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THE TOXCICITY OF THE INFLUENZAL VIRUS

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We will summarize in this brief paper, the results of research effected during the last 2 years and which have been the subject of two articles published in the Annales de l'Institue Pasteur. Their interest resides in that they contribute to the clarification of the physiopathology of influenza and in particular furnish the important explanation of the well known role played in this illness by certain associated bacteria, such importance -each one knows- that one of these bacteria, the Pfeiffer bacillus, has been considered during a number of years as the agent of influenza.

Three fundamental discoveries are the beginning of the progress taken for the experimental study of influenza after 20 years. The first was realized by Andrewes, Smith and Laidlaw, since they recognized in 1933 that influenza is transmissable to the ferret by intranasal inoculation of filtrates collected form the diseased, and that the symptoms present in this animal are strongly analagous to those of the human ill disease; soon after, the mouse was recognized as equally receptive. The second is attributable to Smith who was engaged in the method traced shortly before by Goodpasture on the subject of other viruses, succeeded in 1935 in cultivating the influenza virus in a chicken embryo. The allantoid liquids of the

culture, extremely rich in virus, were provided for searches in valuable materials, particularly propitious for the study of viruses. Finally in 1940, Hirst having ascertained by chance that the agglutinin virus of the red corpuscles is proportional to the binding power of the virulent particles of the liquid is inhibited by the anti-influenza antibodies; he has deduced a method, now classic, of serodiagnostics of influenza. This is the method which, together with the reaction of fixation of alexine, permits the distiction later by their antigenic differences the types A, B, A-prime, C of the influenzal virus. The hemagglutination, which cannot be produced except at a temperature not below the ordinary temperature, corresponds to the fixing of the virus on a globular receptor of a micro-protetic nature probably, which has been the object of many recent works. At 37°C this receptor is distinguished by the enzyme action of the virus, it frees the virus which is eluted. Thanks to the hemagglutination, one can thersfore, by adsorption of virus on globules follow the elution; the separation of the allantois liquid from the culture and concentration of it, the recovery of it in a volume of liquid less than that of the culture.

But, in 1946 , G. Henle and W. Henle have ascertained that innoculation of a massive dose of the influenzal virus had killed the mouse in 8 to 96 hours, before theinfluenza infection has had time to develop and without the appearance of pneu-

monia, characteristic of the infection in the case of the animal. The disease reveals in this case the behavior of intoxication and one finds in the autopsy necrotic lesions diffused through the liver and the spleen. As these authors show, the toxic properties also evident are intimately connected to the virulent particles and are not attributable to a poison which the virus diffues in surrounding milieu.

The choice of guinea pig -not receptive to the influenzal infection and in which the virus does not multiply- as a test animal, and of the peritoneal cavity- an asceptic medium and where the effects of an injection could easily be followed by puncture- as a method of inoculation, has allowed us to specify the role attributable to the toxcicity of the virus in the physiopathology of influenza.

As one can expect, the toxic effects observed are a function of the quantity of virus injected, which can be expressed in "hemagglutinat units", where the name, corresponds to the product of the volume of this liquid expressed in cubic centimeters, by the inverse of its concentration limit capable of causing hemagglutination under the conditions which have been specified by the "Committee on standard serological procedures in influenza studies". For example , 5 cm.^{3} of a virulent liquid determines hemagglutination up to the concentration of 1/8000, contains by definition 40,000 hemagglutinat units of virus. The allantois liquid of culture usually contain in cm^3 500 or 1000 hemagglutinat units; after adsorption follows elution, one obtains easily the suspensions of virus of a titer equal to 8000 or 16000 or more.

In our research we have employed most frequently the classic predecessor of the influenza virus PR8, apparently of type A. We have utilized it in the eluted state, in the guinea always weighing between 300 and 325 gr. The intraperitoneal injection of a dose of the size of 16,000 hemagglutinant units killed most of the guinea pigs within 20 to 40 hours. In the autopsy the peritoneal and pleural cavities were full of a sebous exudate. But the appearance of such a peritoneal exudate regularly follows the inoculation which the animal outlives, a dose of virus obstensibly smaller corresponds to 20,000 hemagglutinat units. In this case the most favoravle moment for the study of toxic effects is between 24 and 48 hours after inoculation. If one punctures the peritoneum of the guinea pig at theis moment one witnesses coming out under pressure of the peritoneal exudate. Upon microscopic examination of this exudate one is impressed by the frequency of pycnosis images of the polynucleus.

Often the nucleus of these altered cells appear extremely cut up, in globules where the quantity can exceed 10. This leucocytal pycnosis does not manifest itself well in less than 24 hours after the inoculation with virus. Over 48 hours, the altered leucocytes become the prey of macrophages which predominate in the exudate, which becomes more abundant is progressively revealed in the following days.

One cannot fail to be affected by the analogy which

which unites these purely toxic effects, observed in the peritoneal cavity of the animal not receptive to the influenza infections, to the lesions produced by the virus in the course of the human disease; this similitude permits, in particular, the explanation for the simple toxic action, the pathogeny of forms so called fulgurant of the influenza, which carry the infection in a few hours, show in the autopsy, the lungs filled with a serous exudate.

On the other hand, it is likewise to the toxcicity that he agrees to ascribe the favorable influence exercised by the influenza on certain bacterial infections. This previous research done on mice by Carlisle concerning streptococcus, by Harford, Leidler and Hara in regard to pneumococcus have not succeeded in reproducing experimentally this favorable influence; the influence observed was quite mediocre, does not establish that the condition of making the germs intervene many days after the influenzal contamination, at the moment when the virus which, in this aminal, causes pneumonia, has already caused microscopically discernable liesions; such results scarcely allow foreseeing that the increase of receptivity in regard to the associated bacteria results from the direct toxic action of the virus.

This toxic origin is apparent, on the other hand, after the evidence of experimentation on the guinea pig, in which, in addidition, the increase of receptivity of the associated

germs is very much manifest. In the case that that one inoculates it in the peritoneal cavity of a guinea pig, which has receiced, half an hour earlier, and the same way, 20,000 hemagglutinant units of influenzal virus, the Pfeiffer bacillus kills the animal, by generalized infection, of a dose 250 times less than the fatal dose for the test guinea pig: the minimum fatal dose is, in effect, less than a quarter of the culture on gelose-blood for the test guinea pig , whereas it is less than 1/1000 of culture for the "influenza" guinea pig. In regard to the hemolytic streptococci, the coefficient of increase of receptivity, under the same conditions, seems to be between 100 and 200; it is only about 10 for the pneumococci. On the other hand, the dose of microbic culture capable of causing death by septecemia, is exactly the same in the "influenza" guinea pig and the test guinea pig, when one applies it to a pathogen germ which, such as the colon bacillus, is not associated with influenza. This clearly shows that the increaseof receptivity in regard to assocaiated is not due to a simple accumulation of the respective noxious actions of the virus and the bacteria, but it results from a sensitization of the organism under the toxic action of the virus to the infection by the bacteria. In addition, if one looks at bacteria which, like the pertussis bacteria, does not multiply in the organism of guinea pig, which does from intoxication, one proves that the fatal dose is little influenced by previous inoculation with the virus; it is not, under these conditions, which reduced to

We also emphasize that the properties responsible for the toxic effects which we have described, appear completely independant of the hemagglutinant powers of the virus. While resisting heating to 56°, which surpresses the infectious power of the virus and its enzymztic power, they are nevertheless very sensitive to heat which the hemagglutinat power is not. Heating at 58° for an hour, of a virulent eluat , titrating 8,000 units, lowers the titer to 2,000 units, a quarter of its intial value. But, the intraperitoneal injection of 10cm^3 of this heated eluat does not produce any toxic effects which we have described, whereas these appear with their usual characteristics after the injection of a volume equal to the same eluat used cold, but previously diluted to a quarter, and where the hemagglutinant titers therefore equal to that of the heated eluat. On the other hand, the hemagglutinant powers of a virulent suspension , which one has taken care to strip of its enzymatic properties by heating to 56°, is considerably reduced by the ovomucine which owes without doubt to the chemical relationship with the globular receptor its properties of saturating the affinities of virus in respect to the hematites; but, the ovomucine does not affect any of the toxic properties of such a suspension.

As we have recalled at the beginning of this article, the immunological studies have lead to classify strains of the influenza in groups distinguished by their antigenic character.

about half, that this can be explained easily, in this case, by the accumulation of the toxic action of the virus and the bacillus.

If one inoculates the virus with a lesser dose, the receptivity to the associated bacterial infection diminished in a way clearly parallel to the importance of the toxic effects determined by the virus. The guinea pigs inoculated with 5,000 hemagglutinat units of virus survive the injection of 1/200 of culture of Pfeiffer bacillus, but they die with the injection of 1/50 of culture, thus proving a receptivity again greater than 10 times that of the test; but if they are again well defined, the toxic effects engendered by this doe of virus do not represent by a quarter of the dose usuallly put in practice, are already appreciably reduced, to suvh a degree of the abundance of the exudate formed that that this concerns the frequency of the leucocytic pycnosis.

The rapididtyof the toxic effects of the virus deserves to be emphasized. The increase of receptivity which it entails, in regard to the Pfeiffer bacillus, proves, in effect, not only when the virus is inoculated shorly before the bacteria, but also if it is an hour, and the same five hours after it. On the other hand, no increase of receptivity is observed in regard to the Pfeiffer bacillus inoculated 24 hours after the virus.; one understands that the effects of the virus are fleeting in the guinea pig. since they do not reproduce the same rarefy rapidly.

Three principal groups distinguished by the letters A, B, and C do not seem to have common antigenic characteristics between them, in this sense they do not cause any crossed immunological reaction as the test used relies on the neutralization of the infectious power, or on the fixing of alexine, or on the inhibition of hemagglutination. The type A' discovered in 1947 and against which the other types are not immune, nevertheless has been designated with this label which does not separate it formally from type A, for the reason that, if the types A and A' don not immunize eachother and they differ completely on the propfs of inhibition of hemagglutination, but on the other hand the fixing of alexine mingles them at least when used as an antigen, not the virus itself but the antigen soluble in the virus; this antigen is soluble, separable by centrifugation of the virus, where it represents without a doubt a product of disintegration, is in reality constituted of particles whose diameter does does not attain 1/10 of that of the virus. Because of its extreme convenience, the proof of inhibiti›n of hemagglutination is the most commonly used for research of the anti-influenza antibodies. In particular it is the one tha one usually resorts to for the serodiagnostics of influenza in man and the determination of the antigenic type to which the virus responsible for the infection belongs.

But one knows in addition that each of these types is far from being homgeneous and that these diverse strains, although

strains of the same type can differ enough one from the other so that there is every reason to consider the exsistance of "sub-types". The antigens capable of varying from one strain to the other doubtlessly do not have the same advantage in the sense that the protective power of the antibodies which correspond to then is probably strongly unlikely. From the point of view of epidemiology and the prophylaxie, it is essential-that goes without saying- to be able to determine in what measure these strains are capable of immunizing eachother and to arrange correlatively an immunologic method which concerns the antigens of most significance in this respect. But, it is not certain that the antigenic differences revealed by the proofs of inhibition of hamagglutination are precisely the most worthy of interest at this point, le relations eventually uniting the virulence to hemagglutinant power are very problematic. As for the proofs of neutralization of the infectious power for the experimental animal, they are very laborious and the application arduous.

Also. having given the role manifestly important which operates the toxcicity of the virus in the influenza, for us there are intersting publications studying the antigenic specificity of the toxic power of diverse strains, in function of their classification in the diverse types and sub-types.

To summarize the results of this study, which has been the object of a paper presented last month at the VI International

Congress of Microbiology in Rome, and which we actually followed, we limit ourselves, -after having pointed out that serum anti-B is without effect on strain A and vice versa - to consider the behavior of four strains where 2 (PR8 and WS) apparently of type A, and two of the A'type: Barratt strain (isolated in 1947) and strain A/Belgium/1/53, isolated in our institute appertaining, according to the nomenclature of Issacs, Gledhill and Andrewes (3), to sub type S (Scandinavian). All of these strains are very toxic, like strain PR8 which has served for the most part for our previous experiments, it causes to appear, with a dose of 20,000 hemagglutinat units, an abundant peritoneal exudate characterized by the frequency of pycnosis images. The corresponding antiserums have been obtained by intraperitoneal inoculation, of the guinea pig, with a dose of eluted virus comprising between 15,000 and 25,000 haemagglutinat units; the serum is collected a month after inoculation.

When one estimates by the inhibition of hemagglutinat e power, the activity of these antiserums of the guinea pig avouches a very clear specificity of strain, each one does not specifically act except on the strain which served to prepare it, with respect to this homologous strain, its titer is 256, that is to say that they ihibit its hemagglutinat action up to the concentation limit of 1/256.

Our tests of neutralization of the toxcicity have been done by inoculating, in the peritoneum of the guinea pig, a mixture, prepared after half an hour, of 20,000 hemagglutinat

units of virus and 30 drops of antiserum or the normal serum of the guinea pig; one punctures the peritoneal cavity 40 hours later and one examines the exudate under the microscope. The neutralization of the toxcicity is apparent by the smaller quantity of the exudate formes and the reduction of the frequency of the pycnosis images; this reduction , very easy to see, allows the accurate estimate of the degree of neutralization. The perfect neutralization (++) corresponds to the formation of a minimum exudate, not showing pycnosis and in which the predominance of mononuclear leucocytes attests to the costliness of the cure; such an exudate is analagous to that which makes the virus appear deprived of its toxcicity by heating. The + sign indicates a very marked reduction of pycnosis images and the ± sign a slight reduction byt nevertheless indubitable. the 0 sign designates the abence of apparent neutralization, the virus added to the antiserum produce in this case the toxic effects which cannot be distinguished form those which determine the additional virus from normal serum, or only inoculated. The results obtained are indicated in the table below:

Strains	Serums anti-			
	PR8	WS	Barratt	A/Bolg./1/53
PR8	++	±	0	0
WS	±	++	0	0
Barratt	0	+	++	++
A/Bolg./1/53	0	+	++	++

One sees that:

1) the toxcicity of each strain is perfectly neutralized by the anti serum which corresponds to it;

2) the strains of type A' are neutralized perfectly by not only the homologous anti-serum but also by the corresponding anti-serum of the other strains of the same type;

3) besides that they both appear to be of type A, the crossed neutralization of the toxcicity of strains PR8 and WS for the respective anti-serums are not very partial; one says that it is the same for the neutralization of the infectious powers with respect to the ferret as Burnet showed in 1937;

4) the anti-serums corresponding to strains of the type A' show themselves devoid of neutralizing powers with respect to type A. reciprocally the serum against PR8 is inactive in regard to the strains A'; on the contrary the serum against WS exercises on the toxcicity of these strains, a neutralizing action which in order not to be partial is nevertheless very pure.

The use of the serum against PR8 collected in the case of the guinea pig inoculated with virus twice whose titer is very high (4,096), indicates furthur the differences observed; the addition of 6 drops of this serum suffices to suppress completely the toxic effects of PR8 virus inoculated; with a dose of 30 drops, this serum neutralizes the virus WS in a way again incomplete but very marked (+) which the serum anti PR8 with a higher titer does not do; the same dose of 30 drops does not exercise an perceptible action with regard to a strain A', such as the Barratt strain.

It goes without saying, finally, that parallel to these results, the inoculation of a strain of virus in the peritoneum of a guinea pig immunized against it does not produce the toxic effectswhich it determines in the new animal.

These results show , in summary, that the actual calssification of strains of influenza virus, no more than it does not exhaust the diversity of their infectious power, does not apply to the very accurate manner of the the antigenic diversity of their toxicicty. Being given the probably essential roles that acts on this toxicicty in the physiopathology of influenza, one can estimate that it would be useful from the point of view of epidemiology and of the prophylaxy of this disease, of joining the toxcicity to proofs actually employed for the immunological studies compared to strains of influenza virus, of tests of neutralization of toxcicity in accord with those described in this article amd where the realization is practically very easy.

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