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(54) A DEVICE FOR USE IN IMMUNOLOGICAL DETERMINATIONS

(71) We, F. HOFFMANN-LA ROCHE & CO., AKTIENGESELLSCHAFT, a Swiss Company of 124—184 Grenzacherstrasse, Basle, Switzerland, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to a device for use in immunological determinations. More particularly, the invention is concerned with a process for applying an immunological material on to a test slide in a self-adhering form. Furthermore, the invention is concerned with a device comprising a test slide treated in this manner and with diagnostic methods using said device.

Diagnostic procedures in humans and animals are making use of immunological principles to an increasing extent. These reactions are used to detect antibodies or antigens in the body fluids of the organism. An antigen is an extraneous substance which, when applied to the organism, activates the formation of certain soluble or cellular substances denoted as antibodies. Any substance, such as for example a protein which is not normally present in a certain organism, can cause the formation of antibodies when it infiltrates into or is applied to an organism under suitable conditions.

The antibodies formed can react in vitro and in vivo with the antigens, whereby they can carry out a protective function in the case of an infection.

Immunological test procedures are based on this antigen-antibody reaction, the presence of an antigen or an antibody being confirmed or determined by bringing the appropriate antibody or the appropriate antigen into contact with a body fluid of the organism, mainly blood serum, blood plasma, urine or a specially treated blood extract. However, other body fluids can also be used. In the presence of the appropriate antibody or antigen in the body liquid of the organism, there is formed an antigen-antibody complex which is made visible, for example, by colour change, precipitation or agglutination, or which can be

determined by radio-activity or fluorescence measurement.

In such procedures, the often sensitive, immunologically reactive materials have to be stored and applied to a test slide. Thus, for example, suspensions of these materials can be used, but these suspensions suffer from the disadvantage that they have a limited stability and must be applied on to the test slide which is expensive for the user. Such suspensions have to be stored, namely in a refrigerator, in use have to be applied on to the test slide using a micropipette or an inoculation loop and have to be subsequently dried. When lyophilised suspensions are used, the stability is substantially improved, but the disadvantage of the time-consuming application on to the test slide is not overcome.

In order to eliminate this latter disadvantage, an attempt has been made to provide the user with a test slide having already dried streaks of an immunologically reactive material. These test slides have the advantage that they are ready-for-use after simple thawing of the streaks. However, such test slides have to be stored at -20°C since otherwise the stability is extremely slight.

In accordance with the present invention, there has been found a process which makes it possible to apply immunologically reactive materials (in a subsequently lyophilised and self-adhering form) to a test slide, the advantages of the lyophilised suspension with respect to stability being combined with the advantages of the dried streaks with respect to simplified and time-saving preparation and handling.

More particularly, the present invention is concerned in one of its aspects with a process for preparing a device comprising an immunologically reactive material applied to a test slide, the immunologically reactive material being in a self-adhering and lyophilised form and said material adhering to the test slide even after several washings with an aqueous solution, wherein a suspension of the immunologically reactive material is applied to the test slide, dried and subsequently lyophilised.

Further, in another of its aspects, the pre-

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sent invention is concerned with a device for immunological determinations, said device comprising a test slide having applied, immunologically reactive material in a lyophilised and self-adhering form.

The expression "immunologically reactive materials" relates to materials as, for example, proteins, peptides, polysaccharides, etc which are of decisive significance for an immunological determination, i.e. the presence of these materials is the determining factor in the immunological test procedure. These materials can be detected in the body fluids of humans and animals using immunological principles or can serve for their detection.

Especially suitable immunologically reactive materials are pathogenic and facultatively pathogenic organisms such as, for example, parasites, protozoa, bacteria or viruses or their immunologically active components, isolated antibodies from humans and animals, serum constituents, toxins, hormones, enzymes, alkaloids cell and tissue extracts, substances with a small molecular weight such as, for example, insulin, angiotensin and urokinase, biogenic amines, blood cells, particles chemically or physically covered with antigens or antibodies, such as, for example, erythrocytes or latex particles.

Particularly suitable immunologically reactive materials for use in the present invention are *Toxoplasma gondii*, *Trypanosoma cruzi* and *Entamoeba histolytica*.

All types of test slides are suitable in accordance with the present invention; for example, test slides (e.g. in the form of films) which are used in the medicinal field or in laboratories. The test slides can consist of any suitable material such as, for example, glass or other synthetic material and are advantageously transparent. Especially preferred test slides are test slides having at least one depression for receiving the immunologically reactive material.

In a preferred embodiment, the test slide is a slide which has corresponding depressions on both the top-facing and bottom-facing surfaces. The depressions on the top-facing surface serve to receive the immunologically reactive material, while the depressions on the bottom-facing surface serve to protect the surface from mechanical damage such as, for example, scratches, which impair the optical properties. The test slide advantageously has a writing area at one end.

The test slide of the preferred embodiment contains several depressions and accordingly enables the qualitative examination of several sera or the quantitative examination of several serial dilutions of a serum. In addition, this test slide is suitable for the application of several different immunologically reactive materials and accordingly for the simultaneous determination of various antigens or antibodies in a test sample.

In accordance with the process provided by the present invention, the immunologically reactive material is suspended in a suitable liquid, applied to the test slide, dried and lyophilised.

Especially suitable as the suspension liquid is an aqueous solution such as, for example, a saccharose or sodium chloride solution. The method of application is not critical, but the suspension is preferably applied dropwise to the test slide. The drying temperature depends on the composition of the applied suspension and the sensitivity of the reagents. However, the drying is advantageously carried out at a temperature between 0°C and 100°C, preferably between 20°C and 40°C. The drying time depends on the sedimentation rate of the suspended material and the temperature, but it usually amounts to at least 5 minutes. The lyophilization is carried out in the usual manner in a conventional freeze dryer. The coated test slide manufactured in this manner can be stored at temperatures above 0°C and accordingly does not require freeze preservation.

The present invention can be used in any diagnostic method where an immunologically reactive material is applied to a test slide in a lyophilised form. However, the test slide in accordance with the present invention is advantageously used in the form of a test slide with lyophilised antigens in an immunofluorescence method. An indirect immunofluorescence method is especially preferred.

For the specific detection of antibodies which are formed in the human or animal organism against exogenous substances (infection defence) or endogenous substances (auto-aggression), the indirect immunofluorescence method has, in fact, proved to be especially good. In such a method, in the first step a test slide having applied antigens is contacted with the corresponding body fluid, incubated and subsequently washed. In this manner, a gammaglobulin (antibody) homologous with the antigen can react with the antigen and accordingly remain attached to the test slide. When such antibodies are present in the sample under examination, in a second step their binding to the antigen is detected with anti-human-globulin which is provided with a fluorescent colouring material. For this purpose, the marked antihuman-globulin is added to the test slide which is subsequently incubated and rinsed. The marked antihuman-globulin reacts with the modified gammaglobulin (antibody) which is bound to the antigen, such that the fluorescent colouring material remains attached to the test slide and the corresponding fluorescence can be detected microscopically. The existence of a fluorescence signifies that antibodies which correspond to the antigens applied to the test slide are present in the sample under examination and thus enables the diagnosis of diseases

which correspond to such antibodies or antigens.

The following Table provides a selection of typical diseases or conditions which can be

determined with the aid of the test slide in accordance with the invention according to the immunologically reactive materials lyophilised on to the test slide.

TABLE

Antigen	Disease
Toxoplasma gondii	Toxoplasmosis
Entamoeba histolytica	Amoebiasis
Trypanosoma cruzi	Chagas
Trypanosoma gambiense/rhodesiense	Sleeping sickness
Leishmania donovani	Leishmaniasis
Schistosoma mansoni	Schistosomiasis
Echinococcus granulosus	Echinococcosis
Filariae	Filariasis
Fasciola hepatica	Fascioliasis
Plasmodia	Malaria
Candida species	Candidiasis
Aspergilli	Aspergillosis
Mycropolyspora faeni/Micromonospora vulgaris	Farmer's lung
Treponema pallidum	Syphilis
Neisseria gonorrhoeae	Gonorrhea
Neisseria meningitidis	Meningitis
Brucella abortus	Brucellosis
Mycoplasma pneumoniae	Pneumonia
Australia antigen	Acute hepatitis
Herpes simplex virus	Herpes simplex
Influenza virus	Flu
Cell nuclei	Systemic lupus erythematosus or Scleroderma
Cryptococci	Cryptococcosis
Torulopsis species	Systemic mycosis

The following Examples illustrate the present invention:

Example 1

0.03 ml of a suspension of *Toxoplasma gondii* (6 million/ml) in a 2.5 per cent aqueous solution of saccharose is added dropwise to each depression of a 1 mm thick polystyrene test slide (76 mm×26 mm) having 8 numbered depressions (diameter 6 mm, depth 0.25 mm) on the top surface and corresponding depressions on the bottom surfaces, as well as a writing area. (The *Toxoplasmae* were prepared according to A. Betz, Bull. World Hlth. Org. 39, 1968 p. 367—374). The test slide coated in this manner is incubated at 37°C for 30 minutes in a drying cupboard with air circulation. Subsequently, the suspension is frozen on to the test slide using dry ice and then lyophilised in a freeze dryer. There is obtained a ready-for-use test slide having self-adhering *Toxoplasmae* which are not detached upon rinsing with water.

Example 2

0.03 ml of a suspension of *Entamoeba histolytica* (5,000/ml) in a physiological 0.85 per cent sodium chloride solution with 2 per cent "Carbowax" is added dropwise to each depression of a 1 mm thick polystyrene test

slide (76 mm×26 mm) having 8 numbered depressions (diameter 6 mm, depth 0.25 mm) on the top surface and corresponding depressions on the bottom surface, as well as a writing area. (The amoebae were prepared according to Morris Goldman, American J. Trop. Med. Hyg. Vol 15 No. 5, 1966 p. 694—700). The test slide coated in this manner is incubated at 37°C for 30 minutes in a drying cupboard with air circulation. Subsequently, the suspension is frozen on to the test slide using dry ice and then lyophilised in a freeze dryer. There is obtained a ready-for-use test slide having self-adhering amoebae which are not detached upon rinsing with water.

Example 3

A serial dilution of a patient's serum is prepared (1/50, 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200 and 1/6400). One drop of each of the dilutions of serum is applied by means of a pipette to each depression of the test slide prepared as described in Example 1. The test slide is then incubated for 0.5 hour at 37°C in a moist chamber and subsequently washed with a buffered sodium chloride solution (pH 7.2). One drop of a commercially available suspension of antihuman-globulin marked with fluorescein isothiocyanate is applied to each depression using a

5 pipette. The test slide is then incubated for
10 0.5 hour at 37°C in a moist chamber and
subsequently washed with a buffered sodium
chloride solution (pH 7.2). The test slide is
then rinsed with distilled water and dried with
a filter paper. One drop of a commercially
available immersion oil is added to each depres-
sion and the fluorescence is determined by
means of an immersion objective in a fluores-
cence microscope. The maximum dilution in
which the fluorescence can still be determined
gives an indication of the relative concentra-
tion of the *Toxoplasma* antibodies.

Example 4

15 Serum samples from 8 different patients
are each diluted five-fold with a buffered
sodium chloride solution (pH 7.2) and num-
bered from 1 to 8. One drop of each of these
20 numbered, diluted serum samples is applied
by means of a pipette to each of the depres-
sions, numbered from 1 to 8, on the test slide
prepared according to Example 1. The test
slide is incubated for 0.5 hour at 37°C in a
25 moist chamber and subsequently washed with
a buffered sodium chloride solution (pH 7.2).
One drop of a commercially available suspen-
sion of antihuman-globulin marked with
fluorescein isothiocyanate is applied to each
30 depression using a pipette. The test slide is
then incubated for 0.5 hour at 37°C in a
moist chamber and subsequently washed with
a buffered sodium chloride solution (pH 7.2).
The test slide is then rinsed with distilled
35 water and dried with a filter paper. One drop
of a commercially available immersion oil is
added to each depression and the fluorescence
is determined by means of an immersion objec-
tive in a fluorescence microscope. Those
40 depressions in which a fluorescence is deter-
mined correspond to serum samples of patients
who have been infected with *Toxoplasmae*.

Example 5

45 A serial dilution of a patient's serum was
prepared (1/50, 1/100, 1/200, 1/400, 1/800,
1/1600, 1/3200 and 1/6400). One drop of
each of the dilutions of serum is applied by
means of a pipette to each depression of the
test slide prepared according to Example 2.
50 The test slide is then incubated for 0.5 hour
at 37°C in a moist chamber and subsequently
washed with a buffered sodium chloride solu-
tion (pH 7.2). One drop of a commercially
available suspension of antihuman-globulin
marked with fluorescein isothiocyanate is
55 applied using a pipette to each depression. The
test slide is then incubated for 0.5 hour at
37°C in a moist chamber and subsequently
washed with a buffered sodium chloride solu-
tion (pH 7.2). The test slide is then rinsed
60 with distilled water and dried with a filter
paper. One drop of a commercially available
immersion oil is added to each depression and
the fluorescence is determined by means of an

immersion objective in a fluorescence micro-
scope. The maximum dilution in which the
fluorescence can still be determined gives an
indication of the relative concentration of the
Entamoeba histolytica antibodies.

Example 6

70 Serum samples of 8 different patients are
each diluted five-fold with a buffered sodium
chloride solution (pH 7.2) and numbered from
1 to 8. One drop of each of these numbered,
diluted serum samples is applied by means of
a pipette to each of the depressions, num-
bered from 1 to 8, on the test slide prepared
as described in Example 2. The test slide is
then incubated for 0.5 hour at 37°C in a
moist chamber and subsequently washed with
a buffered sodium chloride solution (pH 7.2).
80 One drop of a commercially available suspen-
sion of antihuman-globulin marked with
fluorescein isothiocyanate is applied by means
of a pipette to each depression. The test slide
is then incubated for 0.5 hour at 37°C in a
moist chamber and subsequently washed with
a buffered sodium chloride solution (pH 7.2).
The test slide is then rinsed with distilled
water and dried with a filter paper. One drop
of a commercially available immersion oil is
added to each depression and the fluorescence
is determined using an immersion objective
85 in a fluorescence microscope. Those depres-
sions in which a fluorescence is determined
correspond to serum samples of patients who
have been infected with amoebae.

Example 7

0.03 ml of a suspension of *Trypanosoma*
cruzi (2 million/ml) in a 2.5 per cent aqueous
solution of saccharose is added dropwise to
each depression of a 1 mm thick polystyrene
test slide (76 mm×26 mm) having 8 num-
bered depressions (diameter 6 mm, depth 0.25
mm) on the top surface and corresponding
depressions on the bottom surface, as well as
a writing area. (The *Trypanosomae* were pre-
pared according to Allain D.S. and Kayan G.,
The Journal of Parasitology, Vol 60, No. 1
February 1974 pages 179—184). The test
slide coated in this manner is incubated at
37°C for 30 minutes in a drying cupboard
with air circulation. Subsequently, the suspen-
sion is frozen on to the test slide using dry
ice and then lyophilised in a freeze dryer.
There is obtained a ready-for-use test slide
with self-adhering trypanosomae which are
not detached upon rinsing with water.

Example 8

120 A serial dilution of a patient's serum is
prepared (1/50, 1/100, 1/200, 1/400, 1/800,
1/1600, 1/3200 and 1/6400). One drop of
each of the diluted serum samples is applied
by means of a pipette to each depression of
the test slide prepared as described in Example
7. The test slide is then incubated for 0.5

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hour at 37°C in a moist chamber and subsequently washed with a buffered sodium chloride solution (pH 7.2). One drop of a commercially available suspension of antihuman-globulin marked with fluorescein isothiocyanate is applied to each depression by means of a pipette. The test slide is then incubated for 0.5 hour at 37°C in a moist chamber and subsequently washed with a buffered sodium chloride solution (pH 7.2). The test slide is then rinsed with distilled water and dried with a filter paper. One drop of a commercially available immersion oil is added to each depression and the fluorescence is determined by means of an immersion objective in a fluorescence microscope. The maximum dilution in which the fluorescence can still be determined gives an indication of the relative concentration of the *Trypanosoma cruzi* antibodies.

Example 9

Serum samples of 8 different patients are each diluted five-fold with a buffered sodium chloride solution (pH 7.2) and numbered from 1 to 8. One drop of each of these numbered, diluted serum samples is applied by means of a pipette to each of the depressions, numbered from 1 to 8, on the test slide prepared as described in Example 7. The test slide is incubated for 0.5 hour at 37°C in a moist chamber and subsequently washed with a buffered sodium chloride solution (pH 7.2). One drop of a commercially available suspension of antihuman-globulin marked with fluorescein isothiocyanate is applied by means of a pipette to each depression. The test slide is then incubated for 0.5 hour at 37°C in a moist chamber and subsequently washed with a buffered sodium chloride solution (pH 7.2). The test slide is then rinsed with distilled water and dried with a filter paper. One drop of a commercially available immersion oil is added to each depression and the fluorescence is determined by means of an immersion objective in a fluorescence microscope. Those depressions in which a fluorescence is determined correspond to serum samples of patients who have been infected with *Trypanosoma cruzi*.

The word "Carbowax" used herein is a Trade Mark.

WHAT WE CLAIM IS:—

1. A device comprising, immunologically reactive material applied to a test slide, the immunologically reactive material being in a lyophilised and self-adhering form and said material adhering to the test slide even after several washings with aqueous solution.

2. A device according to claim 1, in which the test slide has one or more depressions.

3. A device according to claim 2, in which depressions are present on the top-facing surface and also on the bottom-facing surface of the test slide.

4. A device according to claim 1, in which the test slide is in the form of a film.

5. A device according to any one of claims 1 to 4 inclusive, in which the test slide is of synthetic material other than glass.

6. A device according to any one of claims 1 to 3 inclusive, in which the test slide is of glass.

7. A device according to any one of claims 1 to 6 inclusive, in which the immunologically reactive material comprises antigen.

8. A device according to any one of claims 1 to 6 inclusive, in which the immunologically reactive material comprises antibody.

9. A device according to any one of claims 1 to 6 inclusive, in which the immunologically reactive material consists of protein.

10. A device according to any one of claims 1 to 6 inclusive, in which the immunologically reactive material consists of peptide.

11. A device according to any one of claims 1 to 6 inclusive, in which the immunologically reactive material consists of polysaccharide.

12. A device according to any one of claims 1 to 6 inclusive, in which the immunologically reactive material consists of *Toxoplasma*.

13. A device according to any one of claims 1 to 6 inclusive, in which the immunologically reactive material consists of amoebae.

14. A device according to any one of claims 1 to 6 inclusive, in which the immunologically reactive material consists of *Trypanosoma*.

15. A process for the manufacture of a device as set forth in any one of claims 1 to 14 inclusive, wherein a suspension of the immunologically reactive material is applied to the test slide, dried and subsequently lyophilised.

16. A process according to claim 15, wherein an aqueous saccharose solution is used as the suspension liquid.

17. A process according to claim 15, wherein a sodium chloride solution is used as the suspension liquid.

18. A process according to any one of claims 15 to 17 inclusive, wherein the drying is carried out at a temperature between 20°C and 40°C.

19. A process according to any one of claims 15 to 18 inclusive, wherein the drying is carried out for at least 5 minutes.

20. A process for the manufacture of a device as set forth in claim 1, substantially as

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hour at 37°C in a moist chamber and subsequently washed with a buffered sodium chloride solution (pH 7.2). One drop of a commercially available suspension of antihuman-globulin marked with fluorescein isothiocyanate is applied to each depression by means of a pipette. The test slide is then incubated for 0.5 hour at 37°C in a moist chamber and subsequently washed with a buffered sodium chloride solution (pH 7.2). The test slide is then rinsed with distilled water and dried with a filter paper. One drop of a commercially available immersion oil is added to each depression and the fluorescence is determined by means of an immersion objective in a fluorescence microscope. The maximum dilution in which the fluorescence can still be determined gives an indication of the relative concentration of the *Trypanosoma cruzi* antibodies.

Example 9

Serum samples of 8 different patients are each diluted five-fold with a buffered sodium chloride solution (pH 7.2) and numbered from 1 to 8. One drop of each of these numbered, diluted serum samples is applied by means of a pipette to each of the depressions, numbered from 1 to 8, on the test slide prepared as described in Example 7. The test slide is incubated for 0.5 hour at 37°C in a moist chamber and subsequently washed with a buffered sodium chloride solution (pH 7.2). One drop of a commercially available suspension of antihuman-globulin marked with fluorescein isothiocyanate is applied by means of a pipette to each depression. The test slide is then incubated for 0.5 hour at 37°C in a moist chamber and subsequently washed with a buffered sodium chloride solution (pH 7.2). The test slide is then rinsed with distilled water and dried with a filter paper. One drop of a commercially available immersion oil is added to each depression and the fluorescence is determined by means of an immersion objective in a fluorescence microscope. Those depressions in which a fluorescence is determined correspond to serum samples of patients who have been infected with *Trypanosoma cruzi*.

The word "Carbowax" used herein is a Trade Mark.

WHAT WE CLAIM IS:—

1. A device comprising, immunologically reactive material applied to a test slide, the immunologically reactive material being in a lyophilised and self-adhering form and said material adhering to the test slide even after several washings with aqueous solution.

2. A device according to claim 1, in which the test slide has one or more depressions.

3. A device according to claim 2, in which depressions are present on the top-facing surface and also on the bottom-facing surface of the test slide.

4. A device according to claim 1, in which the test slide is in the form of a film.

5. A device according to any one of claims 1 to 4 inclusive, in which the test slide is of synthetic material other than glass.

6. A device according to any one of claims 1 to 3 inclusive, in which the test slide is of glass.

7. A device according to any one of claims 1 to 6 inclusive, in which the immunologically reactive material comprises antigen.

8. A device according to any one of claims 1 to 6 inclusive, in which the immunologically reactive material comprises antibody.

9. A device according to any one of claims 1 to 6 inclusive, in which the immunologically reactive material consists of protein.

10. A device according to any one of claims 1 to 6 inclusive, in which the immunologically reactive material consists of peptide.

11. A device according to any one of claims 1 to 6 inclusive, in which the immunologically reactive material consists of polysaccharide.

12. A device according to any one of claims 1 to 6 inclusive, in which the immunologically reactive material consists of *Toxoplasmae*.

13. A device according to any one of claims 1 to 6 inclusive, in which the immunologically reactive material consists of amoebae.

14. A device according to any one of claims 1 to 6 inclusive, in which the immunologically reactive material consists of *Trypanosomae*.

15. A process for the manufacture of a device as set forth in any one of claims 1 to 14 inclusive, wherein a suspension of the immunologically reactive material is applied to the test slide, dried and subsequently lyophilised.

16. A process according to claim 15, wherein an aqueous saccharose solution is used as the suspension liquid.

17. A process according to claim 15, wherein a sodium chloride solution is used as the suspension liquid.

18. A process according to any one of claims 15 to 17 inclusive, wherein the drying is carried out at a temperature between 20°C and 40°C.

19. A process according to any one of claims 15 to 18 inclusive, wherein the drying is carried out for at least 5 minutes.

20. A process for the manufacture of a device as set forth in claim 1, substantially as



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hereinbefore described with reference to
Example 1, Example 2 or Example 7.

- 5 21. A device as set forth in claim 1, when
manufactured by the process claimed in any
one of claims 15 to 20 inclusive.

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