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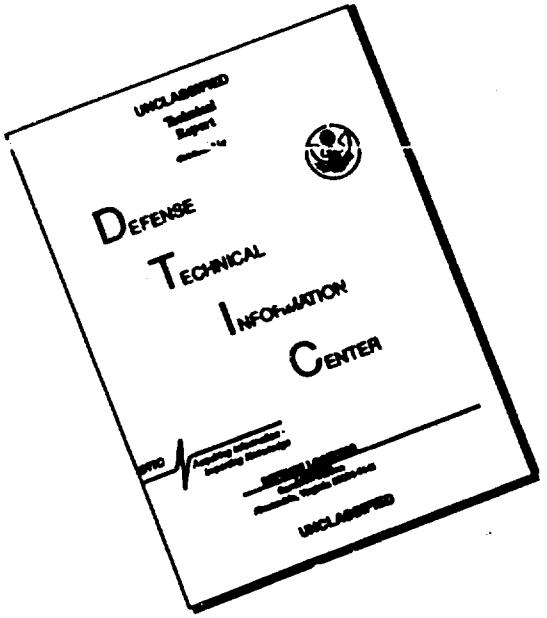

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IMMUNOFLUORESCENCE IN INFRAMICROBIOLOGICAL RESEARCH

Studii si Cercetari de Inframicrobiologie
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A. Mihail

New methods introduced into science are spread rapidly or slowly depending upon the utility and ease of their application; in general, at the beginning it is difficult for a new method to spread and even more difficult if the application of the new method is connected with the use of relatively complex equipment or substances which do not currently exist in the laboratories. As it spreads, however, the method is improved; each researcher tries to improve it and at the same time to simplify it, to make it more accurate, more accessible, which, consequently, makes the method itself easier to spread.

It is seldom a matter of a completely new method, but rather, usually, if we read in the literature of the field we find numerous works directed toward the same findings in which outlines of the new method are found. For example, there is the case of the trypsinization method which was introduced into the current practice of cell cultures in 1954 by the works of Dulbecco, Vogt and Younger; however, the first works in this problem were written 40 years previously by Rous and Jones.

In light of these aspects, it is very difficult, often even impossible to establish a single paternity and a date of origin of some new work methods. If, nevertheless, we should wish to draw up a birth certificate for the method of antibodies tagged with fluorescent substances, we would list the date as 1942 and the parents as Albert Coons, the god parents being Berliner, Creech and Johnes. The place of birth -- Harvard University; the circumstances -- the study of the presence of the pneumococcal polysaccharide in mouse tissues.

The method of fluorescent antibodies is based on two different phenomena, the first belonging to immunology, that is, the precipitation of the antigen-antibody complex, and the second physical, the phenomenon of fluorescence.

The precipitation of the antigen-antibody complex was known as early as the end of the 19th century through the works of Kraus; the phenomenon of fluorescence even earlier, before even hearing of antigens and antibodies, in the pharmacological works of Poterius in 1624.

Without going into details about the phenomenon of fluorescence, we do mention that fluorescence microscopy, developed by Lehman in 1911, was regularly used after that date in microbiology and was used in 1917 by Kaiserling to facilitate the observation of the Koch bacillus. In the field of microbiology, fluorescence microscopy gained wide usage through the works of Hagemann, Himmelweit and Hoffmann in 1937 and was developed fully in the hands of Levaditi, who studied vaccine virus with this method.

We can see that two component elements of the technique existed: the merit of Coons is that he combined them into a single method, with multiple possibilities for utilization in theoretical and applied immunology, in microbiological and virusological research, or in current diagnostics through the possibilities in this method for following the antigens introduced into the organism or of determining their place of formation.

It is not, however, a question here of a virgin field; research in this direction had been made much earlier with other methods.

Thus, in 1897, Mechinikov determined the proportion of tetanic toxins in different organs by observing the toxicity of the extracts from these tissues by innoculations of mice; similar work with different toxins were made -- a century later -- by Bieling and Gattscholk.

A step forward in the direction of following antigens in the organism was made by Haurowitz and Breini, who innoculated rabbits with horse serum tagged through bonding with arsen and determined the arsen content of the different organs. Compared to previous methods, the progress was important since from that time it was possible to follow any antigenic substances, not only toxins, through coupling them with arsen.

The work of Heidelberger improved the method by coupling a coloured compound to the protein-antigen, which made it possible to observe it directly in a microscope in the form of coloured granules, without having to make chemical determinations.

These methods, however, have some major disadvantages, among which the most important is the modification in the specificity of the antigen, as a result of the coupling; secondly, they are useful only in following the fate of an antigen introduced from the outside and do not permit the disclosure of the appearance of an antigen in the organism or the localization of the antigen in the cell tissue.

Through the Coons' method of immunofluorescence, the disclosure of the antigens is realized only physiologically, through their property of reacting and the fixing of the homologous antibody, tagged in advance by coupling it with a fluorescent substance. This tagging has no effect on the functional specificity of the antibodies.

Among the modern techniques which can be used for following and localizing antigens, we mention the method of tagging with radioactive isotopes. This method has a much greater sensitivity than that of immunofluorescence, being able to show up substances in concentrations of only 10.3 x 10 to the minus 3rd power microgram/gram, while the former is limited to only 1 microgram/gram tissue. Even more closely allied is the method of localizing antigens using antibodies tagged with ferritine, used in electronic microscopy.

Fragments of the tissue studied to disclose the antigen are covered with serum containing antibodies of the particular antigen. This method does allow the antigen to be fixed, while the excess of unfixed antibodies is eliminated by washing. Proof of the antigen-antibody reaction is made by examination of the preparation in ultraviolet light; the presence of the antibody, which was coupled ahead of time with a fluorescent substance, is indicated by a fluorescence which permits not only the recognition of the existence of the antigen, but also the localization of the antigen in the tissue and even in the cell. This is the technical schematic through which Coons 20 years ago developed the basis for immunofluorescence. The method has now progressed in all directions, both in regard to the materials which enter into reaction, especially the tagged fluorescent substance, and also in regard to the actual reaction itself. Without going into details related to the preparation of the necessary materials, we will describe briefly the elements that enter into the reaction, the conditions for realizing the reaction, and the examination of the preparations.

After a number of preliminary tests in which the antibodies were coupled with anthracene, Coons, in collaboration with Creech, Johns and Berliner, synthesized a derivative of fluorescein, isocyanate of fluorescein, which was capable of reacting with proteins and of joining with them through the phenyl radicals which formed carbamidic bonds with the lysine in the proteic molecule.

In ultraviolet light the isocyanate of fluorescein gives an easily visible yellowish-greenish luminescence, corresponding to the maximum chromatic sensitivity of the human eye.

The isocyante of fluorescein still has the difficulty of being a difficult preparation involving the use of phosgen in its gaseous state, known to be toxic; at the same time the substance is slightly unstable.

At the present time, the isocynate of fluorescein is tending to be replaced by another derivative, isothiocyanate of fluorescein, which was synthesized by Riggs. In comparison with the other derivative, this substance has the advantage of being easier to prepare and, of special value, has a good stability so that it can be preserved for a long time.

At the same time, tests have been made using other substances than derivatives of fluorescein. Good results have been achieved in the use of lissamin-rodamine, rovamine and 5-dimethyl-1-naphthalene-sulfonilcloride. After the coupling of the fluorescent substance under well determined pH and temperature conditions, an important stage in the immunological serum or the globuleinic fragment of this serum is the elimination of the residues of the fluorescent substance which remained unbonded and which are generated by unspecific fluorescence. Instead of extensive dialysis, lasting 6-8 days, used up to now, greater and greater use is now made of purification with the aid of adsorbtion of the anionic resins, filtering with jellies of the Sephadex type, which have the property of letting through the bonded protein and holding the colourant unfixed by it, or adsorption with active carbons. Through these methods, purification can be accomplished within 1-2 hours, under optimum conditions. Also, for elimination of the unspecific fluorescence, the bonded antibodies must be adsorbed with powders by different organs, among which the most frequently used is the liver, and, in some cases, the bone marrow.

The test tissues must be prepared in such a way that not only is degradation of the antigen avoided, but also the mobilization of the antigen in the place where it is located. In the case of sections of organs, the tissues congeal right after they are taken out at minus 70 degrees or more in a carbonic snow or liquid propane. The sectioning is accomplished in the congealed state with special instruments in a cooled chamber or after drying the congealed tissue. When these conditions cannot be realized, it is possible to make prints of the test tissue, also on cooled plates. The problem is much easier when working with cells cultivated in vitro in a monostratum, since sectioning is no longer necessary. The fixing is made with fixatives which do not change the antigenibicity and which accentuate the autofluorescence of the tissues. The best results have been achieved with alcohols, especially acetone.

Examination of the preparation is made with a standard microscope, with a light source that is rich in ultraviolet. Instead of an arc lamp, almost exclusive use is made of high pressure mercury vapor lamps with quartz tubes, which are much easier to use. The intensity of these lamps must be sufficiently great to provide a fluorescence not only easily distinguishable to the eye, but also to the photographic film. They are currently working with lamps between 200 and 1,000 watts.

The quartz optics are not indispensable, since the ultraviolet rays with lambda equals 360 microns are not retained by the optic plate. Use is made of clear or darkened base condensors, which ensure a greater contrast between the fluorescent elements and base, but which requires, on the other hand, a much greater source of light. Of great significance are the filters, that is, the excitation filter or filters situated between the lamp and the microscope which must stop the rays in the field of visibility and permit the passage of the ultraviolet rays or the rays at the beginning of the blue spectrum; the second filter, for stopping, is located between the preparation and the eye of the examiner or the film and allows the passage of only light in the field of visibility, stopping the ultraviolet rays not absorbed by the preparation.

At the present time, immunofluorescence knows many variations, all connected, however, with the same reaction of the antibody with the antigen.

The simplest form and also the initial form of this reaction as developed by Coons is the direct reaction.

The test preparation is covered with several drops of serum which contains antibodies coupled with fluorescent substances. After an incubation of 30-60 minutes, the serum is taken off and the preparation is washed to get rid of the unfixed antigens, and then the preparation is examined under the microscope. The presence of the antigen and its localization is marked by the fluorescence of the antibodies which have been fixed by it; the cells or portions of cells which do not contain antigens and which therefore have not fixed the antibody coupled with the tagged substance remain dark.

This method has the advantage of simplicity, but requires the preparation of as many types of coupled immunological serums as there are different types of antigens studied.

An improvement of the method, developed by Coons and Weller, is the indirect technique or sandwich technique. The test preparation is covered with an immunological serum which is uncoupled, but including a fluorescent substance. In this case also the antibodies will be fixed by the antigen present in the preparation. After washing the unfixed antibodies, the preparation is treated with a new serum which contains antibodies directed against the globulins of the species from which the first serum was taken. This second serum, in contrast to the first serum, is coupled in advance with the fluorescent substance. For clarity, we will give a concrete example.

We are trying to show the herpetic antigen in a section of tissue. We cover the preparation with a rabbit serum that is antiherpetically immunized, then, after getting rid of the unfixed antibodies, we treat the preparation with sheep serum that is antiglobulinally immunized from

rabbits and coupled with fluorescent substance. The antibodies of this serum will react with the antiherpetic antibodies -- rabbit globuline -- and will be fixed by it. Thus, there will be formed an immunological sandwich, a superpositioning of antibodies connected by the two reactions: herpetic antigen -- antiherpetic serum and rabbit globuline antigen -- antiglobuline rabbit antibody.

Although this modification in the method appears more complicated, in fact it represents a simplification of it, since it is no longer necessary to prepare both the coupled serums and equal number of antigens under study; it is only necessary to have simple immunological serums which are uncoupled prepared for a certain specie and a single, uncoupled, anti-specie, immunological serum from which the first serum were prepared. The advantage of this method appears obvious to us if we think of one of the possibilities of working with this method, that is, the disclosure of antibodies in human serums. Instead of coupling each of the test serums with the fluorescent substance, it is sufficient to have a single antiglobuline, coupled, human serum.

In addition to this, the indirect method also gives better results in respect to the specificity and intensity of the fluorescence. This is explained by the phenomenon of multiplicity in the coupling of the antibodies to the antigen.

Finally, a new modification in the method has been introduced by Goldwasser and Shepard. As in the indirect technique, the preparation is treated with a specific, uncoupled human serum that is mixed with fresh, normal guinea pig serum or purified complement. This is coupled with the antigen-antibody complex formed in the preceeding reaction. Localization of the antigen is accomplished with an antiglobuline guinea pig serum coupled with the fluorescent substance, which will show the place where the complement was fixed, and therefore the place where the antigen-antibody reaction took place. This technique reduces the number of coupled serums to only one, the antiglobuline guinea pig serum. Actually, if in the direct technique each serum used had to be coupled with the fluorescent tag, while in the indirect technique it was necessary to have one coupled serum for each specie for which immunological serum was being prepared, in the Goldwasser technique, through the introduction of the complement and, in the final instance, the disclosure of it, there is no longer any importance to the specie from which the first immunological serum comes.

The application of immunofluorescence is not, however, restricted only to the disclosure of antigens, but can also be used to disclose antibodies.

Two techniques, one direct and one indirect, have also been described in this case, but here the difference between them does not consist only in the modality of the disclosure, but in what is disclosed.

Actually, through the direct technique, which consists of covering the test preparation (for example, a print of rabbit spleen) with an anti-gammaglobuline rabbit serum coupled with the fluorescent substance, it will be possible to disclose the cells that contain gammaglobuline, regardless of their immunological specificity.

The indirect technique, as in the case of the disclosure of the antigens, involves the formation of an immunological sandwich. The test preparation is covered with a layer of antigens which are homologous to the antibodies which one desires to disclose. The antigen will be fixed by those cells and in those cell zones in which the antibody is present. After getting rid of the unfixed antigen excess, the preparation is covered with a coupled serum containing antibodies from the antigen in the preceeding layer. In turn, these antibodies are fixed by the antigen of this layer, thus marking through their fluorescence the place of the antibodies in the cell. In contrast to the direct method, the indirect method not only generally localizes the antibodies, but also determines the antibodies in light of their immunological specificity and determines the presence of the antibodies in relation to a well determined definite antigen.

These are briefly the principles of the technical modality of immunofluorescence. Depending upon the goal desired, there have been described numerous possibilities and methods which have, however, less interest from the standpoint of immunobiological research.

Before proceeding to discussion of some of the results achieved, it is necessary to dwell on an especially important aspect and one which often is no less thorny, that is, the interpretation of the specificity of fluorescence and the elimination of unspecific fluorescence.

First of all, it must be pointed out that there exists a natural fluorescence or autofluorescence of tissues which can be accentuated by certain factors such as fixing with certain substances, including formol, preservation under unsuitable conditions, etc.

In general, this autofluorescence of a light bluish colour is not too strong and can be easily differentiated from the yellowish-greenish fluorescence of the fluorescein, the orange of the rodamine, the yellow of the dimethyl-naphthalene-sulfenil chloride, etc.

Much more difficult, however, is the differentiation and elimination of the unspecific fluorescence produced by the fluorescent substance itself.

The principal causes of the unspecific fluorescence are:

-- the presence in the conjugated serum of the fluorescent substance unconnected to the molecule of the antibody;

-- the presence in the conjugated serum of antibodies that react with the cellular substratum, even in the absence of the antigen being tested;

-- an adsorption of the antibodies conjugated by cells, even in the absence of an immunological reaction, usually owing to electrostatic forces.

Various procedures have been imagined for elimination of the causes which generate the unspecific fluorescence. Thus, for elimination of the uncoupled fluorescent substance, the serum is subjected to a dialysis of 6-8 days, or, using more modern techniques, is filtered through a Sephadex jelly which permits the elimination of the uncoupled substance in a much shorter time. Also, for this purpose some authors recommend adsorption through active carbon, diatomic powders, or DEAE cellulose powders.

The elimination of the heterologous antibodies and those which tend to be adsorbed unspecifically is accomplished through the [illegible] the conjugated serum with powders from the liver or bone marrow of different species of animals; also, tests have been made on blocking the unspecific adsorption points of the cells with another protein, for example, simple or linked albumine, and as with another fluorescent substance of another colour, in order to create a contrast. This latter method also has the advantage of allowing a better observation of the cytoarchitecture and an easier determination of the localization of the fluorescent zone.

In order to be able to consider that it is a matter of a specific fluorescence, any staining procedure involves a number of controls, among which are the following:

-- test for the absence of fluorescence in a preparation not having the test antigen, placed in contact with the serum or serums which are coupled for working;

-- test for the absence of fluorescence in a preparation containing the test antigen, but treated with a coupled heterospecific serum;

-- test for the inhibition of fluorescence in a preparation which contains antigens, but which is treated in advance with uncoupled homospecific serum and then with coupled serum. This test shows that by blocking the antigen with the uncoupled antibodies, the coupled antibodies will no longer be able to react with it.

Since the start of the introduction of the technique of immunofluorescence and up to the present, extremely numerous works have been realized in all fields of inframicrobiology, both in diagnostic and research work. For this reason, we have preferred to treat only a few

aspects, to present just some of the results achieved, and, in particular, those which have in turn brought up new aspects in the virus-cellular relationship or which have raised special problems.

First of all, it is necessary for us to try to answer the problems brought up by the new method of immunofluorescence in relation to other research methods.

With the aid of this method, we can penetrate through the limits imposed by the classical methods, deeper into the heart of the phenomena of infection and the synthesis of viruses in the cells. From this point of view immunofluorescence represents for inframicrobian immunology what the electronic microscope represents for inframicrobian morphology. The fate of the virus, when it has passed into the cell, can no longer be followed until a new generation of viruses come out of the cell through the classical immunological means. With the aid of immunofluorescence, however, it is possible to follow the viral biosynthesis, even before the point is reached where the formation takes place of the elementary, completely infected corpuscles, and much later it is possible to determine the place of the viral synthesis in the cell for the different components of the virus.

This has been shown especially clearly by the tests made by Maassab and collaborators on some mixed viruses: thus, in the case of the influenza, parainfluenza 3 and pestes aviar viruses, it has been possible to bring out the following sequences in the synthesis of the viruses: the viral nucleoprotein is synthesized in the nucleus within only 4 hours from the infection, while the hemagglutination appears in the cytoplasm of the cells no earlier than 12 hours after infection. Other mixed viruses, however, such as the urlian or Newcastle's disease virus, are synthesized exclusively in the cytoplasma.

The synthesis of the vaccinal viruses also appears to be exclusively intracytoplasmitic. Thus, Job and Riggs succeeded in determining the successive appearance of three different antigens, components of this virus. The first to appear, 4 hours after infection, is the proteic LS antigen, followed 6 hours later by the appearance of the NP nucleoproteic antigen containing ADN, and, finally, in 10-12 hours, the hemagglutinating antigen. Only after all of these antigens have appeared is it possible to show the infectant virus; this is proof that they are real and necessary components of the viral particle.

In relation to the initial appearance of the fluorescence, Fresner divides viruses into 5 groups as follows: group 1 which has a strictly nuclear localization of the antigen and which includes the viruses of canine hepatitis, Shope papilloma, and type 4 adenovirus; the 2nd group with initial nuclear localization which then passes, however, into the

cytoplasma, including among others the viruses of influenza, herpes, some adenoviruses, and foaming simian virus.

The 3rd group has the antigen appear initially in the cytoplasma and then in the nucleus, as in the case of measles, polio, and, according to some authors, the vaccinal viruses. Group 4 includes the viruses whose antigen appears simultaneously in the nucleus and in the cytoplasma, including the distemper virus and mouse leukemia, while group 5 includes the viruses which give an exclusively cytoplasmic fluorescence, such as urlian, Newcastle, Sendaym ECHO, psittacosis, encephalytic viruses, yellow fever, etc.

This division, made on the basis of observations made by various authors, also contains contradictory data, for instance, the inclusion of one virus in a number of groups.

In the interpretation of the localization of an antigen consideration must be given to a number of possibilities for error, including, for example, the migration of the antigen from one compartment into another at the moment of cellular architectonic injury through the cytopathic effect or even in the process of preparing the preparation; the possibility that the fluorescent zone does not represent the place of synthesis of the antigen, but rather its point of concentration, with the antigen being formed in another zone where it is in such small concentration that it is not disclosed, or, finally, as we pointed out above, it might not be a question of antigen formed ex nuevo from the cell, but rather phagocyted.

Consideration must also be given to the possibility of the appearance in the cell, under the influence of the infected viruses, of antigenic substances which do not enter into the composition of the virus, as was pointed out in the case of the Newcastle virus.

In certain cases, the virus-cellular relations found could be studied only incompletely with the classical methods and many aspects remained incomprehensible or contradictory.

Thus, for example, it is known that the Newcastle virus is fixed by Ehrlich ascitic cells and has a cytolytic action on them; without, however, being able to bring out a multiplication of the virus in these cells, with the formation of active, infecting virus or even with hemagglutinate properties or complement fixing properties.

What is the mechanism of this cytolytic action without virus multiplication? Is it a question of a toxic, inhibiting action or a real infection followed by the synthesis of a new virus.

In tests made with the aid of fluorescent antibodies, Price and Ginsberg showed that the Ehrlich cells placed in vitro in contact with the Newcastle virus adsorb this virus on their surface, a fact which is evidenced through a peripheral fluorescence of the cells; however, regardless of the quantity of virus adsorbed and the conditions (of time and temperature) in which they are incubated, the cells do not show intracellular cytoplasmic or nuclear fluorescence which would indicate the penetration of the virus (and therefore of the viral antigen) into the interior of the cell or the synthesis of it. If, however, these cells are innoculated intraperitoneally in mice and then taken out at different intervals, it is observed that after a period of complete disappearance of the fluorescence, the fluorescence reappears localized by this time in the interior of the cell or in the cytoplasma. The fact that the fluorescence appears after a period of not being visible and that it increases with the passage of time indicate, without doubt, that this is a question of a synthesis of viral antigen in the cell. Also, the fact that the lysogenesis of the ascitic Ehrlich cells appears only if these cells are introduced into the peritoneum, that is, under the conditions under which this synthesis takes place and not in vitro, indicates that the cytolosis is directly connected with this synthesis and not with a toxic action. This synthesis, however, is imperfect or incomplete and does not succeed in reaching up to the formation of active, infectant virus, hemagglutinate or complement fixer.

Also connected to this problem of aborted, viral synthesis, we must mention a number of studies made in the field of the problem of cellular susceptibility, which many times is only apparent. Working with the pestos aviar virus, Schaier showed that in the cells of the chicken embryo it is possible to show the synthesis of the viral nucleoprotein in the nucleus and of the hemagglutinate in the cytoplasma. After the appearance of both fragments, the nucleoprotein migrates from the nucleus into the cytoplasma and is included, in the vicinity of the cellular periphery, with the hemagglutinate then being given off from the cell as a complete virus.

In contrast with the cells of the chicken embryo, the fibroblastic mice cells in the L stem are not susceptible in respect to the pestos aviar virus, since the innoculation is not followed by the formation of a new virus.

With the aid of fluorescent antibodies, however, it has been proven that this non-susceptibility is apparent, since -- as also in the case of the bird cells -- in these cells also it is possible to show the synthesis of the viral nucleoprotein in the nucleus and the hemagglutinate in the cytoplasma; in this case, however, the movement of the nucleoprotein from the nucleus into the cytoplasma does not take place and thus there is no formation of a completely new virus, creating the false appearance of non-susceptibility.

Interesting information on the host virus-cellular relations was also found in the case of infections of hamster BHK 21 cells with poliomyelitis virus, with a part of these cells being able to attain malignant characteristics and producing tumors when they were reinoculated into the respective animals. This transformation occurred, however, in only a relatively small number of cells, approximately 1-4%, even when massive viral innoculations of up to 1000 UFP/cell took place. Fraser and Gharpure, with the aid of fluorescent antibodies of innoculated mouse virus, tried to see if there existed a relation between the number of cells infected and the cells which suffered malignancy; they found that 5 hours after innoculation almost all of the cells showed a cytoplasmic fluorescence, with the nuclei remaining dark. This fluorescence, however, diminished and disappeared after 3-4 days, in a rate equal to the rate at which the cells continued to divide. Concommitantly, however, a nuclear fluorescence appeared in some cells. The authors interpret the initial cytoplasmic fluorescence as a passive fluorescence due to the presence of the viral antigen in the cytoplasma as a result of its phagocytosis, and not its synthesisization, while the disappearance of the fluorescence is due, they claim, to the dilution of this antigen as a result of successive cell division. The true viral synthesis should take place only in those cells which show late, nuclear fluorescence. The authors feel that only these cells also suffer malignancy, a fact also sustained by the coincidence between their frequency and that of cells capable of producing tumors in vivo. These observations, apart from their interest in the problem of the oncogenous role of some viruses, also show the lack of homogeneity of apparently homogeneous cell populations, in which because of the fact that the virus penetrates into all of the cells, only some of them are capable of reaching the point where they can synthesize viruses.

The influenza viruses have formed the object of numerous studies with the aid of fluorescent antibodies. We will treat only a few of these, also in connection with the problem of the host virus-cellular relations, especially in connection with the importance of the multiplicity of infection and the number of viral corpuscles which infect each cell. Watson's studies, made by innoculating influenza virus into coriolantoid membrane and, with the aid of fluorescent antibodies, following the appearance of viral antigens in the cells, have shown that the different localizations of the virus in the cell, depending on the massiveity of the innoculation. Working with doses varying both on 1,000 corpuscles for a cell and one corpuscle for 1,000 cells, the author found that with the small doses the viral antigen appeared only in the cytoplasm and never in the nucleus, while with the large doses the antigen or viral nucleoprotein appeared initially in the nucleus and then moved to the cytoplasma. In both cases, however, the elementary viral corpuscles could be brought out only with the aid of an electronic microscope in the periphery of the cells, with the components of the virus which were "unassembled" not being able to be distinguished from

the normal cellular constituents. We point out once again that in contrast to the electronic microscope, with the aid of fluorescent antibodies it is possible to bring out non-morphological antigen elements.

The distribution and existence of viruses in the different organs, studied with the aid of immunofluorescence, has provided new data and has succeeded in detecting viruses within the very tissues in which they existed in very small quantities, so that they could be brought out through innoculation or other methods. Thus, Chu and collaborators found that 96 hours after innoculation of monkeys with urlian virus there was virus present not only in the parotid gland, but also in the white substance of the marrow and in the 4th ventricular plate of the brain.

The urlian virus innoculated into the amniotic cavity of the embryo egg is then detected only in the cells in which there is amniotic and coriocalantoid membrane and in the pharyngian and peritoneal cells of the embryo. The same distribution is also found in the case of innoculations of eggs with influenza virus.

The process of adapting the virus to a new host can similarly be followed with the aid of immunofluorescence; for example, Gadelle studied the localization of ECHO 9 virus injected into newborn mice and showed that in the first passage the antigen was found only in the skeletal musculature, while after a number of passages it could also be found in the meninges, and then, after a very large number of passages, in the perivascular cells and in the cells behind the nervous parenchyma.

One of the modern methods of titrating viruses, the method of units formed of [PLAJE] under agar, is known in many cases to yield results which do not match those expected, in light of the number of elementary corpuscles in the titration suspension, with the number of [PLAJE] formed units in general being much less than the number of elementary corpuscles. In this regard also immunofluorescence has made available to researchers a method of increased accuracy and precision. Spendlase and Lennette, making a parallel titration of vaccinal viruses by the method of forming [PLAJE] under agar under the usual conditions and with the aid of fluorescent antibodies, showed that through this method it was possible to bring out 3-4 times more [PLAJE] than through direct reading. Indeed, it was found that while some [PLAJE] increased up to visible dimensions so that they could be counted with the naked eye, others remained localized in a very small number of cells, so that in order to see them it was necessary to use the fluorescence of the infected cells under a microscope. This technique, however, does not assure only an exact enumeration of the [PLAJE], but also permits the titration of the viruses which do not cause the death of cells and the formation of [PLAJE], the antigen being present in the cell, however, is easily seen with the aid of fluorescent antibodies.

In regard to bringing out serum antibodies, we must point out that the reactions of neutralization or fixation of the complement are more sensitive, the titrations of the serums being much less in the technique of immunofluorescence than in that of neutralization of complement fixation. On the other hand, through fluorescence, in addition to greater rapidity, it is possible to bring out some difference between the circulating antibodies. Thus, Riggs and Brown showed that it was possible to differentiate in children the actively inherited antibodies and the passively inherited antibodies from disease or vaccinations of maternal antibodies. In new born children who had an increased antipoliomyletic neutralizing titre and who did not respond to antipoliomyletic vaccination, with the aid of fluorescent antibodies through the indirect method it was shown that where the neutralization method yielded large titres, the serums of these children yielded a much weaker fluorescence or even none at all, a phenomenon which can be explained by the process of degradation of the gammaglobuline antibody passively acquired, a process that modifies the specificity of the antigen globuline before that of the antibody globuline.

In the problem of the disclosure of the antibodies in the cell, there are numerous works in immunology which localize the antibody-producing cells with the aid of direct or indirect techniques, using, however, soluble antigens as the intermediary layer. We still have not found -- for instance -- a work which shows the disclosure of the formation of anti-viral antibodies using the homologous virus as the intermediary layer.