEM (pH at 7.3 to 7.4) and washed 3 times by the centrifugation procedures described above. The cell pellet was then resuspended in Eagle's MEM supplemented with 10% heat-inactivated (56° for 30 min) fetal calf serum, penicillin (50 µg/ml), and streptomycin (50 µg/ml). The leukocyte count and viability were determined, and the cell concentration was adjusted to 2 × 10⁷ leukocytes/ml.

Migration Inhibition Test. The antigen extract to be tested was added to Eagle's MEM supplemented with 10% fetal calf serum, penicillin, and streptomycin. The cell suspension was drawn into 25-µl sterile micropipets by capillary action. The micropipets were completely filled, and one end of each was closed with sealing clay. They were centrifuged at 200 × g for 5 min. Each micropipet was quickly cut with a vial file just below the cell-liquid interface and placed in a Mackness culture chamber which was filled with Eagle's MEM supplemented with 10% fetal calf serum, penicillin, and streptomycin. All chambers were incubated on a level shelf at 37° for 24 hr in a humidified 5% CO₂ incubator. After incubation of the antigen-leukocytes mixtures, the areas of migration were projected with a microscope (x10). Although both inner and outer zones of migration were usually observed, only the outer zone was measured. The final areas of migration were measured by planimetry. The migration index (MI) was calculated by the following formula:

\[
MI = \frac{\text{Mean area of migration of 2 duplicates in the presence of antigen}}{\text{Mean area of migration of 2 duplicates in the absence of antigen}}
\]

The variation of migration area in duplicate tests was almost always within 10%. Normal individuals were often retested at different times, and the variation in MI's on different tests was also usually within 10%. All tests, with or without antigen, were performed in duplicate. Usually, each test included leukocytes from normal donors and from patients with hepatoma, and antigen extracts from hepatoma or normal liver tissue.

RESULTS

Migration Inhibition of Leukocytes from Hepatoma Patients. Among 18 hepatoma patients tested, 15 were proved histologically; 3, for whom biopsy was not feasible because of a rupture of hepatoma that caused internal bleeding or technical difficulty due to a tendency to bleed, were diagnosed by clinical, laboratory, or operative findings. Leukocytes from hepatoma patients were run against soluble hepatoma and normal liver antigens, and all tests were allogenic except those of 3 hepatoma patients who were tested with autologous antigen extracts. Leukocytes from normal individuals or from patients with gastric or colon cancer were tested against hepatoma antigen. In order to eliminate the effect of nonspecific migration inhibition caused by high concentration of antigen, the concentration of antigen versus MI of normal individuals was plotted (Chart 1). Since the nonspecific effect of migration inhibition could be avoided at an antigen concentration of 400 µg/ml, such a concentration was adopted for this experiment.

In all, 25 tests were performed on 18 hepatoma patients. The migration inhibition (MI < 0.87) was observed in 13 tests (Chart 2), and only 5 tests were in the normal range (MI = 0.87 to 1.21). Migration stimulation was observed in 7 tests. In the control groups, leukocytes of 15 normal individuals showed migration stimulation in 7 of 25 tests, and 16 of 25 were within the normal range; migration inhibition was demonstrated in only 2 of 25 tests. Leukocytes of 7 patients with gastric or colonic cancer disclosed migration stimulation in 7 of 15 tests, and 7 of 15 were within the normal range; only 1 test disclosed migration inhibition. When leukocytes of hepatoma patients reacted with the antigen extract of normal liver tissue, migration stimulation was observed in 5 of 13 tests, and only 8 of 13 were within the normal range.

These results suggested that the soluble 3 M KCl extracts of hepatoma tissue contained specific TAA of hepatoma and that this antigen was shared by some hepatoma patients.

Migration Inhibition and Migration Stimulation of Leukocytes from Hepatoma Patients with Autologous Hepatoma Antigen. When leukocytes of 3 hepatoma patients were tested with autologous hepatoma antigen, the results revealed migration inhibition in 2 instances and migration stimulation in 1 instance. However, migration inhibition was not observed when autologous normal liver antigens were used (Table 1).

It was evident from the above data that TAA was contained in the soluble 3 M KCl extracts of hepatoma tissues, and that it has immunogenicity to sensitize patients' leukocytes.

![Chart 1](chart1.png)

**Chart 1.** Concentration of soluble hepatoma antigen versus MI of leukocytes of normal individuals.
Migration inhibition and migration stimulation of leukocytes from hepatoma patients with autologous hepatoma antigen

Table 1

<table>
<thead>
<tr>
<th>Case</th>
<th>Autologous hepatoma antigen</th>
<th>Autologous liver antigen</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>0.52</td>
<td>1.33</td>
</tr>
<tr>
<td>2</td>
<td>0.75</td>
<td>1.17</td>
</tr>
<tr>
<td>3</td>
<td>1.25</td>
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DISCUSSION

Although the LMI assay has been widely used as an easy way to detect the CMI reaction of experimental animal or human cells in vitro, there is still some controversy over its technical feasibility (10). However, if one follows several technical points made by Bendixen and Søborg (2) and Rosenberg and David (16), the LMI assay may be considered a reliable test.

The antigen concentration of 400 µg/ml was used in this experiment because the nonspecific effect of migration inhibition is eliminated at this level of antigen concentration (Chart 1). The normal range was adopted at MI = 1.04 ± 0.17. However, the phenomenon of migration stimulation (MI > 1.21) was observed. It has been suggested that the outcome of the migration inhibition test depends upon the sensitivity of the sensitized cells at the fixed antigen concentration (17), i.e., a highly sensitive culture being inhibited and a less sensitive culture being stimulated or perhaps apparently uninfluenced. Since migration stimulation has been more or less observed both in leukocytes of control groups and in leukocytes of hepatoma patients, there should be some unknown antigen other than TAA included in 3 M KCl extracts. It is not likely that the common antigen that induced migration stimulation is the HL-A antigen. Because most of the patients and normals did not have a history of previous sensitization, such as blood transfusion or pregnancy, the leukocytes had not been previously sensitized by HL-A antigen. An explanation of the nature of the antigen included in 3 M KCl extracts and present in both controls and hepatoma patients seems beyond our present knowledge. Toxicity of the KCl solution could be a cause, but this requires further investigation. To avoid the phenomenon of migration stimulation in the LMI assay, the test with the higher concentration of antigen, such as that with between 800 and 400 µg/ml used in our experiment, could be more ideal, as suggested by Søborg (17).

Since most of the advanced cancer patients demonstrated anergy in the delayed cutaneous hypersensitivity test (9), the relationship between the stage of hepatoma in the patients and the LMI assay should be considered. However, most of our hepatoma patients had either resectable localized tumors surrounded by satellite-like metastatic nodules (3 cases, Stage II) or unresectable tumors with metastasis to the opposite lobe (13 cases, Stage III), and only 2 patients had suffered from rupture of hepatoma that had been classified as Stage IV. However, the clinical staging and the results of the LMI assay were not correlated; i.e., the different stages of patients showed the migration inhibition of leukocytes by the same antigen.

The solubilization of tumor tissue by 3 M KCl could usually obtain 15 to 40% of antigenic activity present in the viable tumor (14); our experiment obtained the same results with the LMI assay. Nonetheless, our preparation was still crude.
Further purification and immunological study of this soluble hepatoma antigen are needed in order to characterize it.

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