CONVENTION APPLICATION FOR A PATENT

R & Z VERMÖGENSVERWALTUNGSGESELLSCHAFT MBH,

WE:

(Use BLOCK letters)

4 2 3 4 9 / 7 8

of Magdeburger Strasse 37, D-4150 Krefeld, West Germany,

hereby apply for the grant of a Patent for an invention entitled

IN VITRO TESTING METHOD FOR THE DIAGNOSIS OF MALIGNANT TUMOURS

which is described in the accompanying complete specification. This application is a Convention application and is based on the application numbered P. 27 55 363 for a patent or similar protection made in West Germany.

12 December 1977.

Our address for service is SANDERCOCK, SMITH & BEADLE, Patent Attorneys,
203 Riversdale Road, (P.O. Box 410) Hawthorn, 3122, Victoria, Australia.

Dated this 7th day of DECEMBER 1978.

To:

THE COMMISSIONER OF PATENTS

(Signature)

BY: SANDERCOCK, SMITH & BEADLE

This form must be accompanied by a complete specification (Form 10 and a true copy) and the appropriate lodgment fee.

The address for service stated in this form must be an address in Australia.

F. D. ATKINSON, Government Printer, Canberra
1. An in vitro testing method for the diagnosis of malignant tumors, preferably human tumors, comprising determining the mobility variation of cellular blood components contacted with a migration inhibition factor (MIF), wherein the MIF is secreted from lymphocytes being sensitized against tumor tissue, characterized by subjecting, as said cellular blood components, the lymphocytes as a part of a leucocyte suspension to be examined proper to a known per se migration inhibition test wherein the lymphocytes are contacted with an antigen capable of stimulating them to produce MIF, and inhibiting and fixing the leucocyte migration after a predetermined period of time.
11. A test plate for carrying out the method according to any one of claims 1 to 10, characterized by a planar carrier plate or dish of an inert material (e.g. glass) having applied thereon a solidified agar layer of a thickness of from 2 to 5 mm, a capillary migration layer being between surface of the plate or dish and agar layer, said migration layer having a thickness of between 1 - 20 \( \mu m \), and said agar layer being provided with at least two substantially circular punched holes (11) having a diameter of from 0.5 to 5 mm, preferably 2.5 mm.
Complete Specification

Class:
Int. Cl.:

Application Number: Lodged:

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   Accepted:
   Published:

Priority:

Related Art:

TO BE COMPLETED BY APPLICANT

Name of Applicant: R & Z VERMÖGENSVERWALTUNGSGESSELLSCHAFT MBH

Address of Applicant: Magdeburger Strasse 37,
D-4150 Krefeld,
West Germany.

Actual Inventor: DR. FRIEDRICH DOUWES

Address for Service: SANDERCOCK, SMITH & BEAD..E, Patent Attorneys,
203 Riversdale Road, (P.O. Box 410); Hawthorn, 3122, Victoria, Australia.

Complete Specification for the invention entitled: IN VITRO TESTING METHOD FOR THE DIAGNOSIS OF MALIGNANT TUMOURS

The following statement is a full description of this invention; including the best method of performing it known to the applicant.* US:

*Note: The description is to be typed in double spacing, p. type face, in an area not exceeding 250 mm in depth and 160 mm in width, on tough white paper of good quality and it is to be inserted inside this form.

11710/76-L F. D. Atkinson, Government Printer, Canberra
The present invention relates to an in vitro testing method for the diagnosis of malignant tumours, preferably human tumours, comprising determining the mobility variation of cellular blood components contacted with a migration inhibition factor (MIF), wherein the MIF is secreted from lymphocytes being sensitized against tumour tissue. Such MIF is not secreted when the lymphocytes come from patients who do not suffer from malignant tumours (malignoma) since these lymphocytes are not sensitized and, therefore, fail to release MIF when contacted with tumour antigen.

It is known from literature (Douwes, F. R. et al.; Verh. Deutsche Ges. inn. Med., Vol. 82, J. F. Bergmann-Verlag, Munich) that the early diagnosis of malignant diseases can be made by means of the so-called electrophoresis mobility test (EMT). The principle of the EMT is based upon the fact that the velocity of migration (mobility) of specific bodies (i.e. macrophages, granulocytes and other blood corpuscles) visible in the electric field of a special microscope (zytophrometer) is only varied when these bodies come into
contact with lymphokines of sensitized lymphocytes. It is hereby assumed that lymphocytes which are sensitized against a specific antigen (so-called T-lymphocytes) release a series of soluble factors, i.e. the so-called lymphokines. An important one among these factors is the migration inhibition factor (MIF). This factor affects the capability of migration of blood corpuscles, especially of macrophages and granulocytes, and provides for their arresting and accumulation at the place where the antigen inducing the sensibilization is present. In vivo, this represents a possibility to destroy the tumour.

Lymphocytes of patients suffering from malignoma react in specific manner by releasing the so-called soluble factors, when contacted with a basic protein obtained from the brain substance on the one hand, or from carcinoma tissue on the other hand. The basic proteins causing the specific reactions of the lymphocytes, are termed encephalitogenous factor (EF) and basic protein from carcinoma tissue (CaBP). They are produced in accordance with known per se methods (Caspary, E. A. et al., Brit. Med. 1971, pages 613 - 617, and other publications; compare Douwes F. R., loc. sit.).
The results of blood test with the EMT as described in literature reveal that the test allows an almost 100% differentiation of the samples to be made so as to determine whether the samples have been taken from an organism affected by malignant (tumour) diseases or from a healthy organism or an organism affected by non-malignant diseases, respectively. All of the patients tested and suffering from (malignant) tumour provided a positive lymphocyte response, i.e. the lymphocytes yielded a factor resulting in a measurable and characteristic variation of the mobility of indicator cells (compare Douwes, F. R., loc. cit.). Control diagnoses with patients affected by non-malignant diseases did not provide such lymphocyte response. Incorrect results could be traced back to specific causes. Accordingly, the EMT can be considered to be the presently most sensitive test for cancer.

It is considered to be disadvantageous in the performance of the test that qualified control of an instrument being relatively complicated to operate, i.e. the zytopherometer, is required. The personnel observing and measuring the speed of migration must be highly trained and supervised very carefully. Owing to diminishing personnel attention, the operators can be
engaged with the work for a limited period of time only. Accordingly, the use of the EMT is restricted to highly specialized institutes. The analysis of the test is made while the blood corpuscles are in motion, i.e. dynamically. "Stopping" of the experiment is not possible; therefore, repeated measurements cannot be taken, either.

It is the object of the present invention to provide a testing method for the diagnosis of malignomae, which method in as general and unspecific as possible a manner with respect to the type of the malignomae and with a high degree of accuracy or reliability signals the presence of malignomae, but owing to its principle may be used or performed in any medical laboratory, while lending itself particularly for use in mass examination for precautional diagnosis of cancer. The result of the test should be readily verifiable. The test should be adapted to be analyzed statistically and recorded without any intermediate protocolling.

These objects are solved in a testing method as defined above by subjecting as said cellular blood components the lymphocytes as a part of a leucocyte suspension to be examined proper to a known per se migration inhibition test wherein the
lymphocytes are contacted with an antigen capable of stimulating them to produce MIF, and inhibiting and fixing the leucocyte migration after a predetermined period of time.

Various migration inhibition tests are known which are principally suitable for the examination in accordance with the basic object. Among other methods, the so-called capillary technique by SØBORG and BENDIXEN is known. For the realization of the inventive idea, it is necessary to detect the mobility of the indicator cells by "freezing" a state of migration reached after a given period of time.

According to the invention, preferably the propagation or variation of mobility of the leucocytes within an extremely fine "open" capillary layer of about 10 microns (\(\mu m\)) thickness is measured. A test system has been found to be particularly suitable, which comprises a dual-layer element, namely a carrier or substrate made of an inert medium such as e.g. glass, and an agar-agar being saturated with nutrients for blood cells. A particularly suitable agar is one sold under the tradename Agarose as produced by the firm Behring-Werke of Marburg (Germany).
Preferably, the intermediate layer between the agar and the carrier layer is partially filled with liquid. This layer has a thickness of about 1 to 20 microns. Advantageously, an incubation period in the MIT of between 10 and 30 hours is chosen.

The test is performed with a sample of leucocyte-rich supernatant phase ("buffycoat") obtained from human blood and containing a fixed number of leucocytes of between $10^4$ and $10^7$ per sample. These standard values are approved and tried values which do not require an excessive quantity of blood to be taken from the patients.

The buffycoat sample is mixed with an antigen solution in a ratio of from 1 : 50 to 1 : 10. To this end, antigen solutions may be used which have been obtained by a method disclosed by CASPARY and FIELD or by means of a 3 M KCl extraction according to MELTZER. As the starting material, human malignoma tissue from surgical operations or human brain substance are employed. However, it is also feasible to yield antigen solution from tumour cells grown in vitro.

The testing method according to the invention is not only applicable to an unspecific malignoma examination,
but may be extended also to an organo-specific examination. If antigens are employed which have been recovered from carcinomous tissue of specific organs, lymphocytes of patients affected by the same organic carcinomae react in the indicated manner. It has been disclosed in literature that antigen solution obtained, for instance, from mammary carcinoma according to CASPARY and FIELD or MELTZER, result in a specific reaction exclusively with respect to lymphocytes coming from persons affected by mammary carcinoma.

In the case that a positive immune response in a general test is present, i.e. in the case of suspected carcinoma, it is also possible to perform a series of tests by using organo-specific antigens, and thereby to determine within a relatively short period of time, what organs might be affected by carcinoma.

It is of great importance that the so-called immune response, i.e. the production of the slowing effect in the movement of the indicator cells in the course of existing tests, can be suprisingly enhanced by adding to the sample solution a solution containing the so-called transfer factor. Such factor is known
from immunology (compare e.g. B.M. Rosenthal; "Der Transferfaktor und seine therapeutische Anwendung"; Schweiz. med. Wschr., Vol. 104, 1974; pages 1501 - 1506). The transfer factor may be produced from normal leucocytes of the blood. This factor represents a subcellular, dialyzable, non-immunogeneous leucocyte factor which is responsible for the T-lymphocyte responsive reaction. Transfer factor preparations are commercially available (SCHURA, Krefeld/Germany) which have been prepared by fractionation in liquid oxygen and subsequent gradual ultrafractionation. One commercially available unit contains transfer factor of about \(5 \times 10^{10}\) leucocytes. The preparation is stabilized by means of 1% solution of human albumin.

It is found that the addition of transfer factor may yield an increase in the immune response. Thus, the discrimination of the various sample tests is substantially improved by the addition of the transfer factor.

Further, the present invention relates to a test dish or plate for carrying out the method, which plate comprises a planar carrier plate of an inert material, e.g. glass, having coated thereon a solidified agar
layer of from 2 to 5 mm thickness and having at least two substantially circular punched holes of a diameter of the order of from 0.5 to 5 mm (preferably from 1.1 to 3.0 mm). Each punched hole has a capacity of from 5 to 20 and preferably 8 microliters of test substance.

It is essential that the punched holes are cut with such a configuration that uniformity of the capillary migration layer is provided even in the transition area between the punched hole and the layer.

Below, the invention is explained in Examples, and furthermore, the following Figures are added:

Figure 1 shows in plan view a test plate according to the invention; and

Figure 2 shows a test plate in cross-sectional view.

Example 1

A) Preparation of the leucocyte suspension

30 ml of human blood are heparinized with 300 USP units of Na heparinate. heparinized human blood
(from a patient) is substrated within a plastic ferrule with 8 ml of 6 percent by weight dextran solution in physiological normal salt solution (molecular weight of the dextran: 75,000). Thereupon, the erythrocytes are allowed to sediment (settle) in a heating cabinet for about 45 minutes at $37^\circ$ C. The leucocyte enriched supernatant phase (buffycoat) is pipetted off and mixed with an equal volume of 0.9 percent by weight NaCl solution and then centrifuged at a centrifugal force of 750 G's. The pellet deposited in the centrifuge is again washed for two times in 10 ml of Hank's solution (pH 7.2) each and suspended in a Tc 199 medium (producer: Serva, Heidelberg). During washing and suspending, the cells are agitated by means of a pipette until lumps of cells are no longer visible to the naked eye. Upon dyeing with Türk's solution, the leucocytes are counted in the Neubauer compartment and adjusted to $2 \times 10^5$ cells/microliter in Tc 199 medium. In the average, the sample contains 25% of mononuclear cells (lymphocytes and monocytes) as well as 75% of granulocytes. The erythrocyte contamination is less than 1 to 1.

B) Preparation of the antigen solution

For the preparation of the antigen solution, the
encephalitogenous factor (EF) or the basic protein of carcinoma tissue (CaBP) has to be isolated.

The tumour or the brain, respectively, are processed as rapidly as possible under sterile conditions after the surgical removal. Connective tissue and healthy tissue are removed from the tumour. Thereafter, the tumour or brain tissue is cut into small pieces with sterile scissors (scalpel) and further disintegrated in a bath of ice in a homogenizer. The tumour tissue must not be heated during such processing. The homogenate is suspended in four times the volume of distilled water and then centrifuged at a force of 23,000 G's. The thus produces pellet is again suspended in a fourfold volume of 0.9 % NaCl, homogenized and centrifuged at 23,000 G's for 30 minutes. The pellet again suspended in four times the volume of distilled water is hereafter freeze-dried. The freeze-dried powder is blended with a mixture (2 : 1) of chloroform and methanol in a ratio of 1 : 10 and agitated for 30 minutes. The mixture is centrifuged for 10 minutes at 600 G's. The supernatant phase is discarded.

This process step is repeated twice. The pellet is
then air dried. The dried pellet is resuspended in the fivefold volume of a 5% NaCl solution in water, and centrifuged at 23,000 G's for 30 min. By adding N/100 NaCl, the pellet again resuspended in a fivefold volume of distilled water is adjusted to a pH of 3.5. The suspension is slightly shaken and thereafter again centrifuged at 23,000 G's for 30 minutes. The supernatant phase is discarded. Again, the pellet is resuspended in a fivefold volume of distilled water which is adjusted to a pH of 2.6 with N/100 HCl, and allowed to stand for from 3 to 18 hours. The pH value is not allowed to vary during this period. Then, the suspension is centrifuged at 23,000 G's for 30 minutes. The supernatant phase is stored, and the pellet is again washed in N/100 NaCl at a pH of 2.6 and centrifuged. The supernatant phase resulting from this step is stored. The two lastmentioned supernatant phases are combined and then dialyzed against distilled water. Hereupon, the product is subjected to freeze-drying.

An antigen test solution is obtained by dissolving 0.1 mg/ml of the freeze-dried substance in distilled water. Principally the same process of preparation is employed for obtaining a solution of the basic protein.
from tumour tissue (CaBP).

C) **Preparation of the test plate**

(compare also Figures 1 and 2)

0.5 g of a specific culture agar (agarose; producer: Behring-Werke of Marburg) are weighted into a 50 ml Erlenmeyer bulb or flask. 22.5 ml of sterile aqua bidestillata are added to the mass.

22.5 ml of the double concentrated Tc 199 medium and 5 ml of plasma obtained from human blood are charged into a second Erlenmeyer bulb. The agarose is dissolved in a boiling water bath by being supported by a bracket, under slight circulatory agitation. Hereby, the Erlenmeyer bulb is sealed with a film or stopper to prevent a part of water from evaporating.

When the agarose is completely dissolved, the substance is cooled to 47°C in a water bath. In the same bath, the medium-plasma mixture is preheated and added to the agarose under shaking, such that there are now present 50 ml of 1% agarose in plain Tc 199 medium containing 10% of plasma.
With the aid of a preheated discharge pipette, 5 ml each of the agar are filled into circular tissue culture dishes (Petri dishes) 10 having a diameter of 50 mm, which dishes are shown in plan view in Figure 1. Upon solidification of the agar, holes 11 of 2.5 mm diameter are punched out from the agar by means of a telescoping punching canule (Behring-Werke, Marburg) with the aid of a template. In the punching work, care has to be taken that the agar layer is not released from the glass layer in the punching area, in order that the capillary layer is not increased such that migration of the leucocytes would no longer be possible.

The pattern of the holes 11 appears from Figure 1. Of course other configurations are imaginable. Figure 2 illustrates an enlarged sectional view of the lower part of dish 10. As indicated, the agar layer 1 is separated from the glass layer of the dish 10 by a thin capillary layer 3. The various columns of holes are provided e. g. for the following purposes:

(A) Control (standard)
(B) Test suspension containing general antigen
(C) Test suspension containing organo-specific antigen
(D) Test suspension containing organo-specific antigen and transfer factor.

D) Reaction, incubation and fixation

The following preparations are provided for a test:

1) A preparation containing each 100 microliters of leucocyte suspension according to (A) and 8 microliters of antigen suspension according to (B);

2) a preparation containing each 100 microliters of leucocyte suspension and 8 microliters of N/100 NaCl solution (for reference value).

The mixed preparations 1 and 2 are subjected to incubation for 60 minutes in a heating cabinet at 37°C and at 100% humidity. Then, 8 microliters of leucocyte- and antigen-containing substance or standard substance, respectively, of the incubated solution are filled into each punched hole. Depending on the configuration of the test plate (number of punched holes), from 2 to 5 measurement results and from 2 to 5 reference values are available for the analysis of the leucocyte migration and the effect of the factors produced by the lymphocytes and delaying the migration. The cells contained in the suspension and introduced
into the punched holes at the start of the test, migrate into the intermediate layer 3 between the agar and the surface of the dish. By incubating the dishes at a temperature of 37°C in the heating cabinet for a period of 18 hours, such migration is maintained across a definite distance. The leucocytes do not migrate into the agar, but rather take their nutrients therefrom. Incubation is effected in a humidified cabinet at a relative humidity of 100%.

Following the incubation, the plates are coated with methanol for 15 minutes, and subsequently with formalin solution (37 percent by weight of methanal) for 10 minutes. Thereafter, the agar is carefully stripped from the bottom of the dish as a membrane. The cells migrated in the intermediate layer are now fixed to the surface of the dish. Upon drying, the cells are dyed in accordance with the Pappenheim method, whereby dark-violet halos 4 (migration halos) are formed on the plate surface (compare Figure 1).

E) Measuring and determining the results

Using a graduated magnifying glass (Bausch & Lomb), the diameter of the migration halo is measured. In
such measurement, the polymorpho-nuclear cells discretely positioned in the periphery should be detected, too. The arithmetic average of a plurality of measuring steps each is formed.

The quotient of the migration area $F_1$ with antigen and of the migration area $F_2$ without antigen yields a "migration index" $MI$:

$$MI = \frac{F_1}{F_2}.$$

A migration index smaller than 0.85 means inhibition of the leucocytes to significant degree and, thus, a positive cell response. The following Table illustrates one example of measurement:

<table>
<thead>
<tr>
<th>Column</th>
<th>$\phi$ (mm)</th>
<th>$F$ (mm$^2$)</th>
<th>MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>6.75</td>
<td>$F_1 = 35.8$</td>
<td>0.80</td>
</tr>
<tr>
<td>A</td>
<td>7.55</td>
<td>$F_2 = 44.75$</td>
<td>(1)</td>
</tr>
</tbody>
</table>
Initial experiments with a total of 97 female patients were conducted to demonstrate the effectiveness of the migration inhibition test in the case of mammary carcinoma. 27 among 46 female patients tested and affected by mammary carcinoma showed an inhibition higher than 15 % (= 58.7 %), i.e. the migration index MI is less than 0.85. Among 23 female patients tested and suffering from mastopathia, only four patients showed such inhibition (= 17 %), whereas in the case of female patients affected by fibroadenoma (fibroma), one female patient out of five (= 20 %) showed an inhibition. The following Table provides a general summary:

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number of patients (n)</th>
<th>Migration index (inhibited)</th>
<th>Migration index (normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>20</td>
<td>0 (0 %)</td>
<td>20 (100 %)</td>
</tr>
<tr>
<td>Mastopathia cystica</td>
<td>23</td>
<td>4 (17 %)</td>
<td>19 (83 %)</td>
</tr>
<tr>
<td>Fibroadenoma</td>
<td>5</td>
<td>1 (20 %)</td>
<td>4 (80 %)</td>
</tr>
<tr>
<td>Mammary cysts</td>
<td>4</td>
<td>0 (0 %)</td>
<td>4 (100 %)</td>
</tr>
<tr>
<td>Other malignant diseases</td>
<td>9</td>
<td>3 (33 %)</td>
<td>6 (67 %)</td>
</tr>
<tr>
<td>Mammary carcinoma</td>
<td>46</td>
<td>27 (58.7 %)</td>
<td>19 (41.3 %)</td>
</tr>
</tbody>
</table>

Example 2

Employment of the transfer factor for increasing the immune response.
It has already been noted that an increase of the immune response may be obtained by the addition of a solution containing transfer factor (producer: SCHURA, Krefeld). It is found that with such addition the factor MI is more accentuated as compared to Test Example 1.

The following test compositions were prepared:

1) 100 microliters of leucocyte suspension + 8 microliters of antigen solution

2) 100 microliters of leucocyte suspension + 8 microliters of antigen solution + 20 microliters of transfer factor solution (prepared by SCHURA; 1 microliter corresponding approximately to the transfer factor of $5 \times 10^5$ leucocytes)

3) 100 microliters of leucocyte suspension

4) 100 microliters of leucocyte suspension + 20 microliters of transfer factor solution as in composition 2).

Incubation and preparation of the dish are made in the same manner as in Example 1. A dish having four times five punched holes (diameter 2.5 mm) is prepared. One column of 5 holes each is charged with 10 microliters of test composition: 1 to 4.
Results (Average of five values each):

<table>
<thead>
<tr>
<th>Composition</th>
<th>Column</th>
<th>Diameter (mm)</th>
<th>F (mm²)</th>
<th>MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B</td>
<td>6,90</td>
<td>37,4</td>
<td>0,79</td>
</tr>
<tr>
<td>2</td>
<td>C</td>
<td>6,05</td>
<td>28,7</td>
<td>0,61 (incl. transfer-factor)</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>7,75</td>
<td>47,2</td>
<td>1,0 standard</td>
</tr>
<tr>
<td>4</td>
<td>D</td>
<td>7,70</td>
<td>46,6</td>
<td>0,99</td>
</tr>
</tbody>
</table>

The low MI of columns B/C indicates a malignoma case.

Results of the migration inhibition test by using the transfer factor in the determination of gastrointestinal tumours:

Measurements were made in accordance with the measuring instruction according to Example 2. No appreciable inhibition was found in a (healthy) group of patients; the migration index was of the order of 1.02 ± 0.1. The transfer factor did not affect these results. In the group of patients affected by colonic carcinomae,
the migration index amounted to $0.65 \pm 0.2$. Actually, 11 out of 15 cases tested showed a migration index smaller than 0.85. Upon addition of the transfer factor, the migration index was of the order of $0.61 \pm 0.09$.

Furthermore, 14 out of 15 patients exhibited an MI of less than 0.85 after the addition of the transfer factor, such that a significant improvement of the test result was obtained in this instance, too. No substantial inhibition could be observed in other groups tested.

Example 3

An antigen solution may be extracted also from tumour cells which have been grown in vitro in a fermentation culture, with the aid of a buffer and with repeated disintegration of the tissues.

Cells of cerebral tumour are grown in vitro as follows: Special care has to be exercised to maintain sterile operating conditions. Preferably, the cells are washed for about 30 seconds in a Hank's balancing solution. Agitation should be avoided. All walls and surfaces of the bulb, preferably a 250 ml bulb of glass, are washed carefully. The solution is made free of the cells
by centrifuging under cold conditions for a period of 10 minutes. The medium is poured into a beaker glass. A small quantity of buffered proteinase enzyme solution is added and purged rapidly in order to avoid decomposition of the cells. In the preferred method, up to 2 ml of trypsin solution (EDTA) are added in a bulb. The trypsin solution is poured away after a purging period of 10 seconds.

Subsequently, the same volume of fresh trypsin solution is added. Incubation is conducted until it can be seen by observation under the microscope that the cells separate from the walls of the chamber. This takes normally from 5 to 10 minutes. A suitable growing medium is added, e.g. 50 ml of a solution of 7 to 10% solution of the serum of calf foetuses in 100 ml of F-10 nutrient.

25 ml of the fresh medium including the cells are transferred into a new growing chamber (bulb) for reproduction. Both chambers are placed into an incubator at 35°C for about seven days. In accordance with the process of this Example, a culture of the artificial tumor cells is divided up to this point into a pair of fresh cultures approximately every seven days.
The mode of process may be repeated in intervals of seven days. Accordingly, the number of cells grown in vitro doubles every seven days.

The medium containing the cells is transferred into a centrifuge tube and centrifuged at 3,000 rpm (± about 2200 G's) in the cold for 10 minutes. The medium is discarded. The cells remaining in the growing chamber are scraped from the chamber walls and washed or purged into the centrifuge tubes with a neutral buffer solution. The cells are washed twice with a neutral buffer solution and then again centrifuged under cold conditions at 3,000 rpm (± about 2200 G's). The medium is disposed of. The washed cells are suspended in 10 ml of neutral phosphate buffer until they are prepared for extraction. Then, the washed cells are treated under cold conditions with a sonic device for 20 seconds in order to break up the cells. However, denaturation of the proteins must be avoided as far as possible. Following the acoustic irradiation, the remains of the cells are centrifuged at 30,000 rpm (± about 220 000 G's) for 30 minutes. The supernatant product is decanted. 10 ml of buffer solution are employed for washing the remaining cell residues. Another acoustic irradiation and centrifugation are performed, and the supernatant products
are combined. This method is repeated once. The combined supernatant product is prevaporized in order to reduce the volume from about 30 ml to about 6 to 7 ml. A corresponding portion is removed for the total protein analysis.

By means of a liquid column chromatograph, and with filtering through Millipore Filters, the preparation is than made free of contaminants. Details of the respective process may be gathered from Example 2 of the laid-open German patent application 2,666,257. A polypeptide having a molecular weight of about 10,000 may be obtained. An antigen solution is prepared having a concentration of about 100 mg (milligrams) of the antigen per liter.

The experiments according to Examples 1 and 2 are carried out with the artificially produced antigen. It is found that the artificially produced antigen provides the same effect as the one obtained from body tissue.

Example 4

Use of an organo-specific antigen.
A) The production of the leucocyte suspension is effected in the same manner as in Example 1 - A.

B) Preparation of an organo-specific antigen solution.

Preferably fresh tumour tissue of a mammary carcinoma is cleaned of blood, fatty tissue and binding tissue, and cut into small cubical pieces or strips. All of the subsequent method steps are performed at a temperature of 4°C. The tissue pieces are disintegrated to pieces of about the size of peas in a threefold volume of a 3 molar potassium chloride solution. The pH of this coarse suspension is adjusted to 7.4. Thereafter, the tissue is disintegrated in an Ultra-turrax (Trademark) homogenizer (20,000 rpm). The homogenisate is allowed to stand for 24 hours. Then, the homogenisate is centrifuged. The supernatant phase obtained after 60 minutes of centrifugation at 4000 G's is dialyzed for 2 hours against a tenfold volume of aqua destillata, and thereafter dialyzed for further 24 hours against a 50-fold volume of 0.9 NaCl solution. The proteins are precipitated from the dialysate by adding 2 molar ammonium sulfate during a period of 1 hours, and then separated by centrifugation for 20 minutes at 4,000 G's. Upon suspending the proteins
in a 0.9 % NaCl solution, a dialysis against the five-fold volume of aqua destillata for one hour, and a further dialysis against the 50-fold volume of 0.9 % NaCl are carried out. Following a sterile filtering step through a 0.45 Millipore Micropore filter, the antigen extract is filled into small ampules and stored at \(-20^\circ\) C. Prior to use, the protein content of every charge is determined by the Biuret method after a preceding TCA precipitation.

C) The same test plate as in Example 1 is employed.

Three compositions are prepared for the test, as follows:

1) Each containing 100 microliters of leucocyte suspension from patient blood according to Example 1 - A, + 8 microliters of N/100 NaCl solution (for column A, standard value).

2) Each containing 100 microliters of leucocyte suspension + 8 microliters of antigen suspension according to Example 4 - B.

3) Each containing 100 microliters of leucocyte suspension + 8 microliters of antigen suspension
according to Example 4 - B + 15 microliters of transfer factor solution.

The test plate having a hole diameter of 2,5 mm is loaded with 8 microliters each of the test substance 1 to 3 incubated in accordance with Example 1. The results, each representing the average of five measurements, are as follows:

<table>
<thead>
<tr>
<th>Composit.</th>
<th>Column</th>
<th>Diameter</th>
<th>F (mm²)</th>
<th>MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>7,70</td>
<td>46,5</td>
<td>1,0</td>
</tr>
<tr>
<td>10</td>
<td>C</td>
<td>7,00</td>
<td>38,1</td>
<td>0,82</td>
</tr>
<tr>
<td>3</td>
<td>D</td>
<td>6,10</td>
<td>29,3</td>
<td>0,63</td>
</tr>
</tbody>
</table>

The result indicates a mammary carcinoma since the MI is clearly below 0.85 and the result becomes still more prominent when the transfer factor is employed.
Further examinations show that the results in the case of female patients, as explained in Example 1, vary still more significantly. Female patients affected by mammary carcinoma, when the method is applied with the aid of the transfer factor, for more than 90% show a migration index smaller than 0.80. It is only in 10% of the patients affected by mammary carcinoma that the migration index is above 0.85.

Example 5

The mobility change of lymphocytes, foreign macrophages and other indicator cells is determined in a capillary test according to SØBORG and BENDIXEN. To this end, an open capillary of 10 microns thickness is used. The results correspond to those according to Examples 1 and 2, wherein principally the same preparations or compositions are used.

Concluding remarks

The testing method cannot be applied to certain aspects of case. Incorrect by negative results were obtained in each case for so-called leucoses. At present,
this phenomenon cannot be explained because it is just leucemic cells that contain plenty of basic proteins. Also, the cellular immunity of such patients is not affected so remarkably that this result could be explained with an affection of the immune system. Thus, it is also striking that the chronic lymphatic leukemia, same as the acute lymphatic leukemia, fail to yield a reaction whereas lymphosarcomae and lymphogranulomatoses show the same reaction as other malignomae. Prior to the chemotherapy, however, the patients showed a distinct inhibition. This inhibition is eliminated, however, already after the first chemotherapy cycle, and converted into a incorrectly positive value in the second cycle. Further, wrong results are obtained from patients in tumour cachexy. Patients with rapidly progresively growing tumours, or patients in the tumour cachexy exhibit wrong results in part, as the cellular immunity is affected in such instances. Experiments to clear up this question are still being carried out.

Incorrectly positive results were obtained in first line in the case of inflammatory and degenerative neurologic diseases, for instance multiple sclerosis. The reason was seen by Caspary and Field in a cross
reaction between the EF and the sensibilization of the lymphocates by brain tissue.

However, wrong results may be easily eliminated by pre-examination such that the abovementioned diseases do not represent any impediment to using the test.
THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An *in vitro* testing method for the diagnosis of malignant tumours, preferably human tumours, comprising determining the mobility variation of cellular blood components contacted with a migration inhibition factor (MIF), wherein the MIF is secreted from lymphocytes being sensitized against tumour tissue, characterized by subjecting, as said cellular blood components, the lymphocytes as a part of a leucocyte suspension to be examined proper to a known per se migration inhibition test wherein the lymphocytes are contacted with an antigen capable of stimulating them to produce MIF, and inhibiting and fixing the leucocyte migration after a predetermined period of time.

2. The testing method according to claim 1, characterized by measuring the rate of migration of the leucocytes in a capillary intermediate layer having a thickness of from 1 to 20 microns and being at least partially filled (saturated) with liquid.

3. The testing method according to claim 2, characterized in that said intermediate layer is bounded,
on the one hand, by an inert medium (e. g. glass) and, on the other hand, by an agar layer saturated with nutrients.

4. The testing method according to any one of claims 1 to 3, characterized by performing the test with a sample including leucocyte-rich supernatant phase ("buffycoat") obtained from human blood and having a fixed number of leucocytes of between $10^5$ and $10^7$ per sample.

5. The testing method according to claim 4, characterized by adding to said sample an antigen solution according to CASPARY and FIELD or a 3 M KCl extract of tumour tissue according to MELTZER in a ratio (volume sample to antigen solution) of from 1 : 50 to 1 : 1/9.

6. The testing method according to claim 5, characterized by adding an organo-specific antigen solution to said sample.

7. The testing method according to claim 4, characterized by adding to said sample an antigen solution obtained from tumour cells grown in vitro, said
solution containing between 50 and 200 mg of tumour antigen per 1000 ml of a suitable aqueous solvent.

8. The testing method according to any one of claims 1 zu 7, characterized by further adding to said sample, in addition to said antigen solution, a transfer factor solution.

9. The testing method according to claim 8, characterized in that the number of leucocytes from which the added transfer factor quantity or volume is obtained, has a ratio of from 1 : 1 to 1 : 100 to the number of leucocytes present in said sample.

10. The testing method according to any one of the preceding claims, characterized in that the incubation period in the migration inhibition test is between 10 and 30 hours, at a temperature of from 35 to 37.5°C and a relative humidity of from 90 to 100%.

11. A test plate for carrying out the method according to any one of claims 1 to 10, characterized by a planar carrier plate or dish of an inert material (e.g. glass) having applied thereon a
solidified agar layer of a thickness of from 2 to 5 mm, a capillary migration layer being between surface of the plate or dish and agar layer, said migration layer having a thickness of between 1 - 20 μm, and said agar layer being provided with at least two substantially circular punched holes (11) having a diameter of from 0.5 to 5 mm, preferably 2.5 mm.

12. The test plate according to claim 11, characterized in that said punched holes are cut such that uniformity of the capillary migration layer exists even in the region of the transition from the respective punched hole to said capillary layer.

13. An in vitro testing method substantially as hereinbefore described.

14. A test plate substantially as described with reference to the accompanying drawings.

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R & Z VERMÖGENSVERWALTUNGSGESELLSCHAFT MBH
By its Patent Attorneys:
SANDERCOCK, SMITH & BEADLE