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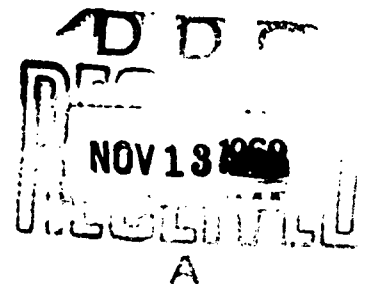
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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

PILOT EXPERIMENT ON THE DETECTION OF THE PATHOGENIC AGENTS
OF THE FIESSINGER-LEROY-REITER SYNDROME

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Paul Galistin

I - Introduction

In 1934 Marrach demonstrated the possibility of fixing certain fluorescent compounds on antibody molecules without causing any modification in the immunological properties. The basic principle of the technique was developed by Coons and Kaplan in 1950. It involves the optical demonstration of an immunological complex by chemical linking of the antibody with fluorochrome. Since the studies by Marshall, Eveland and Smith (1958), fluorescein isothiocyanate has been used. It is easy to handle and does not alter the immunological properties of the antibody.

In the case of accine, this technique permits detection of the antibody in the serum of animals.

We decided to make use of the technique in the diagnosis of inclusion blennorrhoea.

We utilized the indirect technique in two steps:

First, serum from the patient which contains viral antibodies is made to act on the antigen. An antigen-antibody complex is thus formed.

Secondly, human gamma globulin serum is reacted with the coloring agent. The fluorescent antibodies are fixed to the antigen which then appears fluorescent.

In our particular case, we attempted to demonstrate the antigen, i.e. the viruses. (These were patients with viral urethritis, where the

viral inclusion had been detected in the urethral epithelial cells and, by cultures, in the chorioallantoic membrane and the vitelline sac).

However, this indirect technique can obviously also be used for the detection of antibodies in the serum of patients who are presumed to have the disease.

II - Material

In the case of the vaccine, the antigen consisted either of commercial vaccine or an inoculation in the vitelline sac or the chorioallantoic membrane (smear).

Antibodies were found in the pure undiluted serum of inoculated rabbits.

In the case of inclusion blennorrhoea, the antigen consisted of:

urethral smears from patients with nongonococcal urethritis, or

smears from the vitelline sac and chorioallantoic membrane of embryonic eggs inoculated on the seventh day (with the product of urethral secretion from patients exhibiting viral inclusions in the urethral epithelial cells, Giemsa stain, positive).

III - Technique

We shall not describe the conjugation techniques, which are classic and were recently described by Nairn. The techniques comprise several steps of which we shall take up a number of points:

So far as the vaccine was concerned, we used normal anti-rabbit serum (goat anti-rabbit globulin conjugated - microbiological associates, USA).

For inclusion blennorrhoea, we utilized human antiglobulin serum, either in the form of a specially prepared serum or as serum 1299 from the Pasteur Institute. It was obtained by prolonged hyperimmunization of horses or rabbits. The antibodies produced by immunization are contained in the gamma globulin fraction. This fraction is isolated by precipitation with ammonium sulfate in the cold. Then the following steps are performed:

Dialysis of the globulin solution and determination of its nitrogen content;

Conjugation in the cold with fluorescein isothiocyanate;

Elimination of the excess stain not fixed to the antibodies by gel filtration with Sephadex G 25.

Absorption of the fluorescent conjugate on powdered mouse liver to remove the nonspecific fluorescence.

This method is simple:

The smears are fixed for 15 minutes at laboratory temperatures with a mixture of pure acetone. They are then kept in a second bath of 90% alcohol for 15 minutes. The slide is dried around the smear area.

Several drops of the patient's serum which contains antibodies are placed on the flat slide. Contact is maintained for 30 minutes and evaporation prevented. Any possible antibodies will become fixed to the virus during this time. The slide is then washed for ten minutes with cold buffered physiological salt solution (pH 7.4) and dried around the smear area.

The latter is recovered with one or two drops of the fluorescent conjugate.

Contact is maintained for 30 minutes at laboratory temperature. The slide is washed a second time with cold buffered physiological salt solution. The smear which is still damp is then covered again with a drop of buffered glycerin and a cover glass and examined under the microscope.

I - The fluorescence antibody technique

The May-Grunwald-Giemsa stain consists of a specific fluorescence which colors the inclusion elements blue.

Acridine orange

The use of histochemical methods made it possible to ascertain that desoxyribonucleic acid is found in the inclusions of the largest viruses, e.g. vaccine. Other authors have allegedly found DNA in all the viral inclusions.

Two principal theories can be used to explain the nature of these inclusions: they may be the abnormal products of the cellular metabolism following viral modification, or actual colonies of viral particles agglomerated or supported by a matrix of cellular origin.

For neurovaccine: phosphorus and nucleoprotein metabolism stops in the cytoplasm while large quantities of proteins and DNA accumulate in the nucleus.

For viral blennorrhoea: cultivation on embryonic egg, on the chorio-allantoic membrane or in the vitelline sac and on the HeLa cells. Formation of ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) and the particles of these acids are observed under the fluorescence microscope.

Observation of the slide takes place in a dark room equipped with a mono or binocular microscope with a dark base and immersion objective. An Osram HBO 200 mercury vapor burner with an anticaloric filter and a Zeiss EG 12 excitator filter is the ultraviolet light source. The stop filter is a combination EG 23-OG 5 (Zeiss). We used a high-sensitivity Iktachrome (Kodak) film for microphotography. Daylight. Exposure time, 30 to 70 seconds. Observation of the slide with the commercial vaccine revealed a strong yellow fluorescence. The smears from the patients with inclusion blennorrhoea, those from cultures of the chorioallantoic membrane and vitelline sac exhibited yellow fluorescence.

Staining with Acridine Orange

- 1) Fix in Carmoy for five minutes at laboratory temperature.
- 2) Passage in absolute alcohol, then in a series of 90%, 80% and 70% alcohols and finally in water.
- 3) Five to ten minutes in McIlvaine's buffer at pH 4.
- 4) Five to ten minutes in acridine orange, 0.01% solution in the buffer.
- 5) Rinsing for three minutes in the buffer.
- 6) Dry slightly on blotting paper.
- 7) Mount between disks in the buffer and then inspect under microscope in ultra-violet light.

Acridine orange stains ribonucleic acid (RNA) red and desoxyribonucleic acid yellow orange. Nevertheless, the developmental stage of the lesions must be taken into consideration because the intensity increases rapidly and reaches a maximum and then drops rapidly, finally leaving a hyaline inclusion. After four to eight hours of incubation at 35°, it is very interesting to observe a sort of diapedesis, i.e. a change of position in the cytoplasm and nucleus. A large particle of ribonucleic acid, which is usually in the nucleus, is seen in the cytoplasm. After a period of 10 to 14 hours, there are two particles. As the incubation period continues, the particles increase and contain initial and elementary factors.

After infection with vaccinal virus, RNA in the cytoplasm of the chorioallantoic cells and the vitelline sac and HeLa cells is stained with

acridine orange which is very visible two hours after infection. Two to ten hours after infection, DMI is observed to be fluorescent in the nucleus and there is a very strong color, which is probably due to pyknosis.

This staining technique is the fastest for vaccine and the other pathogenic agents.

Discussion in terms of viral etiology:

It seems appropriate to recall that the viral etiology was predicted a very long time ago. Linder, Fritch and Hofstaetter found it in the maternal vagina and the human urether (1910).

In 1931, Dr. Morax said: "If we remove a little epithelium from the conjunctive surface of the tarsus, fix it in absolute alcohol and stain it with Giemsa solution, the protoplasm of the epithelial cells will reveal inclusions that are identical to those of the initial trachoma." (Current theory, already old and forgotten).

Harkness (1945), who might be called the father of nongonococccic inclusions emphasizes the importance of studying these syndromes and their pathogenicity in his remarkable book "Nongonococcal Urethritis".

In 1949, Durel and Siboulet performed remarkable investigations on nongonococccic inclusions.

Mollaret incorporated these in the group of urethral myagawanellosis.

In 1961, 1962, 1963 and at present, this author together with Siboulet performed investigations which are still in progress. We presented our virological theses on the basis of our preliminary results, based on cultures in positive embryonic eggs and those of KB, HeLa and ape kidney strains. We worked with urethro-conjunctive-synovial smears. Embryonic anomalies were ascertained, viral antigens were observed in immunofluorescence (Siboulet Galistin and Hurez) and now in Giemsa and acridine orange fluorescence. This latter reaction permits super rapid diagnosis of the viral disease.

In 1959 and 1962, studies by Collier, Jones and Smith demonstrated the existence of a common antigen for the trachoma and inclusion blennorrhoea virus, as foreseen by Morax in 1931. Unfortunately he had written: "It seems that there is nothing to justify this hypothesis", and the struggle continues.

Conclusions

Subject of this communication: The technique of immunofluorescence and acridine-orange fluorescence.

This practical technique appears to permit very rapid diagnosis. It was successfully applied to vaccine, to two cases of Fiessinger-Leroy-Reiter syndrome, to one case of bullous ectodermic necrolysis, pluriorificial erosive ectodermosis with Lyell's syndrome (patient treated by Prof. P.-F. Merklen, Hôpital Saint-Louis), which will be taken up in a subsequent communication, and one case of Behcet's syndrome (patient treated by Prof. Offret, Hôpital Cochin, subsequent publication).

The aim of this communication is to draw attention to the technique of immunofluorescence, to Giemsa immunofluorescence and to acridine-orange fluorescence. This technique provides valid arguments for the diagnosis of viral diseases, which are probably as reliable as the electron microscope with its pictures which are much less valuable. The only valid solution is in vivo culture of the virus with the immunofluorescence and Giemsa and acridine-orange fluorescence techniques.

Summary

The chicken embryo is inoculated with vaccine, which is injected into the vitelline sac on the seventh day or into the chorioallantoic membrane on the eleventh day.

The same technique is utilized for vaccine.

The smears, which were exposed to vaccinated rabbit serum, made it possible to demonstrate fluorescent particles corresponding to viral antigens. The same smears did not exhibit any fluorescence when they were put in contact with normal sera.

Comparative pilot technique.

The smears, which were prepared from urethral secretion of patients with viral urethritis or from vitelline sac or chorioallantoic membranes of embryonic eggs inoculated with urethroprostatic secretions from these patients (positive culture) on the seventh day, were processed according to the technique of immunofluorescence and Giemsa and acridine-orange fluorescence. Serum from a patient with a urethro-conjunctivo-synovial syndrome of viral origin was utilized.

Subject of this communication:

Rapid practical technique applied successfully to vaccine, in the Fiessinger-Leroy-Reiter syndrome and in Behcet's disease.