Specific Immunodiagnosis of Hepatoma by Tube Leukocyte Adherence Inhibition Assay and a Modified Method of Repeated Tube Leukocyte Adherence Inhibition Assay

Toshio Morizane, Naoki Kumagai, Kanji Tsuchimoto, Tetsu Watanabe, and Masaharu Tsuchiya

Department of Internal Medicine, School of Medicine, Keio University, 35-Shinanomachi, Shinjuku-ku, Tokyo, Japan

ABSTRACT

Tube leukocyte adherence inhibition (LAI) assays were performed with normal liver extract as nonspecific antigen and with hepatoma extract as specific antigen in patients with hepatoma. Titration experiments revealed that the optimal extract concentration was 400 μg/ml when expressing the results in terms of a nonadherence index. The results of tube LAI assays were positive in 26 of 40 cases (65%) of hepatoma. The results were negative in all cases of other liver diseases and other cancers.

The tube LAI assay was repeated after discarding the nonadherent cells in the initial tube LAI assay with normal liver extract. The nonadherence index of the repeated tube LAI assay we devised was significantly higher than that of the original tube LAI assay (p < 0.001) in patients with hepatoma. Ten of 12 patients with hepatoma in whom the results of the original tube LAI assay were negative showed positive results in the repeated tube LAI assay. The present study suggests that the problem of false-negative results in tube LAI assay can be solved by repeating the tube LAI assay.

INTRODUCTION

Since the discovery of the LAI3 phenomenon by Halliday and Miller (13) in 1972, much evidence has accumulated to support the claim that the LAI assay can reliably detect the tumor-specific immune response of the tumor-bearing host (9, 14, 15, 26). Powell et al. (29, 30) and recently Koppi et al. (19) have demonstrated that sensitized T-lymphocytes release a lymphokine which inhibits leukocyte adherence on glass in the LAI assay (using a hemocytometer) originally developed by Halliday and Miller (13). In contrast, the tube LAI assay developed by Grosser et al. (9) was based on the reaction of cytophilic IgG antitumor antibody bound to the Fc receptor of monocytes on tumor extract (25). They found that monocytes are the main effector cells which react with the tumor antigen, resulting in a loss of its ability to adhere to glass (11).

In previous reports (2, 12, 21, 27, 38), it was demonstrated that LAI is abrogated in advanced cancer patients by excess circulating tumor antigen, and the results of the LAI assay become negative. However, the results of the tube LAI assay might be negative even in the reacting patients if the proportion of monocytes in the leukocytes applied to the assay is small.

The results of tube LAI assays are generally expressed in terms of NAI, which is calculated from the number of nonadherent cells that react with specific antigen and nonspecific antigen (9). It is well known that the LAI is highly dependent not only on the specific cell-mediated immune response but also on the protein content in the medium (7, 17, 26, 31, 37, 39). The number of nonadherent cells increases by the addition of a small amount of nonspecific antigens. Therefore, it is probable that, if the extent of nonspecific LAI reaction to nonspecific antigens exceeds that to specific antigen, the results of the LAI assay will be negative even in patients in whom the monocytes have the potential to react with TSA.

In the present study, LAI response was examined in patients with hepatoma by tube LAI assay with various concentrations of specific and nonspecific antigens in an attempt to find the difference between specific and nonspecific LAI response. The tube LAI assay was repeated after discarding the nonadherent cells in the initial tube LAI assay with nonspecific antigen. We have found that the value of NAI in the repeated tube LAI assay that we devised is higher than that in the original tube LAI assay and that the incidence of false-negative results can be decreased by the repeated tube LAI assay.

MATERIALS AND METHODS

Subjects. Forty cases of hepatoma which were diagnosed by high titer of α-fetoprotein, scintiscan, and angiography, as well as various control groups, were tested with tube LAI assay before surgery or any other treatment. The diagnosis was confirmed pathologically by specimens obtained at autopsy in 9 cases of hepatoma; in 4 cases, it was confirmed at surgery. The control groups included 9 cases of liver cirrhosis, 3 cases of acute hepatitis, 13 cases of stomach carcinoma, 7 cases of pancreatic carcinoma, 2 cases of biliary tract carcinoma, 1 case of cholangioma, and 22 healthy subjects. In all of these malignant cases in the control groups, the diagnosis was confirmed pathologically by examining specimens obtained at surgery or biopsy. The investigators performed tube LAI assays without previous knowledge of the subjects, and the specimens were coded prior to the preparation of mononuclear cells. When the assay was done in patients with hepatoma, healthy subjects and patients with other diseases were always tested simultaneously.

Tissue Extracts. PBS extracts of hepatoma and normal liver were prepared as described by Grosser et al. (9). These samples were obtained at surgery and autopsy. Extracts from the same samples were used in this study. The tissues were minced by scissors in PBS after removing the connective and necrotic tissues; then 20% (w/v) homogenates made by a Teflon homogenizer were centrifuged at 20,000 × g for 30
min. Protein concentrations of the supernatants were determined by the method of Lowry et al. (23) with bovine albumin as a standard. The supernatants were divided into small aliquots and stored at —80°.

Tube LAI Assay. The tube LAI assay was performed as described by Grosser et al. (9). Heparinized blood was drawn from the antecubital vein. Mononuclear cells were separated by density gradient on Ficoll-Hypaque (4). The separated cells were washed and centrifuged in Hank's balanced salt solution 3 times and then suspended in TC-199 (pH 7.3) unsupplemented by serum to the cell concentration of 1 x 10^7/ml. The cell concentration was determined by Celltrak (Biorad, Minneapolis, Minn.), a simplified model of the Coulter counter (Coulter Electronics, Hialeah, Fla.). A 50-μl aliquot of cell suspension was diluted by 5 ml of PBS in a 10-× 50-mm test tube and then applied to Celltrak. The cell number was counted 3 times, and the mean value was calculated. Cell suspensions (0.1 ml; 1 x 10^6 cells) were poured into each glass tube (16 × 150 mm; Iwaki Glass, Tokyo, Japan) with 0.3 ml of TC-199 and 0.1 ml of tissue extract. The glass tubes were placed horizontally so that three-quarters of the area of each tube was covered by cell suspension; then the cell suspensions were incubated at 37° in a 5% CO2 humidified atmosphere. After 2 hr, the tubes were vertically placed, and samples of the nonadherent cells were gently suspended by Pasteur pipet and counted by Celltrak as mentioned above. The results were expressed as NAI (9). All tests were done in duplicate, and the mean value was calculated. NAI was calculated according to the formula:

\[ \text{NAI}(\%) = \frac{\text{Nonadherent cells in presence of hepatoma extract} - \text{Nonadherent cells in presence of normal liver extract}}{\text{Nonadherent cells in presence of normal liver extract}} \times 100 \]

To determine the dose-response relationship between concentrations of tissue extracts and LAI response, tissue extracts were diluted with PBS to concentrations of 2.0, 1.0, 0.5, and 0.25 mg/ml and added to yield final concentrations in the medium of 400, 200, 100, and 50 μg/ml. As a control, 0.1 ml of PBS was added instead of tissue extracts. The results were expressed as the percentage of nonadherent cells (percentage of nonadherence) in the total number of cells per tube (1 × 10^6) and NAI at each concentration. The results were also expressed as the percentage of increase rate in numbers of nonadherent cells, which was calculated by the following formula at each concentration of tissue extract:

\[ \% \text{ of increase rate} = \frac{\text{Nonadherent cells in presence of hepatoma extract or normal liver extract} - \text{Nonadherent cells without extract}}{\text{Nonadherent cells without extract}} \times 100 \]

Repeated Tube LAI Assay. After the initial tube LAI assay with normal liver extract or hepatoma extract, the test tubes were placed vertically, nonadherent cells were counted, and all of the nonadherent cell suspensions were discarded. Then, 0.4 ml of TC-199 and 0.1 ml of hepatoma extract were added to each tube, the tubes were placed horizontally so that the same side of the tube was covered by the medium, and the tube LAI assay was carried out again according to the method described above. The number of nonadherent cells was counted in the repeated tube LAI assay. In order to express the change in the number of nonadherent cells calculated in the 2 assays, the number of nonadherent cells with normal liver extract or hepatoma extract in the initial tube LAI assay was divided by the number of nonadherent cells with hepatoma extract in the second tube LAI assay, then multiplied × 100; i.e., the change in the number of nonadherent cells was expressed as a percentage of ratio of nonadherent cells in the second tube LAI assay to those in the initial tube LAI assay (Chart 1A).

Another combination of extracts in the initial tube LAI assay and the repeated tube LAI assay was as follows. After the nonadherent cells by the initial tube LAI assay with normal liver extract were discarded, the repeated tube LAI assay was done in paired glass tubes with normal liver extract or hepatoma extract to calculate NAI in the repeated tube LAI assay. These results were then compared with the NAI in the original tube LAI assay [calculated as described above (Chart 1B)].

Percentage of Monocytes in Mononuclear Cells. The percentage of monocytes in mononuclear cells separated by density gradient on Ficoll-Hypaque was calculated by counting microscopically the cells in which the peroxidase was stained on the smears prepared after separation (35). On the same samples of mononuclear cells, the percentage of nonadherence was counted in the tube LAI assay with PBS (without extract), with normal liver extract and with hepatoma extract with a final concentration of 400 μg/ml in the medium.

Statistical Analysis. Statistical analysis was made by Student's t test.

RESULTS

Percentage of Monocytes and Nonadherent Cells. The mean values (± S.D.) of the percentage of nonadherence without extract, with normal liver extract, and with hepatoma extract at a concentration of 400 μg/ml in 19 healthy controls, as well as in 17 patients with hepatoma, are shown in Table 1. The percentage of nonadherence with hepatoma extract in patients with hepatoma (p < 0.02) was significantly higher than either the percentage of nonadherence with normal liver extract.

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tract, or that without extract in these patients ($p < 0.05$), but the difference was not significant between this value and the percentage of nonadherence with hepatoma extract in healthy controls.

The difference in the percentage of monocytes in mononuclear cells between healthy controls and patients with hepatoma was not statistically significant (Table 1).

The results shown in Table 1 suggest that adherent cells include not only monocytes but also a portion of lymphocytes. If only the monocytes adhered to the glass and the lymphocytes did not, the percentage of nonadherence would not reach the values shown in the table.

**Dose-Response Relationship.** In healthy controls, nonspecific LAI was observed with hepatoma extract as well as with normal liver extract. Representative cases are shown in Chart 2. Nonadherent cells increased by increasing the tissue extract concentration; although the increasing rate was low, the slopes of the dose-response curves were gentle with both extracts, and percentage of nonadherence was almost the same at each concentration with both extracts. NAI was low and did not change remarkably at high concentrations, even in healthy Control 4, in whom the slope of dose-response curve of the percentage of nonadherence was rather steep.

In patients with hepatoma, nonspecific LAI was also observed with normal liver extract. The dose-response relationship between the percentage of nonadherence and extract concentration was similar to that in healthy controls (Chart 2). The percentage of nonadherence with hepatoma extract exceeded that with normal liver extract by increasing the extract concentration. NAI was sufficiently high at 400 µg/ml in all cases. In some cases (Patients 1 and 2), NAI was sufficiently high at 100 or 200 µg/ml; but in other cases, NAI was high only at 400 µg/ml. These results suggest that the optimal concentration is 400 µg/ml in most cases.

The difference between specific and nonspecific LAI is more marked when the dose-response curves are expressed by a percentage of increase rate (Chart 3). The subjects shown in Chart 3 include the patients and healthy controls shown in Chart 2, as well as other patients and healthy controls. Although the dose-response curves shown by the percentage of increase rate demonstrated almost the same dose-response relationship between nonadherent cells and the extract concentration as shown in Chart 2, the percentage of increase rate of nonadherent cells from the base of nonadherent cells without extract was greater than 65% in most cases (Patients 1 and 2), NAI was sufficiently high at 100 or 200 µg/ml; but in other cases, NAI was high only at 400 µg/ml. These results suggest that the optimal concentration is 400 µg/ml in most cases.

The difference in the percentage of nonadherence with hepatoma extract in 4 patients with hepatoma and 4 healthy controls. The results are expressed as percentage of nonadherence with hepatoma extract ($\theta$), percentage of nonadherence with normal liver extract ($\phi$), and NAI (C) at each concentration. These results indicate that the optimal extract concentration is 400 µg/ml in most cases.

**Table 1**

<table>
<thead>
<tr>
<th>Percentage of monocytes and nonadherent cells</th>
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<tr>
<td>Nonadherent cells were counted at a 400-µg/ml concentration of tissue extracts. The difference between $h$ ($p &lt; 0.02$) and $g$ or $f$ ($p &lt; 0.05$) was statistically significant, but it was not significant between $h$ and $d$. The difference between $d$ and $c$ or $b$ was not significant, and the difference between $a$ and $e$ was not significant.</td>
</tr>
<tr>
<td>% of monocytes in mononuclear cells</td>
</tr>
<tr>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Healthy controls ($n = 19$)</td>
</tr>
<tr>
<td>Patients with hepatoma ($n = 17$)</td>
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</tbody>
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*a* Mean ± S.D.
Tube LAI in Hepatoma Patients

The percentage of increase rate in the number of nonadherent cells from the base of nonadherent cells without extract emphasizes the difference between specific and nonspecific LAI. In patients with hepatoma, the percentage of increase rate increased rapidly by increasing the concentration of hepatoma extract, and at 400 µg/ml, the percentage of increase rate was more than 150% in every case of hepatoma.

Increased gradually by increasing the concentration of normal liver extract. In these nonspecific LAI response, the percentage of increase rate never exceeded 150% even at a 400-µl/ml concentration of tissue extracts. On the other hand, in patients with hepatoma, the percentage of increase rate increased rapidly by increasing the concentration of hepatoma extract. The difference of the percentage of increase rate between specific and nonspecific antigen was marked at 400 µg/ml.

Because of these results, all assays were performed with 400-µg/ml extract concentrations for both extracts in the following investigations.

Tube LAI Assay in Patients with Hepatoma. Because NAI’s were all below 50% in healthy controls, NAI’s above 50% were interpreted as positive. The results of tube LAI assays were positive in 26 of 40 cases (65%) of hepatoma.

There was no positive case in the control group, which consisted of patients with other liver diseases and other cancers (Chart 4).

The results of tube LAI assays seemed to be independent of serum α-fetoprotein.

Repeated Tube LAI Assay. In patients with hepatoma in whom the results of tube LAI assay were positive, the change in the number of nonadherent cells was over 100% when normal liver extract was added in the initial tube LAI assay and the hepatoma extract was added in the repeated tube LAI assay. The change in the number of nonadherent cells was below 100% in one patient in whom the result of the tube LAI assay was positive. In this case, however, the percentage of nonadherence was over 50% in the initial tube LAI assay with normal liver extract. In the same combination of antigens, the number of nonadherent cells did not increase in the repeated tube LAI assay in control groups or in the patients with hepatoma in whom the results of tube LAI assay were negative, because the change in the number of nonadherent cells was near 100% or below. The control groups consisted of 10 healthy subjects, 6 patients with liver cirrhosis, 3 patients with pancreatic carcinoma, and 7 patients with stomach carcinoma. When the hepatoma extract was added in both the initial assay and the repeated assay, the change in the number of nonadherent cells was near 100% or below in all patients with hepatoma and in the control groups (Chart 5).

The above results indicate that cells that are reactive with

Pancreatic carcinoma, and 7 patients with stomach carcinoma.
specific antigen can be distinguished from those that are reactive with nonspecific antigen; furthermore, the cells that are reactive with specific antigen remain adherent to the glass after they are incubated with nonspecific antigen.

The following assays were performed mainly in the patients in whom the results of the tube LAI assay were negative. These assays were performed in order to assess whether the problem of false-negative results could be overcome by repeating the tube LAI assay following the procedure shown in Chart 1B. When the repeated tube LAI assay was done with normal liver extract and hepatoma extract after discarding the nonadherent cells collected by the initial tube LAI assay in paired test tubes with normal liver extract, the NAI significantly increased in individual cases of hepatoma compared with the NAI of the original tube LAI assay. The difference was statistically significant between the NAI of the original tube LAI assay and that of the second tube LAI assay in 15 patients with hepatoma (p < 0.001). In most of the healthy controls and in all patients with other diseases, the NAI of the second tube LAI assay was lower than that of the original tube LAI assay, and the difference was not statistically significant. In all the control subjects, the NAI of the repeated tube LAI assay was significantly lower than that of the original tube LAI assay (p < 0.05) (Chart 6). These results also indicate that the cells reactive with specific antigen remain adherent to the glass after they are incubated with nonspecific antigen in advance. If an NAI above the upper limit in the control group is interpreted as positive, 10 of 12 patients with hepatoma in whom the results of the original tube LAI assay were negative showed positive results in the repeated tube LAI assay.

DISCUSSION

The LAI assay has been used successfully to detect the cell-mediated immune response in cancer-bearing hosts (6, 8, 21, 23, 32 33, 36), transplantation models (16, 39), and other antigen systems (1, 5, 40). (The key studies in this field appear in Ref. 10).

The present study shows that the mononuclear cells separated by density gradient on Ficoll-Hypaque included about 20 to 30% monocytes on an average. These results coincide with those of previous reports (3, 18, 34, 35). The percentage of nonadherence with normal liver extract was about 20% on an average in patients with hepatoma. As demonstrated by Koller et al. (18), the majority of mononuclear cells that adhere to glass in the absence of serum are lymphocytes, some of which are monocytes, but the ratio of lymphocytes to monocytes does not differ from that of mononuclear cell suspension. On the other hand, in the presence of serum, the majority of cells that adhere to glass are monocytes. Koller et al. (18) hypothesized that the effect of serum appeared to be modification of the glass surface. This speculation is supported by Kumagai et al. (20). However, it is not known how normal liver extract affects the adherence of mononuclear cells to glass, because we did not directly determine the percentage of monocytes in nonadherent cells or in adherent cells with normal liver extract. The results of the repeated tube LAI assay suggest that the monocytes might remain adherent to glass after incubation with normal liver extract, because the cells that react specifically with TSA are monocytes as described by Grosser et al. (11). Therefore, it is reasonable to speculate that most of the monocytes would remain adherent to glass and that the majority of nonadherent cells might be lymphocytes when mononuclear cells are incubated with normal liver extract. If the proportion of monocytes is too small, it is probable that the NAI will have a very small value, even if all monocytes react with TSA. For example, if the percentage of monocytes is 5% and the percentage of nonadherence with normal liver extract is 30%, the percentage of nonadherence with hepatoima extract theoretically would be 35% when all monocytes react with TSA, resulting in a loss of their property of adherence. In this case, NAI amounts to 17% [(35 — 30) + 30 x 100], and the result should be regarded as a false negative. Because the monocyte percentage in mononuclear cells separated by density gradient on Ficoll-Hypaque is variable from subject to subject, it seems likely that the tube LAI assay results in a false-negative value when the percentage of monocytes is very small.

The standard tube LAI assay is performed in serum-free medium. Prior investigations (26, 37, 39) have demonstrated increased nonadherence or decreased adherence in control donors when the leukocytes were incubated with tissue extracts. They have also demonstrated the same results in cancer-bearing patients when the leukocytes were incubated with normal tissue extracts or with tumor extracts of different tissue types from those borne by the patients. The tested tissue extracts in these studies include melanoma, hepatoma, breast cancer, normal breast tissue extract, etc. Decreased adherence was found even with fetal calf serum (26). The present study also provides evidence of increased nonadherence with
normal liver extract and hepatoma extract in healthy donors, as well as with normal liver extract in patients with hepatoma. These results prove that the tube LAI assay is highly dependent on protein concentration. However, the mechanism of this nonspecific LAI is not known. It will be necessary to investigate which components of tissue extracts cause the nonspecific LAI.

The titration experiments show that the number of nonadherent cells increases when the protein content of the extracts is increased. This dose-dependent relationship was demonstrated in cancer-bearing patients as well as in healthy subjects by many investigators (7, 26, 29, 31, 37). Our data also demonstrate the similar dose-dependent relationship between the percentage of nonadherence and the protein concentration in the medium. The dose-response curves expressed by percentage of nonadherence either with normal liver extract or hepatoma extract in healthy donors did not differ fundamentally from those in patients with hepatoma. However, the percentage of nonadherence with hepatoma extract exceeded that with normal liver extract in patients with hepatoma. When the dose-response curves were expressed by the percentage of increase rate from the baseline of the number of nonadherent cells without extract, the shapes of the dose-response curves with hepatoma extract in patients with hepatoma were entirely different from those in healthy controls or those with normal liver extract in the patients. Although the results of the tube LAI assay have been expressed by NAI in general, our data suggest that it is possible to express them by the percentage of increase rate at 400 µg/ml of hepatoma extract from the baseline of the number of adherent cells without extract, because the percentage of increase rate in all tested patients with hepatoma was higher than the upper limit of the percentage of increase rate in healthy donors. However, it will be necessary to prove the reliability of expressing the results as the percentage of increase rate by testing more patients.

In a study of patients with malignant melanoma, Marti and Thomson (26) noted that the difference in reactivity to the specific and nonspecific antigen in the tube LAI assay was not observed at protein concentrations of less than 75 µg/tube (150 µg/ml) or greater than 200 µg/tube (400 µg/ml); extract concentrations of 75 to 150 µg/tube (150 to 300 µg/ml) were optimal, although they observed that the titration curves of different patients varied. In another report, Thomson et al. (37) demonstrated that the optimal extract concentration was about 100 µg/tube (200 µg/ml). They used the tumor extract of other tissue types as nonspecific antigen. In a study of patients with breast cancer, Fujisawa et al. (7) noted that the optimal concentration was 200 µg/ml; at greater concentrations, there was no significant difference in the positive rate of the LAI assay using a hemocytometer between breast cancer patients and patients with other cancers. Fujisawa et al. (7) used normal breast tissue extract as nonspecific antigen. The data presented here show that a protein concentration of 400 µg/ml was optimal in the tube LAI assay when it was performed with hepatoma extract as the specific antigen and normal liver extract as the nonspecific antigen in patients with hepatoma, although some variations were observed. The cause of the discrepancy among optimal extract concentrations reported here and in previous studies is not known. It might be dependent on the sources of tumor extract, the type of tissue used as nonspecific antigen, and the type of cancer. Therefore, it is necessary to titrate the tissue extracts carefully before applying them to the routine work.

Crude tissue extracts, i.e., the supernatants of the tissue homogenates spun at 20,000 × g, have been used successfully as test antigens, and it has been suggested that they contain TSA. It should be noted that they contain many proteins (24) which might suppress leukocyte adherence to glass nonspecifically, although direct evidence showing which proteins are involved has not yet been obtained. Decreased adherence with tumor extracts in control donors, as mentioned above, clearly shows that these crude tumor extracts induce the nonspecific LAI response. Therefore, it is reasonable to assume that the LAI for hepatoma extract in patients with hepatoma would be the sum total of the nonspecific LAI by the proteins other than TSA in the extract and the specific LAI by TSA. The results of tube LAI assay have been expressed by NAI in general. The high or low value of the NAI is determined by 2 values: (a) the number of nonadherent cells with nonspecific antigen; and (b) the difference between the number of nonadherent cells with specific antigen and the number of nonadherent cells with nonspecific antigen. For this reason, it is very important to know which tissue extract is used as nonspecific antigen and to titrate the different tissue extracts and use a protein concentration that induces a similar number of cells from control subjects to be nonadherent in the range of 20 to 30% nonadherence [as described by Marti and Thomson (26)]. Ideally, purified TSA should be used as specific antigen at a concentration which does not induce the nonspecific LAI response. The second best way seems to be to use the tissue extract as nonspecific antigen in which all components except TSA are the same as the specific antigen and to express the results by the NAI. In their study of patients with breast cancer, Fujisawa et al. (7) have concluded that, when studying alloge neic leukocyte responses to tumor extracts, one must use normal tissue extract as the control. We agree, because in the present study normal liver extract was used successfully as the nonspecific antigen to distinguish between the LAI response of patients with hepatoma and that of control donors.

The repeated tube LAI assay is entirely different from the ‘‘blocking LAI’’ described by Grosser and Thomson (12). They demonstrated that the reactive leukocytes lost their reactivity when they were incubated with the serum of advanced cancer patients before they were applied to the tube LAI assay. They aimed to block the cytophilic antitumor antibodies by the free tumor antigenic determinants in the serum. In contrast, we designed the repeated tube LAI assay in order to eliminate the leukocytes involved in nonspecific LAI before applying them to the assay. Our data show that the cells that react specifically with TSA remained adherent on the glass after they were incubated with nonspecific antigen. The values of NAI in the repeated tube LAI assay were significantly higher than those obtained in the original tube LAI assay in patients with hepatoma. We speculate that the proportion of cells that react specifically with TSA (i.e., the monocytes) might increase by this procedure. As described under ‘‘Discussion,’’ the increase of the cells that react specifically with TSA must result in the increase of NAI. Prior investigations have demonstrated that the results of tube LAI assay are negative in patients with large tumor burdens because the excess tumor antigenic determinants in the serum blocked the LAI response (2, 9, 22, 27, 33, 38). Our data suggest that sometimes negative results are
obtained from the tube LAI assay even in the reactive patients, because positive results were obtained by repeating the tube LAI assay in most patients in whom the results of original tube LAI assay were negative. Although direct evidence has not yet been obtained, the percentage of monocytes, i.e., of cells that react specifically with TSA, might be small in the mononuclear cells from those patients, and this percentage might be increased in the repeated tube LAI assay.

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