

DAKAR VACCINE

PREPARATION OF YELLOW FEVER VACCINE AT THE INSTITUT PASTEUR, DAKAR

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One of the first strains of yellow fever virus, whose experimental transmission to a receptive animal opened the way to the important advances made in the study of the disease, was isolated at the Institut Pasteur at Dakar by Mathis, Sellards & Laigret² during the yellow fever epidemic which broke out in Senegal in 1927. This virus had been transmitted to *Macacus rhesus* monkeys by the bites of *Aedes aegypti* fed 24 and 31 days earlier on a patient suffering from yellow fever. Early in 1928 the virulent organs from one of the monkeys infected in this way were transported in a frozen state to Europe and America, where they were placed at the disposal of various laboratories under the name of "French strain".

Theiler,⁹ studying the properties of this strain, showed in 1930 that the white mouse was susceptible to intracerebral inoculation, which induced a yellow fever encephalitis capable of being transmitted in series. After a certain number of passages in this animal, the properties of the yellow fever virus became modified so that when inoculated subcutaneously it no longer caused the death of the monkey but brought about a mild or asymptomatic illness followed by lasting immunity.

The idea then arose of using this modified yellow fever virus for human vaccination. In 1931, Sawyer, Kitchen & Lloyd⁷ carried out the first trials on human beings by simultaneous injection of a suspension of the French strain (105th to 176th mouse passage) and a certain quantity of human immune serum.

The following year, Sellards & Laigret⁸ suggested a method involving the subcutaneous inoculation of the modified French strain alone, without immune serum.

As soon as he had established a technique for this method, Laigret applied it for the first time in French West Africa.¹ The procedure advocated consisted of three successive injections, at intervals of 20 days, of

virus suspensions whose activity had been reduced by exposure for decreasing lengths of time to laboratory temperature.

In 1935, Nicolle & Laigret³ simplified the method by reducing it to a single injection of virus exposed for 24 hours to a temperature of 20°C and coated with a layer of egg yolk.

From 1934 to 1939, 20,000 persons were vaccinated in French West Africa by one or the other of these two methods.

After showing that yellow fever virus maintained in the brain of the white mouse was able to enter the human or receptive animal organism through slight scratches in the skin and then to multiply and bring about the appearance of antibodies in the same way as following subcutaneous injection of the virus, Peltier et al.⁴ applied this form of inoculation to human yellow fever vaccination. In 1939 they carried out a large-scale experiment on almost 100,000 inhabitants of Senegal, whom they inoculated simultaneously, by scarification of the skin, with yellow fever mouse virus and the vaccine.⁵

Out of a total of 1,387 subjects followed up and whose serum had shown no yellow fever antibodies before vaccination, 96.3% gave a positive sero-protection test a month after inoculation.

In 1940 and 1941, this scarification technique was used on 1,910,000 persons in various territories of French West Africa, and, on 10 December 1941, the public authorities decided to make it compulsory for the whole population of these territories. The UNRRA Standing Technical Committee on Health in 1946 (see page 117), and the World Health Organization in 1948,¹⁰ recognized the validity of the yellow fever vaccination method developed by the Institut Pasteur at Dakar.

Like the yellow fever vaccine proposed earlier by Laigret, that of the Institut Pasteur at Dakar consists of the dried brain tissue of white mice inoculated with the virus modified by passages in that animal. The only difference is in the method of treatment of the mouse brains. At the time of vaccination, the brain is made into a suspension and inoculated by means of cutaneous scarification. As this method of inoculation introduces only small amounts of antigen into the organism, the virus vaccine is not subjected to any preliminary treatment which might reduce its potency.

Preparation of the Vaccine

Virus

The virus used is derived from the French strain adapted to white mice by Theiler. It was made available to the Institut Pasteur, Paris, by Sellards and brought to Dakar in November 1931, when it was at the

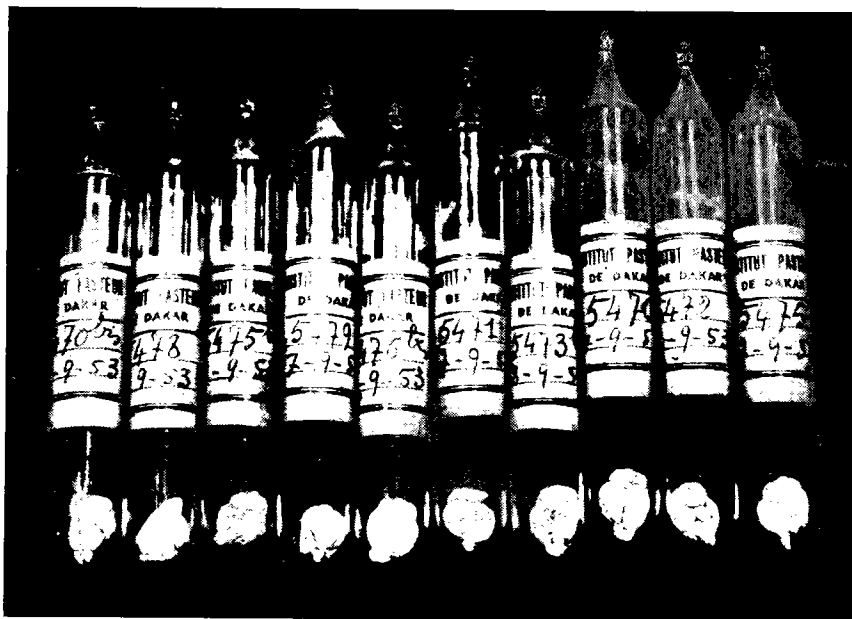
128th passage. From 1931 until the end of 1939 it underwent 121 further mouse passages.

The first trials in inoculating animals and man by the scarification method were carried out with the 237th passage. The method used for the preparation of the vaccine, still in use today with certain changes of detail, was first employed in September 1939, when the virus was at its 249th passage.

Since then, so as to avoid possible variations in the properties of the strain, the frequency of the maintenance passages has been progressively reduced. Thus, in the period between September 1939 and November 1945, nine passages (from the 250th to the 258th) were carried out, and only two (the 259th and 260th) since then. Thus the 258th, 259th, and 260th passages of the virus have been the ones employed during the last eight years in preparing the vaccine.

A large number of brains from these three passages is kept in reserve in the dry state (fig. 1). To husband this reserve of virus, each brain is used to carry out several series of inoculations. At the time of use, one brain is ground up coarsely in a mortar and the fragments obtained are distributed into ampoules, using a measuring spoon containing one tenth of a dried brain, two spoonfuls being placed in each ampoule.

FIG. 1. STORAGE OF YELLOW-FEVER VIRUS IN FROZEN AND DRIED MOUSE BRAINS



The ampoules, which are sealed under vacuum and kept at a temperature of -25°C , are then used as required to inoculate batches of mice intended either for maintenance of the strain or for preparation of vaccine.

When the reserve of virus decreases, the contents of an ampoule from the oldest brain passage are made into a suspension and inoculated into some 50 mice. The brains of the paralysed animals are harvested, immediately frozen, dried at a low temperature, distributed into separate tubes, sealed under vacuum, and stored at -25°C . They constitute a new reserve batch.

Various trials have shown that the average weight of the fresh brains is 0.40 g, which falls to 0.10 g on desiccation.

A 10^{-6} dilution of the virus maintained in this way regularly kills six mice out of six.

Mice

The white mice used are bred in an experimental station established by the Institut Pasteur in 1944 at an isolated site 15 km from the city of Dakar. Animals imported from France were first used for breeding, but in 1947 the stock was entirely renewed from breeders supplied by the Yellow Fever Research Institute at Lagos, Nigeria.

The total number of mice, which includes an average of 2,000 breeders, varies from 6,000 to 8,000. The quantity supplied each year to the laboratories of the Institute varies between 18,000 and 20,000 mice, the animals being used when they have reached the age of $2\frac{1}{2}$ -3 months. Their weight is then approximately 17 g.

No variation has been found at Dakar in the behaviour of mice inoculated with the French strain. Paralysis regularly appears on the fourth or fifth day, and death ensues within the following 24 hours.

Inoculation

On three mornings a week, a batch of one hundred mice is inoculated in order to meet the demand for vaccine.

Ten glass jars are prepared in advance. Each of them is provided with sufficient grain to feed the animals, and given a label indicating the passage number, the number of the corresponding experiment record-sheet, and the date of inoculation.

An ampoule containing one fifth of a dried brain is opened and emptied into a mortar. The fragments of nervous tissue are ground up with a pestle, while at the same time 4 ml of isotonic salt solution are added drop by drop.

The animals are presented one after the other by an assistant, who slightly moistens the skull of each mouse with a tampon soaked in iodized alcohol. The inoculation is administered in the right cerebral hemisphere by means of a syringe graduated in hundredths of a millilitre ; each mouse receives 0.03 ml of suspension. As the virus strain regularly kills six mice out of six in a dilution of 10^{-6} and as a 1 : 50 suspension is employed, the quantity injected represents about 20,000 minimum lethal doses (MLD).

As each mouse is inoculated, it is placed in a jar, ten mice to each jar.

Harvesting

The mice are examined daily up to the end of the fifth day after inoculation. All animals dying during the observation period are rejected.

The brains are harvested on the fourth and fifth days, only those from living but paralysed animals being used. During these two days the jars are visited morning and evening. At each visit, mice which are clearly paralysed are transferred to an isolated, air-conditioned room, where the temperature is maintained at 24°C and the relative humidity at 40%-50% (as against 80%-90% elsewhere in the laboratory).

The animals are killed with chloroform one after the other and pinned to a sheet of cork. The operator is provided with a set of dissecting forceps and fine scissors for each mouse, wrapped up separately and sterilized by dry heat.

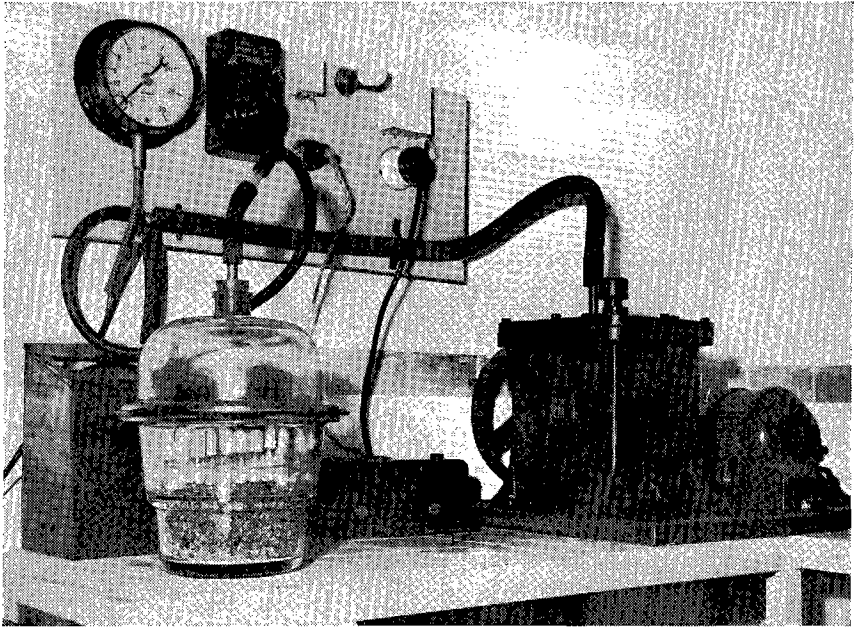
The bared skull is rendered aseptic with a few drops of iodized alcohol. Once the calvaria has been removed, each brain, resting on the flat of the scissors, is introduced into a tube plugged with cotton wool. Each tube is numbered according to the order in which the mice are entered on the corresponding experiment record-sheets, and is immediately placed in a vacuum flask containing ice. The operator then removes a fragment of the medulla oblongata with a platinum loop and seeds it in a tube of broth bearing the same number as the brain from which the sample has been taken.

At the end of each session, the brains harvested are stored in a refrigerator at -25°C, and the tubes of seeded broth are placed in the incubator at 37°C.

Desiccation

On the sixth day after inoculation, all the tubes containing frozen brains from the same batch are quickly placed in a Pyrex desiccator, containing Actigel (silica gel), which has first been chilled to prevent melting (fig. 2

FIG. 2. DRYING OF MOUSE BRAINS



The frozen brains are placed in a chilled desiccator containing silica gel. The desiccator is kept at a temperature of -25°C and connected to a vacuum pump.

and 3). The apparatus is then placed in the refrigerator at -25°C and connected to a rotary-vane pump by means of a rubber tube passing through the wall of the refrigerator. The apparatus is evacuated until the Crookes dark space in a discharge-tube used as a control is 20 mm long, representing a pressure of 0.1 mm Hg. The pump is run for several hours in the morning and evening in order to maintain minimum pressure in the desiccator.

The brains are completely desiccated at the end of three days.

Grinding

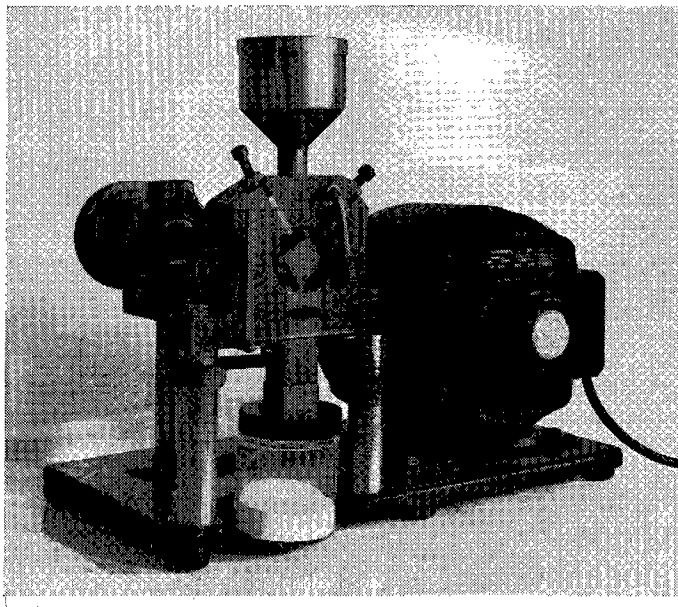
On the morning of the ninth day following inoculation, the tubes of broth seeded on harvesting the brains are inspected and the numbers of those giving a culture noted. The desiccator is then removed from the refrigerator and transferred to the air-conditioned room, where it is opened. The tubes containing the dried brains are then examined, and those bearing a number corresponding to one on the list of contaminated broths are rejected.

FIG. 3. CLOSE-UP OF DESICCATOR



All the other brains are placed together in a mortar. They resemble whitish hazel nuts in appearance and are friable in consistency. They are easily ground with a pestle, and grinding is continued until the powder is quite uniform. (In the near future this operation will be carried out by a Wiley laboratory grinder driven by an electric motor (fig. 4).) A small quantity of the powder is then seeded in broth and spread on agar.

Next, a certain amount of Célite (purified and dehydrated infusorial earth), first filled into tubes holding 0.25 g, is added to the powder. One

FIG. 4. WILEY APPARATUS USED FOR GRINDING DRIED BRAINS

The brains are placed in the hopper. The powder obtained passes through a sieve in the lower part of the grinding chamber and falls into the glass jar.

tube of Célite is required for every three brains. The tubes are emptied into the mortar at regular intervals, while grinding is continued, so as to obtain a uniform mixture. The Célite increases the volume of the powder and reduces any tendency it may have to stick to the glass.

This final operation concludes the preparation of the vaccine. After further seedings in broth and on agar, the powder is transferred to large tubes plugged with cotton wool, which are placed in a vacuum-desiccator at -25°C .

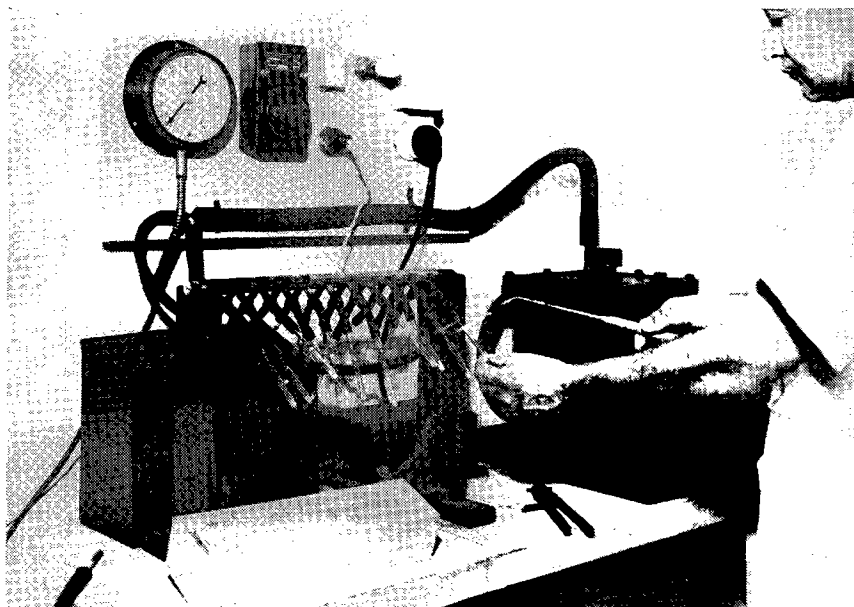
Filling into ampoules

Twenty-four hours later, i.e., on the tenth day after inoculation, the vaccine is dispensed into ampoules. This operation is carried out in the shelter of a Plexiglas cage installed in the air-conditioned room.

One measure of vaccine, corresponding to one tenth of a brain, is introduced into each ampoule. A final check of the culture media is made before the end of dispensing and the ampoules, plugged with cotton

wool, are placed in a vacuum desiccator at a temperature of -25°C . On the following day they are sealed with a blowlamp under vacuum (see fig. 5).

FIG. 5. SEALING VACCINE AMPOULES UNDER VACUUM

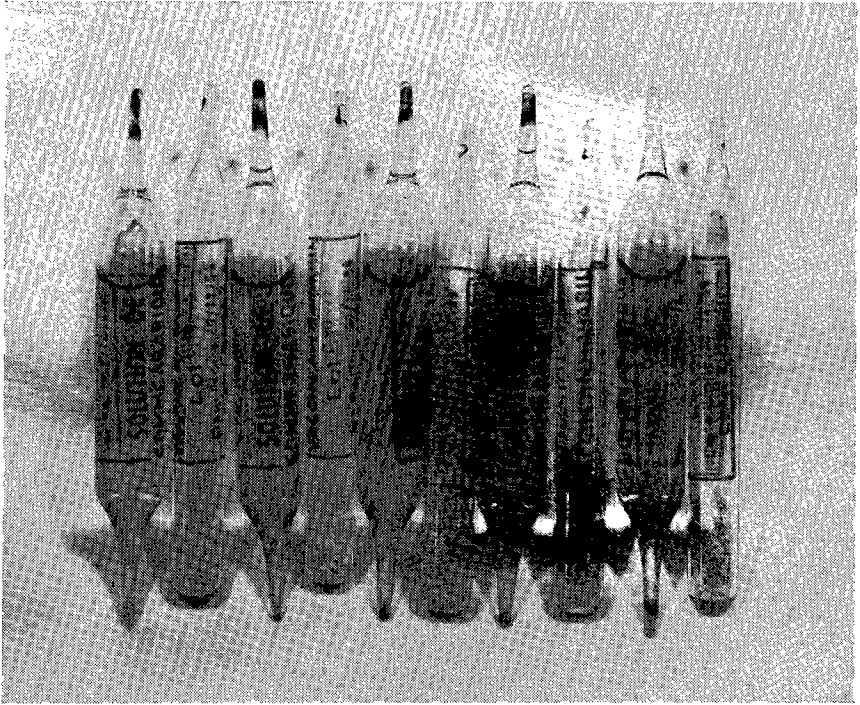


Storage and dispatch

The sealed ampoules are packed in containers bearing the batch number of the vaccine and stored at 4°C (see fig. 6). At the Institut Pasteur itself they can be used for vaccination during a period of one year. Ampoules to be dispatched are passed through an automatic machine which prints the batch number and the expiry date on each. This date is set to ensure that the period of validity of the vaccine does not exceed two months from the day of dispatch.

The vaccine can withstand air temperature for a few days without losing its immunizing properties. It is sent out in ordinary parcels, provided that it does not take more than about ten days to arrive, or by air-mail when its destination is very distant. On receipt, the ampoules of vaccine should again be placed in a refrigerator at a temperature below 5°C and kept there until required for use. The expiry date should not be exceeded if unsatisfactory results are to be avoided.

FIG. 6. AMPOULES OF DRIED VACCINE AND AMPOULES OF GUM ARABIC SOLUTION



Excipient

When wanted for use, the contents of an ampoule of vaccine are suspended in 2 ml of gum arabic solution, prepared as follows :

To 1 kg of powdered Senegal gum weighed out into a glazed dish are added 1,500 ml of tap water. After stirring, the solution is poured into a flask and sterilized for 30 minutes in an autoclave at 105°C.

Two litres of the gum solution are measured out into a 5-litre flask. In another flask, 95.06 g of dry disodium phosphate are dissolved, with gentle warming, in 1,332 ml of distilled water.

The phosphate solution is poured into the gum solution and the two are mixed, sterilized for 20 minutes at 110°C, and then filtered hot through Chardin filter paper.

After cooling, the pH is adjusted to 7.2-7.4 with sodium carbonate solution. The solution is filled into 2-ml ampoules under vacuum in a bell-jar and sterilized by tyndallization.

Vaccine Sterility and Potency Tests

As indicated above, the vaccine undergoes four tests for sterility in the course of its preparation :

- (1) test of each brain removed ;
- (2) test of the powdered brain ;
- (3) test of the brain-Célite powdered mixture ;
- (4) test of the vaccine at the time of filling into ampoules.

Each operation is undertaken only if the preceding test has shown bacterial contamination to be absent.

Occasionally, tests are performed to make sure that the virus strain is pure. For this purpose 1 ml of a 1 : 10 suspension of virus is injected intraperitoneally into each of two guinea-pigs. The animals are kept under observation for 15 days, during which they should not show any appreciable clinical symptoms.

A few ampoules from each batch of vaccine are kept in reserve at the Institut Pasteur for titration and for any tests which may be required. The ampoules intended for titration are kept in the dark at laboratory temperature (an average of 28°C) ; the others are kept at 4°C.

Titration takes place at the beginning of each month, at which time three different batches of vaccine, prepared at intervals of a month, are examined as follows :

- (1) a batch two months old and exposed since then to laboratory temperature (this batch will have already undergone an initial titration immediately after filling into ampoules, and a second titration a month later) ;
- (2) a batch one month old, already titrated immediately after filling into ampoules and kept since then at laboratory temperature ;
- (3) a batch which has just been prepared.

One ampoule from each batch is taken for titration. The ampoule from the first batch is emptied into a mortar and the vaccine is mixed, by stirring with a pestle, with 4 ml of physiological saline containing 10% of normal serum. This amount of vaccine corresponds to one tenth of a brain, i.e., 0.04 g of fresh tissue ; the dilution of the suspension obtained is consequently 1 : 100.

From this suspension 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} dilutions are prepared in physiological saline containing 10% of normal serum. These dilutions are placed in a refrigerator at 4°C until required for the inoculations. The same operations are then repeated with the ampoule of vaccine from each of the other two batches.

When all the dilutions are ready, the inoculations are carried out, 0.03 ml of each of the 10^{-4} , 10^{-5} , and 10^{-6} dilutions being administered intracerebrally to six mice. Titration of each batch of vaccine is thus done on 18 mice, in three jars of six each. The mice are kept under observation for 15 days.

As a general rule, the 10^{-6} dilution of the freshly prepared vaccine kills six mice out of six. Consequently, 0.03 ml of this dilution contains at least one minimum lethal dose (MLD), i.e., 33 MLD per ml.

At the time of vaccination, one ampoule of vaccine (representing 0.04 g of fresh brain) is made into a suspension with 2 ml of gum arabic solution, giving a 1 : 50 dilution. If this vaccine kills six mice out of six in a 10^{-6} dilution, the vaccinal suspension will contain 660,000 MLD per ml. As the 2 ml of suspension serve to vaccinate 100 persons, each separate dose of vaccine contains at least 13,200 MLD.

Appreciably the same results are obtained by using the method of Reed & Muench.⁶ The 2 ml of suspension prepared with one ampoule of vaccine are considered in this case as the unit. They are diluted with 18 ml of physiological saline containing 10% of normal serum. Starting with this 1 : 10 dilution, the successive tenfold dilutions are prepared with 10% of normal serum, up to 10^{-6} , and six mice are inoculated intracerebrally with 0.03 ml of each dilution. The results show that the 50% end-point usually lies between 1 : 20,000 and 1 : 50,000, corresponding to a titre of 660,000 to 1,650,000 MLD per ml of vaccine suspension. Since 2 ml serve to vaccinate 100 persons, each separate dose contains 13,200 to 33,000 LD_{50} .

Titration carried out on ampoules kept at 4°C give similar results. After a year, no appreciable fall in potency is observed, the vaccine always containing at least 13,200 MLD per dose of vaccine.

On the other hand, tests carried out with batches kept at 28°C indicate a small but gradual decrease in the potency of the vaccine. This decrease is not appreciable during the first three weeks, whereas at the end of a month the 50% end-point may fall to 1 : 10,000; the titre of the vaccine is then 330,000 MLD, or 6,600 LD_{50} per dose. The fall in activity subsequently increases and at the end of the second month of exposure to a temperature of 28°C, the titre of vaccine may be as low as 16,500 MLD; each dose will then contain only 330 LD_{50} .

The World Health Organization has stated that for the satisfactory immunization of man each dose of 17D vaccine should contain not less than 500 LD_{50} . This dose, however, is injected subcutaneously and thus introduced in its entirety into the organism. As the Dakar vaccine is administered by scarification, only part of the dose is absorbed. Consequently it is essential for this dose to be very much richer in virus. In

our opinion, vaccine administered by scarification should have a titre of at least 250,000 MLD per ml and the individual dose should contain a minimum of 5,000 LD₅₀.

Strict observation of the conditions indicated earlier for the transport and storage of the vaccine will ensure that at the time of vaccination the dose administered to each individual is sufficient for effective immunization.

From 1939 until 31 March 1954, the Institut Pasteur, Dakar, had supplied over 84 million doses of yellow fever vaccine.

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VACCINATION TECHNIQUE WITH YELLOW FEVER VACCINE OF THE INSTITUT PASTEUR, DAKAR

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The yellow fever vaccine of the Institut Pasteur, Dakar, is in the form of a powder distributed in tubes sealed under vacuum. Each tube bears the number of the batch of vaccine as well as the expiry date for its use and contains an amount sufficient to vaccinate about one hundred persons. A 2-ml ampoule of gum arabic solution is sent out with each ampoule of vaccine.

Every consignment is accompanied by a notice giving the necessary details for use of the vaccine, i.e., concerning storage, preparation of the vaccine suspension, method of vaccination, reactions, and contra-indications.

Preparation of Vaccine Suspension

The following equipment is used at the Institut Pasteur, Dakar, for preparing the vaccine suspension: test-tubes, 15 × 70 mm; glass rods, 6 × 150 mm; watch glasses 70 mm in diameter; Petri dishes 100 mm in diameter; vaccinostyles. All these items are first sterilized.

At the time of vaccination, an ampoule of vaccine is removed from the refrigerator and a file is drawn across the upper part of the ampoule, which is then broken off. The contents are emptied into a small test-tube by inverting the ampoule and gently tapping the bottom.

The two points at either extremity of an ampoule of gum solution are broken off and, while the operator blocks one end with a finger, a few drops of solution are allowed to fall on the vaccine while the mixture is stirred with a glass rod so as to obtain a uniform paste. The remainder of the solution is then added drop by drop, the mixture being stirred constantly. The suspension, which should be free from lumps, is then ready for use.

It is transferred to a watch glass which is placed in a Petri dish to protect it from dust. The suspension should be used within one hour of its preparation.

Method of Inoculation

A vaccinostyle is taken with sterile forceps and mounted on a penholder. It is then charged by immersing it horizontally in the vaccine suspension. Three drops of suspension are placed, a few centimetres apart, on the outer surface of the upper arm.

Three scarifications 8-10 mm in length are made through each of the drops, making certain that very slight bleeding occurs. The vaccine suspension is then carefully mixed with the slightly bloody serous fluid and allowed to dry for five minutes.

The gum arabic solution has the advantage of being less fluid than glycerol. It does not flow and solidifies fairly rapidly. In a few moments it forms a fine film which covers the scarifications and thus keeps the vaccine in place (fig. 1).

Mixed Vaccination against Yellow Fever and Smallpox

Inoculation by scarification makes it possible to combine yellow fever vaccination with smallpox vaccination. This mixed vaccination, by means of which simultaneous immunization against both diseases can be carried out with the minimum of equipment, is particularly indicated when a large number of subjects is involved.

For this purpose it is preferable to employ a dried smallpox vaccine, able to resist the action of the air temperature in hot countries.

All that is necessary is to empty the contents of a 100-dose ampoule of each vaccine into a small test-tube and then to grind them dry with a glass rod, mixing them well. The gum arabic solution is then added drop by drop so as to obtain a uniform suspension. Inoculation is carried out as indicated above for yellow fever vaccine alone.

Vaccination Reactions

Yellow fever vaccination alone does not cause any local reaction. After a few days the scarifications heal up and disappear completely.

After mixed vaccination the local reaction is similar to that caused by smallpox vaccination alone. Pustules develop within the same period of time and follow the same course in both cases.

FIG. 1. FILM OF GUM ARABIC COVERING SCARIFICATIONS



A general reaction may appear in a certain number of vaccinated subjects; in such cases it regularly develops on the fourth or fifth day after inoculation. The reaction takes the form of fever, headache, and stiffness. It usually lasts only 12-24 hours but may continue for several days with varying intensity. Although rare among young persons in good health, the general reaction becomes more frequent with age and the gradual wearing out of the organism and may then affect 30% of vaccinated persons. Negroes appear to be less susceptible than white people, among whom women react less frequently than men.

Experience has shown that after inoculation by scarification, the yellow fever virus multiplies rapidly in the blood of the person vaccinated, where it can be detected on the third to the sixth day. It is during this period that the general reaction appears. The presence of the virus can be demonstrated in about 60%-70% of inoculated persons by intracerebral injection

of their blood into white mice. The general reaction seems to occur only if the multiplication of virus in the bloodstream is intense enough to be detected. However, it does not necessarily arise in all cases where the virus is detectable and immunity may be established without reaction or the appearance of virus in the blood.

Late general reactions, setting in after the tenth day, have been very rare in French West Africa, where more than 48,000,000 yellow fever vaccinations by scarification have been carried out so far. When such reactions have been observed, the picture has been one of meningo-encephalitis, usually moderate in intensity, with fever, headache, stiffness of the neck, presence of Kernig's sign, convulsions in children, restlessness, and increased pressure of the cerebrospinal fluid, the protein content and cell count of which are above normal.

These accidents have always occurred separately and have never constituted foci, contrary to what has been observed in certain other countries. They have not numbered more than a few score out of all the vaccinations performed in French West Africa, and there has usually been complete recovery without sequelae. Fatal cases have been exceptional.

Contra-indications

A poor general condition of the subject or intercurrent complaints, particularly those affecting the liver or kidneys, may constitute temporary contra-indications to yellow fever vaccination by scarification. Because of their sensitivity to neurotropic viruses, we advise against the vaccination of children under two years of age and, generally speaking, we feel that the vaccination of children should be postponed in regions where virus diseases (mumps, measles, encephalitis, etc.) have recently been observed.

Precautionary Measures

Certain rules should be followed to ensure the success of yellow fever vaccination by scarification. These concern the storage of the vaccine, the technique of inoculation, and the supervision of the vaccinated subjects.

Numerous tests have shown that the yellow fever vaccine of the Institut Pasteur at Dakar undergoes a certain loss of potency after exposure for a month to a temperature of 28°C. For this reason we have set at about 10 days the maximum period during which the vaccine can be kept without harm at air temperature. This period is used for shipping the vaccine, and once it has elapsed, the vaccine should be again placed in a refrigerator and stored at 5°C until required for use. If further transport is necessary,

the ampoules of vaccine should be transported as required in vacuum flasks filled with ice or, where this is not possible, in containers wrapped in wet cloth and kept out of the sun. The expiry date for the use of the vaccine printed on the ampoule should never be exceeded. The vaccine suspension should be prepared away from direct sunlight and used within the hour.

Only experienced personnel should be allowed to carry out the inoculations. Since the risk of cutaneous infection is extremely small, no preliminary preparation of the area selected for inoculation is necessary and the use of any antiseptic product capable of deteriorating the vaccine should be forbidden. As indicated above, the scarifications should be sufficiently numerous and deep to ensure the penetration of the vaccine.

Once inoculation has been completed, the subjects should be kept under observation for ten minutes, out of the sun. If there are many of them, care should be taken to prevent crowding and pushing, so as to avoid rubbing of the scarified area. An excellent check consists in making sure, before sending away the vaccinated person, that the film of gum arabic covering the scratches is intact. If it has disappeared, the inoculation is repeated.

Finally, all vaccinated persons should be advised to avoid fatigue and excessive eating during the week following vaccination. If a reaction appears, rest and dieting should be immediately prescribed. Under these conditions the symptoms usually disappear within 24 hours, whereas they continue and become more pronounced in the case of persons who refuse to stop working.

POST-VACCINATION IMMUNITY WITH YELLOW FEVER VACCINE OF THE INSTITUT PASTEUR, DAKAR

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In 1931 Theiler⁹ showed that the antibodies present in the serum of persons immunized against yellow fever have a protective effect when a yellow fever virus suspension mixed with this serum is injected intracerebrally into the white mouse. The adaptation of this biological test to an animal in routine use made it possible to develop immunological research on yellow fever to a very considerable extent.

Two procedures have so far been very widely used for detecting the presence of yellow fever antibody—the intraperitoneal method of Sawyer & Lloyd⁶ and Theiler's¹⁰ intracerebral method. The second of these two has the advantage of requiring only small amounts of serum.

The mouse protection test was used as early as 1932 at the Institut Pasteur at Dakar, where the original technique consisted of adding the serum to be tested to a 1 : 100 suspension of neurotropic virus and inoculating the mixture intracerebrally into six mice. This technique has since undergone various modifications in order to suit it to local conditions and to make the test more sensitive, and has been very widely used, as modified, in surveys of endemic yellow fever in French West Africa and as a check on yellow fever vaccination.

Neutralization Test Method at the Institut Pasteur, Dakar

The neutralization test method employed at the Institut Pasteur, Dakar, consists of mixing the serum to be tested with varying dilutions of yellow fever virus and inoculating batches of white mice with these mixtures.

Virus

The French strain of virus adapted to the white mouse is used. A batch of 40 mice is inoculated intracerebrally with a 1 : 50 suspension of virus from the 258th or 259th mouse passage. The brains of animals paralysed on the fourth and fifth days are harvested, immediately frozen, and dried in vacuo at a temperature of -25°C . They are then mixed and pulverized in the mortar. The powder thus obtained is dispensed into ampoules, 0.10 g being transferred to each ampoule; this weight corresponds approximately to that of one dried brain. The ampoules are sealed under vacuum and stored at -25°C .

Mice

The mice come from breeding colonies which the Institut Pasteur, Dakar, reorganized in 1947 from breeders sent from the Yellow Fever Research Institute at Lagos. The animals used for the tests are $2\frac{1}{2}$ -3 months old and weigh an average of 17-18 g.

Before carrying out the test, the number of mice necessary is collected (9 for each serum under test and 24 for the controls and titration) and one third of them is marked on the back with a red spot, one third with a blue spot, and one third with a yellow spot.

Preparation of serum-virus mixtures

An ampoule containing 0.10 g of powdered brain is emptied into a mortar. The powder is ground with a pestle while 2 ml of 10% normal serum in saline are added with a dropper. The suspension thus obtained is a 1 : 5 dilution, since the amount of virus added corresponds to 0.40 g of fresh brain.

This suspension is centrifuged for 10 minutes at 3,000 revolutions per minute, after which 0.5 ml of the supernatant liquid is removed and added to 4.5 ml of 10% normal serum so as to give a 1 : 50 dilution. From this 1 : 50 dilution are prepared the requisite quantities of the following dilutions: 1 : 500, 1 : 5,000, and 1 : 50,000 dilutions for the tests and 1 : 1,000,000 and 1 : 2,000,000 dilutions for titration of the virus. All these dilutions are made with 10% normal serum.

A quantity of 0.5 ml of each dilution is placed in haemolysis tubes arranged in racks holding three rows of 25 tubes each. The 1 : 50,000 dilution is placed in the back row, the 1 : 5,000 dilution in the middle row, and the 1 : 500 dilution in the front row.

The sera under test are then added. Each transverse line of three tubes corresponds to one serum and bears a serial number. With a pipette 1.5 ml of the first serum are taken up, and 0.5 ml is added to each tube

of line No. 1, beginning with the back tube (containing the highest virus dilution). The other sera are then dispensed in the same way into the three tubes of each successive line, always proceeding from the back to the front row.

When the quantity of serum to be tested is less than 1.5 ml, 0.25 ml of each virus dilution is placed in each of the three tubes to each of which 0.25 ml of serum is then added.

The six tubes of the last two lines are reserved for the control sera. To each of the three penultimate tubes is added 0.5 ml of immune serum from a donkey hyperimmunized by massive inoculations of virulent mouse brain, while 0.5 ml of normal monkey serum is added to each of the last three tubes.

Once the mixtures have been completed, all the tubes are shaken separately by hand, so as to ensure contact of the virus with the sera, and left for half an hour at laboratory temperature (25-30°C).

Inoculations

The serum-virus mixture in each tube is injected intracerebrally into three mice, 0.03 ml being administered to each animal. The same syringe is used for the three mixtures of each serum. The mixture from the back tube (highest virus dilution) is inoculated into three mice marked with yellow spots, the mixture from the middle tube into three mice with blue spots, and the mixture from the front tube (lowest virus dilution) into three mice with red spots. The nine mice inoculated with the three mixtures of each serum are placed together in one jar, marked with the number of the experiment, the registration number of the serum, and the date of inoculation.

The procedure is repeated with the control sera (normal and immune), which are the last to be injected.

When these operations are finished, the check on the virus is completed by inoculating three mice marked with blue spots with a 1 : 1,000,000 virus dilution and three mice with yellow spots with a 1 : 2,000,000 dilution. These six animals are placed in the same jar.

Observation of inoculated animals

The inoculated mice are examined every morning for 10 days. The results of the examination of each jar are entered daily on a printed form of eleven columns, for the days of observation, and nine horizontal rows numbered for the nine mice (see fig. 1 and 2).

The first mouse to fall ill or die in each of the three groups is assigned No. 1 for those with red spots, No. 4 for those with blue spots, and No. 7

**FIG. 1. RECORD SHEET FOR MOUSE PROTECTION TEST,
SHOWING GOOD PROTECTION**

		0	1	2	3	4	5	6	7	8	9	10
Red	1						P	+				
	2							P	+			
	3							P	P	+		
Blue	4											L
	5											L
	6											L
Yellow	7											L
	8											L
	9											L
Result = ++ (good protection)												

P = paralysed L = living + = dead

for those with yellow spots, and so on in numerical order. Death or the onset of paralysis is entered in the row for the mouse concerned under the day of occurrence. Deaths before the fifth day are not taken into account.

Interpretation of results

The use of different virus dilutions makes it possible to assess to a certain extent the protective power of the serum being examined. The various possible results which can be obtained after a 10-day period of observation are summarized in table I.

The mice used for virus control are regularly killed by the 1 : 1,000,000 dilution and sometimes by the 1 : 2,000,000 dilution. It may therefore be taken that each of the animals has received at least 1-2 minimum lethal doses (MLD). With the addition of an equal volume of serum the 1 : 500, 1 : 5,000 and 1 : 50,000 virus dilutions used for the tests become 1 : 1,000, 1 : 10,000 and 1 : 100,000 dilutions. Consequently, mice inoculated with each of these three dilutions have received 1,000-2,000, 100-200, and 10-20 minimum lethal doses per animal, respectively.

FIG. 2. RECORD SHEET FOR MOUSE PROTECTION TEST, SHOWING NO PROTECTION

		0	1	2	3	4	5	6	7	8	9	10
Red	1						P	+				
	2						P	+				
	3							P+				
Blue	4							P+				
	5							P+				
	6							P+				
Yellow	7							P	+			
	8								P	+		
	9								P	+		
Result = 0 (no protection)												

P = paralysed + = dead

Under these conditions, the neutralizing power of a serum examined may fall within one of the following categories. In the case of a serum protecting against a dilution of :

- 1/50,000, 1 ml neutralizes 600-1,200 MLD
- 1/5,000, 1 ml neutralizes 6,000-12,000 MLD
- 1/500, 1 ml neutralizes 60,000-120,000 MLD

Interval between Vaccination and Immunity

Experience has shown that the virus contained in the Dakar vaccine multiplies rapidly in the blood of the vaccinated subject. Its presence may be demonstrated in 60%-70% of cases by intracerebral inoculation of the white mouse with the subject's blood. The virus usually appears on the third day after vaccination, persists for a few days, and disappears at the latest by the seventh or eighth day. It is probable that by the time the virus disappears small quantities of antibody have already begun to form in the blood.

TABLE 1. DEGREE OF PROTECTIVE POWER CONFERRED BY YELLOW FEVER VACCINATION

Ratio of mice surviving to mice inoculated				Symbol	Interpretation *
1 : 500 virus dilution	1 : 5,000 virus dilution	1 : 50,000 virus dilution	total		
3/3	3/3	3/3	9/9	+++	Very good protection
2/3	3/3	3/3	8/9		
1/3	3/3	3/3	7/9	++	Good protection
0/3	3/3	3/3	6/9		
0/3	2/3	3/3	5/9		
0/3	1/3	3/3	4/9	+	Poor protection
0/3	0/3	3/3	3/9		
0/3	0/3	2/3	2/9	?	Inconclusive
0/3	0/3	1/3	1/9		
0/3	0/3	0/3	0/9	0	No protection

* If inconclusive or contradictory results are obtained, the test is repeated.

Certain very sensitive methods for carrying out the mouse protection test have been suggested by Bugher,¹ Whitman,¹¹ and Smithburn.⁷ The use of such methods makes it possible to determine with some degree of accuracy the period between vaccination against yellow fever and the appearance of antibodies. The Institut Pasteur at Dakar has investigated whether this period can also be determined by the technique it has been using for many years, and which has been described in detail above, account being taken not only of the number of surviving mice but also of the average survival time, as advocated by Bugher.¹

Two experiments were carried out for this purpose.

Experiment 1

Three white persons aged about 20 years, Ma, Vi, and Bo, who had recently arrived from France and had never been vaccinated against yellow fever, agreed to undergo the first experiment. A preliminary mouse protection test was negative in all three cases.

On 2 February 1952 they were inoculated with Dakar vaccine (batch B 8). It was administered in three series of three scarifications 8-10 mm long on the external surface of the left arm. There was no noticeable

reaction during the next few days, and none of the subjects interrupted his normal activities.

A blood sample was taken daily from each subject during the period following vaccination. The samples taken on the third to eighth days were injected intracerebrally into batches of mice for detection of virus in the circulatory system, and a mouse protection test was carried out with each of the sera collected from the fifth to the eighteenth day.

TABLE II. RESULTS OF MOUSE PROTECTION TESTS ON SERA OF 3 SUBJECTS INOCULATED WITH DAKAR VACCINE (BATCH B 8)

Time of blood-sampling	Ratio of mice killed to mice inoculated (indicating presence of circulating virus)			Mouse protection tests					
				ratio of mice surviving to mice inoculated			average survival time (days)		
	Ma	Vi	Bo	Ma	Vi	Bo	Ma	Vi	Bo
Before vaccination	—	—	—	0/9	0/9	0/9	5.5	5.1	5.4
3rd day	5/6	0/6	1/6	—	—	—	—	—	—
4th "	5/5	5/6	5/6	—	—	—	—	—	—
5th "	5/5	6/6	4/4	0/9	0/9	0/9	5.1	5.3	4.8
6th "	6/6	6/6	4/6	0/9	0/9	0/9	5.0	5.0	5.5
7th "	3/6	6/6	0/6	0/9	0/9	0/9	5.3	5.0	5.5
8th "	0/6	0/5	0/6	0/8	1/9	0/9	5.1	6.1	5.6
9th "	—	—	—	0/9	0/9	0/9	6.0	6.1	6.2
10th "	—	—	—	1/9	0/9	2/9	7.3	6.5	7.0
11th "	—	—	—	3/9	0/9	1/9	7.4	6.6	7.2
12th "	—	—	—	3/9	2/9	2/9	7.5	7.7	7.6
13th "	—	—	—	2/9	5/9	2/9	7.6	8.6	7.4
14th "	—	—	—	6/9	4/9	4/9	8.7	8.4	8.4
15th "	—	—	—	4/9	3/9	6/9	8.1	8.0	9.3
16th "	—	—	—	8/9	3/9	7/8	9.6	8.1	8.6
17th "	—	—	—	5/9	7/9	7/9	9.0	9.3	9.4
18th "	—	—	—	5/9	8/9	5/9	8.7	9.8	9.0
41st "	—	—	—	6/9	8/9	6/9	9.0	9.5	8.8
6 months	—	—	—	9/9	7/9	8/9	10.0	9.2	9.8

The results of these tests are shown in table II, which indicates for each serum the proportion of surviving animals and the average survival time. This is calculated by the method indicated by Bugher:¹ during the 10-day period of the test, the number of surviving mice in each batch is noted daily, these numbers are added up, and the total obtained is divided by the number of mice alive on the fourth day. Mice which die before the fifth day are not taken into account.

Interpreting the results shown in table II according to the scale of protection indicated in table I, i.e., taking only the surviving mice into consideration, the sera of Ma can be considered positive only from the 11th day (3/9), of Vi from the 13th day (5/9), and of Bo from the 14th day (4/9). However, examination of the average survival time shows that after being low between the 5th and 7th days, the average survival time

begins to increase from the 9th day, and then gradually rises until it approaches 10. The fall at the outset is probably related to the presence of circulating virus which adds to the effect of the virus in the dilutions used for the mouse protection test.

Experiment 2

Using a sensitive mouse protection test method, Smithburn & Mahaffy⁸ have shown that yellow fever antibodies can be detected on the tenth day in most persons vaccinated with 17D vaccine. And the Expert Panel on Yellow Fever of the World Health Organization stressed during its first session¹² "the fact that there was considerable evidence to show that effective immunity was established as early as the seventh day following inoculation..."

The second experiment carried out by the Institut Pasteur, Dakar, was a comparative investigation of the appearance of yellow fever antibodies in two groups of subjects inoculated with Dakar vaccine and 17D vaccine respectively.

Six white volunteers aged about 20 were used for this experiment. They had recently landed at Dakar, none of them had been vaccinated against yellow fever, and their sera, samples of which were taken before the commencement of the experiment, were found to be without protective power.

On 15 July 1952, three of them, Ch, Sa, and Ca, were inoculated with Dakar vaccine (batch B 66) under the same conditions as the subjects of the first experiment. On the same day, the three other volunteers, Cl, El, and Hi, were given a subcutaneous injection of 17D vaccine (batch 52-2) from the Institut Pasteur, Paris. There was no apparent reaction after these vaccinations.

Blood samples were taken from the third to the nineteenth day for detection of circulating virus and then of antibody. Tables III and IV show the results obtained.

According to the degrees of protection shown in table I, the sera tested can only be considered positive from the 10th day (3/9) and from the 11th day (4/9 and 5/9) for the subjects inoculated with Dakar vaccine, and from the 11th day (5/8), the 13th day (4/8), and the 15th day (4/9) for those inoculated with 17D vaccine. Nevertheless, it can be seen that in both groups the average survival time begins to increase from the 7th day.

The average survival time was also calculated by grouping together the sera from the six subjects of experiments 1 and 2 who had received Dakar vaccine and the sera of the three subjects inoculated with 17D vaccine. For this purpose all the surviving mice in each of these two groups were counted daily. The figures obtained were added up for each series

TABLE III. RESULTS OF MOUSE PROTECTION TESTS OF SERA OF 3 SUBJECTS INOCULATED WITH DAKAR VACCINE (BATCH B 66)

Time of blood-sampling	Ratio of mice killed to mice inoculated (indicating presence of circulating virus)			Mouse protection tests					
				ratio of mice surviving to mice inoculated			average survival time (days)		
	Ch	Sa	Ca	Ch	Sa	Ca	Ch	Sa	Ca
Before vaccination	—	—	—	0/9	0/9	0/9	5.6	5.4	5.8
3rd day	1/4	1/4	1/4	—	—	—	—	—	—
4th "	5/5	4/5	1/4	—	—	—	—	—	—
5th "	5/5	2/6	1/5	—	—	—	—	—	—
6th "	4/5	3/6	0/6	—	—	—	—	—	—
7th "	0/6	1/5	0/6	1/9	0/9	1/9	6.4	5.8	6.4
8th "	—	—	—	1/8	3/9	2/8	6.8	7.2	7.1
9th "	—	—	—	2/9	2/9	2/9	7.3	7.2	7.1
10th "	—	—	—	2/9	2/9	3/9	6.8	6.8	7.4
11th "	—	—	—	4/9	5/9	3/9	7.8	8.7	7.6
13th "	—	—	—	3/9	3/8	3/9	7.7	8.0	8.1
15th "	—	—	—	5/9	6/9	4/9	8.7	9.1	8.4
17th "	—	—	—	5/9	5/9	4/9	8.7	8.6	8.4
19th "	—	—	—	6/9	5/8	5/9	9.3	9.1	8.7

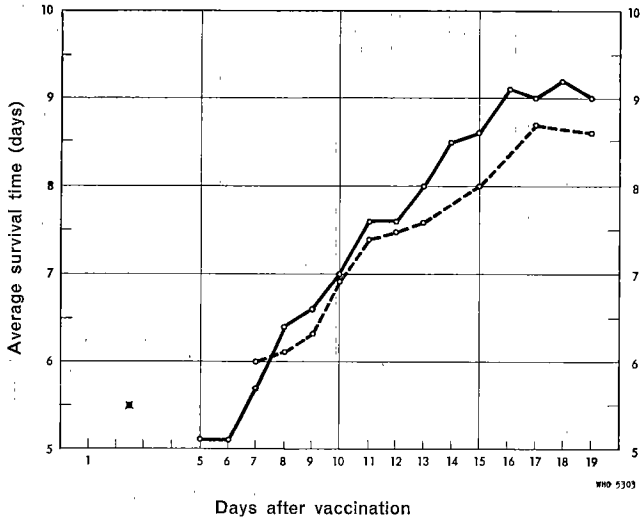
TABLE IV. RESULTS OF MOUSE PROTECTION TESTS OF SERA OF 3 SUBJECTS INOCULATED WITH 17D VACCINE

Time of blood-sampling	Ratio of mice killed to mice inoculated (indicating presence of circulating virus)			Mouse protection tests					
				ratio of mice surviving to mice inoculated			average survival time (days)		
	Cl	El	Hi	Cl	El	Hi	Cl	El	Hi
Before vaccination	—	—	—	0/9	0/9	0/9	5.8	5.6	5.4
3rd day	0/5	0/6	0/5	—	—	—	—	—	—
4th "	0/4	0/5	0/4	—	—	—	—	—	—
5th "	0/6	0/6	0/5	—	—	—	—	—	—
6th "	0/6	0/6	0/6	—	—	—	—	—	—
7th "	0/6	0/6	0/6	0/8	0/9	1/9	6.1	5.7	6.1
8th "	—	—	—	1/9	0/9	1/9	6.7	5.8	5.8
9th "	—	—	—	2/8	1/9	1/9	7.0	6.0	6.0
10th "	—	—	—	2/9	2/9	2/8	6.8	6.8	6.2
11th "	—	—	—	5/8	2/9	2/9	8.5	6.8	7.0
13th "	—	—	—	3/9	4/8	2/8	7.4	7.3	7.1
15th "	—	—	—	3/9	4/9	4/9	8.3	7.7	7.8
17th "	—	—	—	6/9	4/9	5/8	9.0	8.2	9.0
19th "	—	—	—	4/8	5/9	4/8	8.6	8.6	8.5

and each of the two totals was divided by the total number of mice surviving on the fourth day in the corresponding group. The distribution of the averages obtained is shown in fig. 3.

These experiments with Dakar and 17D vaccines both confirm previous notions and show the similarity between the results obtained with the two methods of vaccination.

FIG. 3. AVERAGE SURVIVAL TIME OF MICE INOCULATED WITH SERA OF 9 SUBJECTS VACCINATED WITH DAKAR VACCINE OR 17D VACCINE



- ✕ = average survival time with the 9 serum samples taken before vaccination
- = Dakar vaccine (6 sera)
- - -○- - - = 17D vaccine (3 sera)

The neutralization test method as employed at the Institut Pasteur, Dakar, thus makes it possible to get some idea of the interval between yellow fever vaccination and the appearance of antibodies. With Dakar vaccine, the presence of antibodies on the 7th to the 9th day is revealed by a progressive increase in the average survival time, and then from the 10th to the 14th day by the definite protection of animals inoculated with the 1 : 50,000 virus suspension. The protective power then gradually increases, approaching the maximum towards the end of the third week.

It may be affirmed that Dakar vaccine, like 17D vaccine, confers effective immunity before the 10th day.

Efficacy of Vaccination

Having established that the French strain adapted to the white mouse was able to penetrate into the human organism through slight cutaneous scarifications and to bring about the formation of antibodies, the Institut Pasteur, Dakar, undertook the first large-scale immunization campaign

with this method of immunization in 1939.^{4, 5} More than 100,000 inhabitants of Senegal were inoculated simultaneously against yellow fever and smallpox. Only schoolchildren whose identity had been carefully established at the time of vaccination were used for control purposes. Out of a total of 1,387 subjects, samples of whose serum taken before inoculation had been found to be without protective power against the yellow fever virus, 1,336 (96.3%) gave a positive test 1-2 months later.

The following year, 35,000 Africans on the Ivory Coast and 29,000 in the French Sudan were vaccinated under the same conditions by the Institut Pasteur, Dakar. Controls carried out after an interval of 2-3 months, also on schoolchildren, gave positive results in 91.2% of cases for the Ivory Coast (400 sera tested) and 98.9% for the Sudan (198 sera tested).

In 1945, in an experiment which took place in France under the auspices of UNRRA,³ two groups of 200 white soldiers who had never left France were vaccinated by scarification. Group A received only Dakar yellow fever vaccine, while group B received Dakar yellow fever vaccine combined with smallpox vaccine. Samples of sera taken a month later were tested in three different laboratories (Dakar, Montana, and Rio de Janeiro). Of the group A sera, 98.9% were found to be positive (188 sera tested) and of the group B sera 97.9% (193 sera tested).

All these experiments, the first two of which were carried out on large numbers of subjects under the most unfavourable conditions, clearly showed that on inoculation with Dakar vaccine immunity was conferred in an average of 96% of cases.

The results obtained by the Institut Pasteur at Dakar were confirmed by a test carried out in 1949 in the Republic of Panama, where Courtney² inoculated 24,000 persons with the Dakar vaccine by scarification. Of 150 sera selected at random six months after vaccination, all gave a positive test.

Duration of Immunity Induced by Vaccination

The Institut Pasteur at Dakar has also carried out a certain number of control tests to determine the duration of the immunity conferred by inoculation with Dakar vaccine. The subjects tested were either selected at random from among the inhabitants of various territories of French West Africa or were isolated individuals.

In the first case, the aim was to check the immunity rate among African populations who had undergone systematic vaccination against yellow fever a few years before, during tours made by health service teams. Table V shows the results obtained among the inhabitants of 14 small

villages away from main roads and lines of communication. It should be made clear that samples of sera were taken without there being any absolute certainty that they all came from definitely vaccinated subjects.

TABLE V. RESULTS OF YELLOW FEVER IMMUNITY TESTS IN ISOLATED FRENCH AFRICAN VILLAGES

Number of years since vaccination	Territory	Number of sera tested	Positive results	
			number	%
2	Senegal	25	24	96.0
	Guinea	58	52	89.6
	Ivory Coast	34	34	100.0
	Dahomey	18	11	61.1
	Upper Volta	11	7	63.6
	Niger	50	47	94.0
	Total	196	175	89.2
3	Senegal	25	25	100.0
	Sudan	24	19	79.1
	Ivory Coast	40	40	100.0
	Upper Volta	25	25	100.0
	Total	114	109	95.6
4	Senegal	106	91	85.8
	Dahomey	25	22	88.0
	Total	131	113	86.2
7	Senegal	72	59	82.0

The duration of immunity was also investigated in a number of isolated persons, almost all Europeans who had been vaccinated in various French or African vaccination centres. All these persons were in possession of a vaccination certificate stating the date of vaccination. The results of the control tests were as follows :

<i>Number of years since vaccination</i>	<i>Number of persons tested</i>	<i>Number of persons immune</i>	<i>Percentage immune</i>
1	142	134	94.3
2	89	81	91.0
3	30	28	93.3
4	17	17	100.0
5	16	16	100.0
6	10	9	90.0
7	4	3	} 87.5
8	1	1	
9	7	7	
10	1	0	
11	1	1	
12	2	2	

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