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RESULTS OF IMMUNO-FLUORESCENCE INVESTIGATIONS USING FLUORESCEIN ISOTHIOCYANATE

/Following is a translation of an article by Peter Geck, Mrs. Sandor Horvath and Denes Karasszon in the Hungarian-language periodical Kiserletes Orvostudomany (Experimental Medicine), Vol 14, No 5, Budapest, 1963, pages 513-518_7

Following the pioneering work of Coons and his associates (1), the immunofluorescence methods of investigation for displaying bacterium and virus antigens, gained increasing popularity primarily in the micro-biological laboratories. (2)

Coons and his associates have linked the antibody with a fluorescent dye: fluorescein isocyanate, and used the resulting "conjugatum" as a specific histo-chemical dye. In recent years numerous other fluorescent dyes have been used for the marking of the antibody. Among these, according to domestic experience as well (3, 4, 5,), and one marked Lissamine-Rhodamine B-200 yields excellent results.

According to the data given in literature, the numerous difficulties, associated with the original Coons-method, can be most perfectly eliminated by the application of fluorescein isothiocyanate, perfected by Riggs and his associates, (6) for this reason we have conducted our investigations using the latter method. The indirect dyeing method of Weller and Coons (7) served as the basis. We selected as our models the Clostridium perfingens strain as well as the type 3 attenuated poliomyelitis virus strain.

In the interest of promoting the wide-spread domestic use of this extremely valuable process we wish to publish the results gained in the course of its application.

Methods and Materials of Investigation

I. The Preparation of the Conjugatum

1. To produce anti-rabbit globulin, we have drained the blood of rabbits under sterile conditions, fourn to be healthy during an observation period lasting two to three weeks. From their native serum, having eliminated para-typhoid infection, we precipitated the globulin fraction, using ammonium sulfate: a mixture of 40 weight parts of concentrated ammonium sulfate, plus 60 weight-parts of native rabbit serum was left overnight at a temperature of 4°C, then it was centrifuged for 30 minutes at 3,000 revolutions. Having skimmed off the top, the sediment was diluted with distilled water in the original proportion of the plasma. This process was repeated twice, but we found it more expedient to reduce the second dilution to half of the original quantity. The resulting globulin fraction was dialyzed in a cellophane bag for four days, against a biological solution of common salt at 4°C. The ammonium sulfate content of the dialyzing liquid was checked by precipitation test performed with 10% barium chloride. After the negative result of the precipitation test, on the fourth day, the globulin fraction, and later the globulin fraction emulsified in a quantity in the Freund incomplete adjuvant (in a mixture of 1 part ARLACEL plus nine parts of Bayol), were used for the immunization of a young healthy goat in good condition.

As a basic immunization, we injected a mixture of 5 ml adjuvant plus 5 ml globulin fraction intra-muscularly, followed by intra-muscular injections of 5-5 ml native globulin every second week. For seven days after the third immunizing injection we checked by precipitation test the anti-rabbit titer of the goat serum and found that it will yield positive results even in a 1:7,000 dilution. Out of this goat serum of satisfactory rabbit-precipitation titer, we again precipitated the globulins, using the above described method. After dialyzation, we conserved the resulting globulin fraction with a 1:10,000 solution of merthiolate, and stored it at 4°C until used.

2. Conjugating fluorescent dye with the immunoglobulin, according to the process of Riggs and his associates (6). We perform the micro-Kjeldahl or biuret process in order to determine the protein content of the immunoglobulin to be conjugated. According to our experience, if necessary, the determination can be accomplished satisfactorily,

for practical purposes, with the aid of the Phillips-Van Slyke (8) specific weight determination as well.

Having determined the protein content, we diluted the globulin fraction with a 9 pH biological kitchen salt carbonate-bicarbonate solution containing 10 mg protein per ml.

The next step was to pour into a 50 ml capacity Erlenmeyer flask 10 ml biological common salt solution and a 3 ml 9 pH carbonate-bicarbonate buffer solution, followed by it with dry ice until ice crystals were formed, the, while stirring continuously with a magnetic mixer, added 10 ml of diluted globulin solution of the known concentration. We continued to cool the solution-mixture, then added, while constantly stirring, the needed quantity of fluorescein isothiocyanate (marked: VUFB 101061), dissolved in 1.5 ml acetone, figuring 5 mg dye powder for 100 mg protein. Further conjugation procedure was carried out at a temperature of 4°C: we placed the mixture, together with the magnetic mixer, in a refrigerator, and for 18 hours (over one night) stirred it slowly, avoiding foam formation. Subsequently, we placed it in a cellophane bag and dialyzed it against a 7 pH buffered biological solution of common salt, renewed several times, until the dialyzing liquid no longer fluoresced under blue light. The dialyzate was then transferred into test tubes and stored in a deep freezer

3. <u>The preparation of tissue powder according to Coons' process</u>. We diluted homogenized mouse liver with approximately identical volume of biological solution of common salt. To the resulting suspension we added about four times its volume of acetone, let it stand for a few minutes then skimmed it. The remainder was washed in biological common salt solution several times, put in a centrifuge, the sediment diluted with an identical volume of biological solution of common salt and four times its volume of acetone added to it. This process was repeated a third time, and we finished by straining through filter paper, in a Buchner funnel. The several times washed and extracted liver powder adhering to the filter paper was dried overnight at 37°C, and the finished tissue powder was stored in a well sealed glass vessel at 4°C.

For the purpose of purification, we mixed mouse liver powder with the conjugate in the quantity of 100 mg/ml, we let the mixture stand at room temperature for one hour, then we centrifuged it at 3,000 revolutions for a few minutes.

This process may be repeated two or three times before utilization, and in the interest of more completely exhaustive effect, we may run the centrifuge at 10,000 revolutions.

II. Dyeing

1. Making the bacteriological preparation. A bacterium suspension, diluted with a biological solution of common salt, was thinly smeared on a clean, dry, de-greased slide. The smear was dried at room temperature, then fixed either by heat or by alcohol. In the latter case, the fixing liquid was left on the smear for ten minutes in a moist chamber (see later). After pouring off the fixing liquid, our preparation was dried in a thermostat at 37°C.

Subsequently, we first dripped some non-conjugated homologous rabbit immuno-serum onto the preparation, in 1:10 dilution, this treatment was continued in a moist chamber for 15 minutes, then, after thoroughly washing it in biological common salt solution, we left it to dry without blotting. We dripped the indicated anti-rabbit globulin ("conjugate") onto the dried smear, taking care to cover the entire preparation evenly. Handling time in the moist chamber was 15 minutes, and after the subsequent thorough washing and drying, our preparation was ready for examination.

2. Making virus preparation. We grew a trypsinized monkey-kidney epithelium tissue-culture on a slide, placed it in a potato test tube. On the seventh day, on the occasion of the first changing of the nutrient liquid, we mixed into the nutrient solution 10^{-3} quantity of type 3 attenuated virus strain, taken from domestically produced live anti-poliomyelitis vaccine (10). For the purpose of examination, after the 24th, 48th, and 72nd hours we lifted out the slides which, after having been washed three or four times in a solution of isotonic buffer-salt (PBS), were dried for an hour at 37°C. This was followed, after fixing in acetone for ten minutes at 4°C, by another 30 minutes of drying, then we dripped onto the preparation some immuno-serum produced by rabbits against type 3 poliomyelitis ("Sanket") virus strain, in 1:10 dilution, leaving it there for ten minutes at 37°C. After thoroughly washing it for ten minutes in a buffer solution, we dripped onto the slide some anti-rabbit globulin marked with fluorescent dye ("conjugate") this treatment was carried on for 30 minutes, at 37°C, in a moist chamber, to avoid drying out. We prepared a moist chamber by jamming filter paper, moistened with biological

common salt solution, into the lid of a Perti cup.

After the half hour effect-time had elapsed, there followed 20 minutes of thorough washing in several changes of buffer solution, then we sealed our preparation with glycerin diluted 1:10 with buffer solution.

3. In the course of the bacteriological examinations we used, for control, some foreign bacterium strain (most ofter E. coli), while for the examination of the virus preparations, the control used sometimes was a preparation not contaminated by virus, but otherwise identically handled, at other times the control was a preparation inoculated with virus but not treated with polio immuno-serum.

III. Microscope and Micro-photo

Our investigations were conducted in part with the aid of a Leitz fluorescence microscope marked "Ortholux", and in part with the aid of a Zeiss type "LgOg" microscope illuminated by a Zeiss "L" microscope light. The light is generated in the former by a 150 W, in the latter by a 50 W high-pressure mercury vapor lamp. We used a Schott BG 3/2 blue filter; to prevent the filter from overheating we used a 4% solution of copper sulfate (to eliminated red and infrared rays:; to protect our eyes from ultraviolet rays we employed protective glass filters marked GG9/OGI.

The photographs were made through the above listed filters, using the Leitz Ortholux microscope, with an "EXA" camera. Exposure time was eight minutes and extended developing time was used.

Results of Investigation

Bacteria treated with fluorescein isothiocyanate, using the above outlined procedure, emitted extraordinarily vivid light-green light (Figure 1.) in a fluorescence microscope employing approximately 400 power magnification. We designated the intensity of light emission by crosses. The bacteria were shining clearly, brightly with sharp outlines against the dark, ash-gray background. Impurities in the smears could be distinguished from the specifically dyed bacteria, besides their shapes, by having indistinct outlines of light gray color.

In the course of the investigations of tissue cultures infected with type 3 attenuated poliomyelitis virus, we noted both in the nucleus of the cell and in the cell plasma, diffused fluorescence, as well as the appearance of various sized grains of bright greenish fluorescence (Figure 2.)

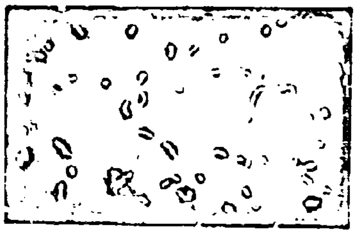

Figure 1. Immuno-fluorescent image of Clostridium perfingens. Fluorescein isothiocyanate, indirect dyeing. Magnification about 400 times.

Figure 2. The immuno-fluorescent image of attenuated poliomyelitis virus propagated for 48 hours in a primary monkey-kidney-epithelium tissue culture. Fluorescein isothiocyanate, indirect dyeing. Magnification: about 400 times.

Having examined the same preparation through both microscopes, we found that using the Ortholux equipment m manufactured by Leitz, the radiance appears stronger, the outlines, particularly when using greater magnification, sharper, and the entire image is more suitable for photographic purposes, if for no other reason that this microscope light is slower to heat and can be left turned on for longer periods. On the other hand, the advantages of the "L" microscope-light manufactured by Zeiss are its simplicity, relatively low price, and its ability to be used with all microscopes, consequently we believe that, particularly for routine diagnostic work, it is excellently suited for use in smaller laboratories, while the larger, high performance equipment is more suitable for research purposes.

We stored bacteriological preparations (smears) by simply drying them we found, however, that it was more expedient to evaluate the preparations, dyed with fluorescein isothiocyanate, within 24 hours, since they proved to be less durable relative to preparations dyed withother fluorescent dyes. This finding is even more valid for virus preparations, these could not be stored without lossof radiance for more than a few days, even though sealed with buffer-glycerin and stored in a refrigerator.

Evaluation of Results

Fluorescein isothiocyanate excels other fluorochroma used in immuno-fluorescent examinations, due to its stability its easy conjugability, and to the color and strong radiation intensity of the preparations dyed with it. Our findings in this respect support in all aspects the similar findings of foreign authors (11).

In the course of conjugating we followed the original Riggs procedure, which we specified in our description, although we know that a simpler method is available by now. We did this primarily for the purpose of testing the dye itself, but also we did so to make the classic process available, in the Hungarian language, for domestic laboratories. The essence of the more recent process is the addition of the fluorescein isothiocyanate -- on the basis of subsequent observations by Riggs -- at 9.0 pH to the serum to be marked, simply in powder form, in the desired quantity. Besides its simplicity, the main advantage of the above method is the complete elimination of the use of acetone, which has a destructive effect on proteins. Our further investigations are being conducted with the use of conjugates prepared according to the more recent points of view.

The tissue-powder absorption aims not only at the elimination of grains of dye that have remained unbound, but it also minimizes to a certain extent the possibilities of non-specific dyeing (12), and, according to certain investigations (13), adequately accomplished absorption even renders dialyzation quite unnecessary. Most recently, the quantitative separation of unbound dye from the marked antibody was accomplished by a gel-filtration process: Sephadex G-50 (14), with encouraging results.

Recently the addition of polyvinyl alcohols to the sealing liquid is being recommended, to increase the durability of the preparations (15).

The indirect method is more suitable, from various points of view, than the simple direct method, particularly for the investigation of viruses (16). When using the indirect method, it is not necessary to prepare a spearate antibody conjugate against each antigen; due to its much higher sensitivity, the indirect process yields successful results using lower order immuno-serums, either laboratory diagnostic serums or convalescent serums.

A further refinement of the indirect process is the displaying of complement binding antigen through the immunofluoresc...ce process (17), this method was successfully applied to the poliomyelitis virus (18), and with its aid the sensitivity of the method can be further increased.

Summary

The authors discuss, primarily from the point of view of methodology, their immuno-fluorescence investigations accomplished with the use of fluorescein isothiocyanate, They give a detailed description of the production of immunoglobulin; the preparation of the conjugate and tissue powder absorption; dyeing; the evaluation of preparations; they describe the practical application of the fluorescence microscope; and they briefly digress to the making of microphotographs. By describing the process to enable anyone to reproduce it, they wish to promote its widespread application.

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