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CONTRIBUTIONS TO RAPID DIAGNOSIS THROUGH IMMUNOFLUORESCENCE OF INFECTIONS AND IMMUNITY IN SMALLPOX VACCINE

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This paper shows the current importance of some rapid methods for detecting the smallpox vaccine antigen in pathological products and of rapid dosing of antibodies circulating in persons who might be immune or receptive. A description is given of a direct technique of immunofluorescence with fluorescent, anti-vaccinal, human serum used in detection of the antigen, as well as an indirect technique of serological titration of human serum antibodies, using a substratum consisting of vaccine and a fluorescent, anti-human serum.

Smallpox, a disease which disappeared for years in most of the European countries, has begun to regain importance. In recent years, smallpox has undergone a significant increase in the world, with the number of cases increasing from 78,000 in the world in 1959 to 93,000 in the world in 1963, and in Europe from 13 to 124 cases (4).

The causes for this growth must be sought in the intensification and acceleration of international traffic, in the discontinuance of effective quarantines, and in the relaxation or failure to apply systematic and effective anti-smallpox vaccinations, the protection from which is shorter than believed.

At the present time, in the majority of European countries (including our country) several questions can be posed for which the answers will depend on a number of organisational and antiepidemic measures.

These are the following:

1. Can the diagnosis of smallpox be invalidated in a very rapid manner?

2. Can the presence of smallpox in a suspect be confirmed in a very rapid manner? (Can the smallpox antigen be detected quickly?)

3. Can it be determined quickly whether a person is receptive or immune to smallpox infection? (Can the anti-smallpox antibodies be detected very rapidly? Also, can the titration of antibodies in a given preparation of gammaglobulins be evaluated very rapidly?)

a) For the first question it is much easier to answer by a simple cytological examination of the product collected from vesicular (or pustulous) cutaneous lesions. The presence of gigantic cells with monstrous nuclei or multinucleated cells, as in herpes, zona, or chickenpox, invalidates the diagnosis of smallpox (11).

b) The smallpox antigen, if found in sufficient quantity in a pathological product, can be identified rapidly by a reaction of fixing its complement or, more slowly, by the technique of precipitating it in an agar gelatin (5). But if the quantity of antigen in the pathological product is quite small, rapid identification can be accomplished only through immunofluorescence, using fluorescent, anti-vaccinal globulins (1), (9).

c) Evaluating the degree of immunity of a person is accomplished clinically by means of vaccination. But the answer is not available for several days and is often still equivocal: if the vaccine takes, it means that the person was receptive, but if it does not take, the conclusion can be drawn that the person was immune. Detection of the antibodies in the serum of patients can be accomplished by the reaction of neutralization, which lasts several days, by the reaction of fixing of the complement, or by the technique of immunofluorescence.

In this paper, we propose on the one hand to present the methods of immunofluorescence designed to detect the smallpox antigen or vaccine in a pathological product, and on the other hand to detect also the presence of the titre of anti-smallpox or antivaccinal antibodies in the blood of persons receptive or immune to these infections, as well as in a random lot of commercial human globulin.

Not having any smallpox virus nor any sera with anti-smallpox antibodies, we limited our studies to the vaccine virus. Since the method that we used (immunofluorescence) is based on the antigen-antibody union and since there is a close antigen relation between the smallpox virus and the vaccine virus, we feel that our tests can also be used in the diagnosis of smallpox.

Materials and Method

The antigen that we used was represented by the vaccine virus. We used vaccine virus from the "Dr. I. Cantacusino" Institute, cultivated from calves and rabbits; the HeLa cell cultures and fibroblasts were from the human embryo, and the vaccine from a human pustule (traditional vaccination).

As pathological products (from pustulous vesicles), we used thin smears on glass plates. The vaccine lymph from the calf (unglycerinated) was diluted 1 to 10 and homogenized with Hanks solution. We also used cell cultures cultivated in Barski tubes and infected with vaccine virus.

For the negative specimen preparations, we used smears from the vesicles or pustules of herpes, chickenpox, zona, etc.

For the serological research, we used vaccine antigen (from calves) diluted 1 to 10 and spread thinly on a glass plate (like a blood smear).

The antivaccine antibodies were obtained from the blood of a man vaccinated and then revaccinated repeatedly at 1 to 2 month intervals with the blood of rabbits immunized by cutaneous and intravenous inoculations of vaccine (9).

The neutralizing titre (compared to the cutaneous inoculation of rabbit vaccine) was over 1/200.

The anti-human antibodies were obtained from rabbits immunized by repeated injections of human globulin with the addition of adjuvants (the technique of Dr. Gh. Stoica) [Note: Personal communication.]

The "negative" sera (of receptive persons) were collected from people who had not been vaccinated against smallpox for at least 25 years.

The "positive" sera (from immune persons) were collected from healthy adults from 18 to 25 years in age who had been successfully vaccinated 2-4 months previously.

The immune globulin was obtained by filtering human blood with DEAE cellulose (Levy's technique (7).)

The fluorochrome used was fluoroscein isothiocyanate (Prolabo, France) for uniting with immune globulin, and Lisamin Rhodamin B_{200} for uniting with bovine blood albumin.

The technique used for uniting with fluoroscein isothiocyanate was that recommended by Marshall (8). Uniting the bovine blood albumin with the acidic chloride of Lisamin Rhodamin B_{200} was achieved by use of the Chadwick technique (2).

Removal of the uncombined isothiocyanate was accomplished was realized by flitering with Sephadex G_{25}, while the Lisamin Rhodamin B_{200} was removed by agitation with active carbon (the Chadwick technique) (2).

Removal of the natural, antivaccine antibodies in the fluorescent, anti-human, rabbit globulin was realized by two adsorbtions repeated with vaccine dried in acetone (an adaptation of the Coons technique (3).) For study of the vaccine antigen in the human-origin product, we used a human antivaccine serum, and there was no need for making supplementary adsorbtions.

Fixing the preparations was achieved, after drying, by using acetone for a period of 20 minutes at room temperatures (the preparations fixed were used immediately or preserved in a freezer.)

The coloration used was either direct (in a stratum) or indirect (in two strata).

We also used an unspecific fluorescent coloration with the bovine blood albumin united with Lisamin Rhodamin B_{200} that we added extemporaneously in a proportion of 1/9 globulin mixed with Smith's fluoroscein isothiocyanate (10).

For study of the antigen in the human-origin pathological product, we used the technique of direct coloration with human, fluorescent, antivaccine globulin. In this way, the adsorbtion of the antivaccine globulin in dry powder (in acetone) of human liver became ineffective. The useful working dilution was 1/4.

As a specimen, we used fluorescent, human, anti-chickenpox globulin.

For the serological reactions, we used the technique of indirect coloration. With the aid of a small matchstick sharpen-

ed and imbedded in a silicon gel, we made a series of small circles of about 6 millimeter diameter on thin plates with vaccine antigen. In each of these small areas, we placed a small drop of the test serum, as well as a drop of absolutely negative specimen serum (receptive serum) and a drop of absolutely positive specimen serum (immune serum). The test serum was diluted. After a contact of 20 minutes in room humidity, the plates were each washed 3-4 times for a period of 3-4 minutes with a 0.15 M sodium chloride solution in a phosphate tampon of 0.01 M at pH 7.2, dried, and then dyed with anti-human rabbit globulin mixed with fluoresceein isothiocyanate (mixed at random in a proportion of 9 to 1 with bovine blood albumin united with Lisamin Rhodamin B_{200}.) After being kept 20 minutes in room humidity, the plates were washed as above, dried, and mounted in a "fluormounting" between the plate and the lamella, and then examined with a number 10 lens in a fluorescence microscope.

We used a mercury vapor Opton lamp at HBO_{75} maximum pressure, with a BG 12/3 excitation filter, a cardiod condensor with darkened field, lenses number 10 and 40, a number 7 eye glass, and a GG 9/1 + OG 1/1.5 dam filter.

For the photography, we used Agfa Izopan 21 DIN film and an exposure time of between 7 and 21 minutes.

Results

Detection of the Vaccine Antigen. To control the method and the ingredients, we first made a study of the detection of the vaccine antigen in cell cultures /See Note./ In the HeLa cell cultures and the human fibroblasts infected with vaccine virus, the vaccine antigen could be detected intracytoplasmatically starting 12 hours after infection of the cells in the form of fine, fluorescent granules grouped especially arounf the perinucleus. In the later hours, the number and especially the size of these granules increased and after 18-24 hours the whole cytoplasm was filled with an intensely fluorescent mass. Later, when the cytopathic effect appeared, the vaccine antigen appeared to have spread into all cell parts (including the nucleus) and into the cellular remains that result from their destruction. /Note: Detailed study of the development of vaccine antigens in different cell lines in immunofluorescence will be the subject of another work./

By comparing the two types of cell cultures, it was found in the fibroblastic cells the phenomena are similar, but slower and less intense (Figure 2).

On smears made with the product of vaccine pustules, the vaccine antigen appeared in the form of intensely fluorescent particles which appeared in the different cells or cell remains (Figure 3).

In the specimen preparations made from pustules of herpes and chickenpox, the staphylococcal or streptococcal pustules could only be detected with diffuse autofluorescence of the mass, but the particles with specific immunofluorescence could not be detected.

It is useful, but not indispensable to make use of fluorescent coloration unspecific to the mass with the bovine blood albumin combined with Lisamin Rhodamin B_{200}.

Detection and Titration of Antivaccine Antibodies

1. Determination of the titration of natural antibodies found in the blood of "receptive" persons.

A dilution is first of all made of the fluorescent, human, anti-globulin antibodies so as to determine the optimum working dilution, that is, that dilution which will no longer produce a fluorescence that is non-specific to the mass, but which nevertheless produces in the presence of human globulins an obviously specific immunofluorescence (+++). We thus found a working dilution of 1/10/

The titration was realized with antinuclear globulin from the blood of a patient with disseminated lupus erythematous fixed on leukocytes.

To determine the titre of the natural antibodies in the negative sera, we made dilutions of each individual negative serum and of a mixture of negative sera; with these we treated the antigen preparations. After washing, treating with fluorescent, human, antiglobulin antibodies in a 1/10 dilution, and the rewashing, the preparations were examined in a fluorescent microscope with a number 10 lens.

This small increase can best be found if the preparation area treated with a certain serum shows or does not show a specific fluorescence. The zones adjoining these areas serve as evidence (Figure 4).

Study of Table 1 indicates that the 1/10 dilution can be considered as the upper limit for bringing out the antivaccine antibodies through our technique in the antibodies of receptive persons.

Figure 1. HeLa cell cultures infected with vaccine virus for 24 hours

Figure 2. Culture of human embryo fibroblasts infected with vaccine virus for 16 hours

GRAPHIC NOT REPRODUCIBLE

Figure 3. Product of vaccine pustule in which the vaccine antigen has been brought out.

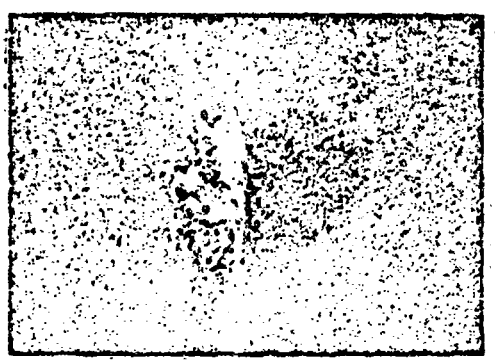

Figure 4. Vaccine smear partially covered with immune serum, then completely treated with fluorescent antiglobulin serum (the microscope field containing the limits between the two regions).

GRAPHIC NOT REPRODUCIBLE

2. Determination of the titre of antivaccine antibodies in the blood of "immune" persons.

For determination of these titres, we proceded as below, with the dilutions, however, being started from a minimum dilution of 1/10.

Table 1

Dilution of "Receptive" Serum

	1/2	1/5	1/10	1/20	1/40
(1) Amestec de ser negativ (receptiv)	++	±	−	−	−
(2) Serul 1	++	±	−	−	−
2	++	−	−	−	−
3	++	−	−	−	−
4	++	−	−	−	−
5	++	±	−	−	−
6	++	−	−	−	−
7	++	−	−	−	−
8	++	++	±	−	−
9	++	++	−	−	−
10	++	−	−	−	−
11	++	++	±	−	−

Key: 1. Mixture of negative (receptive) serum
2. Serum

Study of Table 2 indicates that through the technique that we used it is possible to distinguish a net difference between the "receptive" and the "immune" sera.

Discussion

In view of the antigen relationship between smallpox virus and vaccine, we believe that our results realized with vaccine can also be valid for smallpox.

To detect the smallpox-vaccine antigen in pathological products, the technique of immunofluorescence had proved useful in all cases in which only a very small amount of pathological material was available. The minimum time necessary for preparing the test product and up to the realisation of a result is less than an hour and a half.

Table 2

Dilution of "Immune" Serum

	1/10	1/20	1/40	1/80	1/160	1/320	1/640
(1) Amestec de seruri pozitive „imune"	+++	+++	+++	+++	++	++	±
(2) Nr. 1	+++	+++	+++	+++	+	±	−
2	+++	+++	+++	+++	+	+	−
3	+++	+++	+++	+++	++	++	±
4	+++	+++	+++	+++	+	±	−
5	+++	+++	+++	+++	+	±	−
6	+++	+++	+++	+++	++	++	±
7	+++	+++	+++	+++	+	±	±
8	+++	+++	+++	+++	++	++	±
9	+++	+++	+++	+++	++	++	−
10	+++	+++	+++	+++	++	±	−

Key: 1. Mixture of positive "immune" sera;
2. No.

To detect the "receptives" and the "immunes," research lasts approximately 3-4 hours, a period that includes the time for taking the blood sample, separating the serum, preparing the serum dilution, as well as the actual technique of immunofluorescence in two strata.

The practical determination of the relations that might exist in a given minimum titre of circulating antibodies detectable through immunofluorescence and the state of resistance (immunity) to natural infection still remains to be established by lengthy research which cannot be brought to a proper completion except in regions with endemic smallpox.

In view of the fact that a very small quantity of blood is needed for execution of this reaction, the tests can be made on a droplet of blood taken from the tip of the finger.

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