THE EXPERIMENTAL REPRODUCTION OF INFLUENZA, NEPHRITIS, AND ENCEPHALITIS,

BY INOCULATING SUBCULTURES OF THE ISOLATED VIRUS.

BY

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INFLUENZA.

This investigation is described in detail in papers appearing in the *Quarterly Journal of Medicine* for April. It was an inquiry into the etiological significance of an organism isolated by Captain J. A. Wilson from the blood, pleural effusion, and, in cases complicated by nephritis, from the urine of patients suffering from influenza, and already described in the *BRITISH MEDICAL JOURNAL* and the *Lancet* of February 1st, 1919.

Cultures.

An essential factor in the experimental reproduction of influenza and other diseases has been the interposition of an actual pure culture of the organisms concerned in the transference of the respective diseases from man to animals. Thereafter passage by the inoculation of blood, bile, or urine, as the case may be, has been easy from one animal to another.

In the case of influenza six subcultures were tested, five of the second and one of the third generation, there being no noticeable difference in the effects produced; in particular there was no loss of virulence in subcultures. On the other hand, the days of growth of the individual culture were of great importance, so that four days' growths produced more marked effects than ten days'. Altogether forty-one inoculations were made on monkeys, guinea-pigs, and rabbits between November 12th, 1918, and February 9th, 1919. On two occasions only filtrate of sputum was inoculated, namely, on June 27th, 1918, and November 30th, 1918, the latter being done as a control to the subculture derived from it. The results were trivial as compared with those obtained by the growths.

Methods.

The methods of inoculation were subcutaneous, intravenous, and subdural. The first yielded little or no evidence of a positive result beyond a slight disturbance of the normal temperature curve. The second yielded definite evidence of respiratory trouble during life in monkeys and guinea-pigs, and in the case of one monkey also of the later onset of the clinical symptoms of nephritis. The effects of intravenous injection seemed to stand in direct relation to the size of the dose injected. The most marked and constant results followed subdural inoculation, the incubation period being commonly only twenty-four hours.

Experimental Results.

Of six monkeys two were inoculated with the filtrate of sputum and four with cultures. Of the latter, two died four months later and one was killed when it had developed the clinical signs of acute nephritis. The respiratory symptoms appeared within twenty-four hours and those referable to the kidney on the fifth day. Of the guinea-pigs, one was allowed to die, and eight, being more or less moribund, were killed. So that, out of a total of twenty-one experiments, 50 per cent. of the monkeys and not more than 60 per cent. of the guinea-pigs may have been expected to die. Viewed in conjunction with the mild form of the disease in those surviving, the conclusion is arrived at that the disease was reproduced in an attenuated form.

In passage experiments an enhanced degree of virulence was evident, so that, for example, the mortality in subdural inoculations rose from a probable 4 in 6 in the primary inoculations to a real mortality of 3 in 3.

Reinoculation may aggravate the symptoms or modify them favourably according to the method employed in the first inoculation. Preceding subcutaneous inoculation, while producing little evident effect, modifies the effect of a subdural inoculation, so that the weight of the illness is taken off the lungs, but not off the kidney and liver.

The lesions found in the lungs, liver, kidney, heart, brain, and voluntary muscles are striking reproductions of those found in man when dead of uncomplicated influenza, and are illustrated by a series of coloured and black and white drawings.

The organism has been recovered by Captain Wilson from the blood, bile, and tissues of all the animals so treated, including animals in which the disease had been transferred by passage.

Koch's Law.—On the basis of the evidence Sir John Rose Bradford, Captain Wilson, and myself claim that the filter-passing virus with which we have worked satisfies the requirements necessary before its etiological importance can be regarded as established.

The uniformly severe symptoms following subdural inoculation may support the view that the disease finds entrance via the nasopharynx but does not exclude other modes of entrance. The only possible explanation of the course of the experimental disease after subdural inoculation is that it is primarily a blood infection. The pathogenic action is exerted on the capillaries and small arteries and veins. The lungs, in company with other organs, are attacked from within, so that the whole finely co-ordinated mechanism of respiration ensuring a uniform distribution both of blood and air is disorganized, it may be, within twenty-four hours. Masses of dead and disorganized tissues and blood clot provide foci for secondary infections to develop in, which, in their turn, come to invade the system, producing septicaemias on their own account by a reversal of the process in an individual already weakened by the primary disease.

The detailed study of the pathological process shows that it is primarily an affection of the capillaries and smaller arteries and veins. The vascular endothelium is proliferated, the structure lost, and haemorrhage and thrombosis common; a series of other changes naturally ensues. In the lung they are associated with the pouring out of much fluid of an oedematous nature which may be partly a pressure oedema and partly due to the fluid squeezed out of the numerous thrombi contained in vessels the permeability of whose walls has been altered. In addition there is an active inflammatory exudate. Whether the maximum incidence falls on the lungs because of a selective action on the capillaries there, or is due wholly or in part to the mechanical factor of the constant movement of the damaged organs, remains a matter for further investigation.

NEPHRITIS.

Altogether five subcultures were inoculated from November 9th, 1918, to February 9th, 1919, the source of three being the urine and of two the blood stream. For urine there were two second and one third generation cultures or subcultures. For the blood there was one second and one third generation culture or subculture. With these a total of thirteen primary inoculations were made subcutaneously, intravenously, and subdurally in three *Mucosus rhesus*, nine guinea-pigs, and one rabbit. All three monkeys developed mild and apparently transient nephritis clinically, and were killed when this seemed to be passing off. Of the nine guinea-pigs, five died, two were killed, and two were reinoculated. The figures are small, the outstanding result appearing to be the greater virulence of cultures derived from the blood as brought out more particularly by subcutaneous injection in guinea-pigs. Whereas a culture derived from the urine appeared to have an incubation period of two or more months, for blood culture the incubation period was from two to five days. For second generation subculture fourteen days' growth was less virulent both for blood and urine than six or seven days' growth. Third generation subcultures appeared more virulent than second. Thus while the virulence appeared to diminish with prolongation of the days of growth of the individual subculture it was, on the contrary, improved by subculturing. Intravenous injection yielded very constant and marked results; but an estimation of the relative effects of the three methods of inoculation is impracticable on the basis of the present figures. Since clinical results for the urine were obtained in the monkeys comparable with symptoms seen in man, but not for the guinea-pig, owing to the absence of facilities for collecting the urine in the latter, the pathogenic effects in monkeys may be considered separately.

ENCEPHALITIS.

On November 9th, 1918, two monkeys were inoculated subdurally, one with a drop of emulsion of brain in saline, and the other with a fourteen days' growth of a subculture prepared from the brain. Eleven days later the monkey which received the emulsion was weak and climbed with difficulty. Thirteen days later the monkey that received the subculture was similarly affected; it showed lack of agility, crept about, and did not spring or jump. The condition persisted in the first monkey for five days, when it was killed. The second monkey still showed marked muscular weakness at that time, but seven days after the onset of symptoms appeared to be better. On December 2nd, however, it had relapsed into a weak condition, climbed slowly, did not fetch food, showed no inclination to move, moped in a corner, and looked ill. On January 9th, 1919, it was killed. There were no evident naked-eye lesions in the first monkey. In the second the brain looked milky and was much congested. The arachnoid was distended with fluid. There were numerous discoloured areas in the white matter suggestive of old haemorrhage. The spinal cord was firm, and theca distended with fluid up to mid-dorsal region. The dura was adherent anteriorly with haemorrhagic discoloration. The posterior root ganglia in the cervical region appeared unduly large. A third monkey was inoculated with brain emulsion from the first, and developed symptoms and post-mortem lesions as described. From the brain of all three monkeys Captain Wilson recovered the organism.

Microscopically there was a slight patchy leptomeningitis. The veins showed irregular and eccentric accumulations of round cells in the media. The smaller arteries, both in the brain and cord, were thickened and hyaline-looking. The capillaries appeared normal, but were accompanied by a

moderate number of round cells. There was extensive degeneration of the nerve cells throughout the several layers of the cortex, most marked in the large antler cells, of which groups were completely degenerated, and a similar state of affairs in the cerebellum and cord.

The cervical root ganglia showed a striking picture. While most cells appeared normal, a number scattered throughout the ganglion stood out deeply stained, the nucleus being with difficulty recognizable from the cell body. In others there was marked eccentricity or vacuolation of the nucleus and cytoplasm. There was no accumulation of round cells such as seen in poliomyelitis. No lesions were observed in other organs with the exception of the kidney, which showed glomerular and tubular nephritis, together with hyaline thickening of the arteries.

GENERAL CONCLUSION.

The several diseases were reproduced in an attenuated form in the first place. While not all, certainly many of the lesions described can be referred to the consequences of ischaemia, others appear to be toxic in origin.

In the case of polyneuritis a curious homogeneous or hyaline change was noted in the vessels of the cord, particularly the arteries in a monkey which lived for thirty weeks after inoculation. A similar or closely related change has been noted in polioencephalitis. In influenza and nephritis vascular lesions are the predominating features, and by reinoculation after complete recovery acute and chronic vascular lesions can be produced side by side—for example, in the kidney and lungs. The diseases of vessels are most imperfectly understood, and little is known of the stages which may precede or determine the changes observed clinically or after death by the microscope. In the case of experimental nephritis it appears that the vascular changes are concomitant with the onset of the disease and are not a late development.

It may be that new insight is about to be obtained into the pathology of the blood vessels, the production of oedema, and other phenomena associated with alterations in vessel walls, such as the rashes of the exanthemata, but, until further work has been done in some other diseases and the material already accumulated more fully worked out, it seems inadvisable to enter into a detailed discussion of phenomena which are at present not a little confused and whose relations to one another are not at all clear.

THE BACTERIOLOGY OF CERTAIN FILTER-PASSING ORGANISMS.

BY

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THE filter-passing organisms here discussed have been under investigation during the past ten months. The cultivation of such an organism is no new thing, yet it is possessed of a certain novelty. In 1913 Flexner and Noguchi¹ cultivated a filter-passing organism from the nervous tissues of fatal cases of acute anterior poliomyelitis. Their method was that introduced by Noguchi² in 1911 for the cultivation of the spirochaete of syphilis. The original technique has been modified, but the underlying principle remains the same, and constitutes one of the greatest additions to bacteriological technique within recent years.

It is the "Noguchi technique" which has been employed in the present investigation, but before discussing its details it is necessary to refer to one or two points in the preliminary treatment of the infected material.

Preparation of Materials for Culture.

Blood.—The withdrawal of blood is accomplished in the usual way, 10 to 15 c.cm. being withdrawn from a vein in the arm and added to an equal volume of sterile 2 per cent. sodium citrate in normal saline. For cultural purposes the whole blood, the plasma, or the washed red corpuscles may be used. When it is not convenient to put up the cultures on the day on which the blood is withdrawn there are two possible procedures—namely, the separated plasma and red corpuscles may be mixed with equal parts of sterile 50 per cent. glycerin in citrated saline, or the whole blood on withdrawal may be added to an equal volume of the glycerin solution.

Pleural Fluid and Cerebro-spinal Fluid.—In the case of these two fluids a portion is centrifugalized and the sediment microscopically examined to determine the presence or otherwise of

an added infection. If there is no evidence of other infection the fluids may be used in their natural state, or they may be centrifugalized and the sediment taken for the culture, but it has to be remembered that the latter method increases the risk of contamination. Where other organisms are present in the specimen it may be filtered, or the sediment (obtained by centrifugalization) may be emulsified in three volumes of the 50 per cent. glycerin solution and allowed to stand at room temperature, and in the dark, for a period of three or four days, when the culture may be put up.

Sputum.—The sputum is collected in sterile vessels. The most satisfactory method of excluding the many other organisms present is to filter the specimen, either in its natural state or, if it be too glutinous, after emulsification in normal saline, through Berkefeld N or V filters, these forms having been found most suitable. Where a large number of sputa are under observation the strain on the filters is a heavy one, and it becomes necessary to resort to the glycerin method, one part of sputum being emulsified in three parts of the 50 per cent. glycerin solution. The sputum, however, sometimes contains glycerin-resistant organisms, more especially in cases of bronchopneumonia, and this process entirely fails.

Urine.—The sediment obtained by centrifugalization of a catheter specimen is invariably used. It may be filtered, but emulsification in glycerin is usually an adequate method.

Post-mortem Specimens.—Such specimens being, as a rule, grossly contaminated, require prolonged treatment in the glycerin solution. The method is eminently suitable for nervous tissues from fatal cases of polyneuritis, encephalitis, and rabies, in which diseases the specific organism is resistant over long periods. In nephritis and influenza the glycerin method is not, as a rule, available; it is necessary to emulsify the tissues in normal saline and to filter, as in the case of sputa.

Louse Excreta.—The only other material to be considered is the louse excreta, for which the glycerin method gives excellent results, about one milligram of excreta being emulsified in each cubic centimetre of the glycerin solution. The period required to kill off the other organisms present varies from four to eighteen days.

Technique of Culture.

Into each of a series of sterile test tubes (6 by $\frac{1}{2}$) a fragment, about the size of a pea, of sterile rabbit kidney is placed, and alongside it a piece of the nervous tissue, or a drop or two of the filtrate, whichever is under examination. Serum agar, in the proportion of one part of inactivated serum to ten parts of melted agar, at a temperature between 53° C. and 56° C., is added to each tube in an amount just sufficient to cover the tissue. After solidification of the serum agar has taken place there are added to each tube about 15 c.cm. of serum bouillon, also one part in ten, and at a similar temperature. Complete anaërobiasis is obtained by running on to the surface of the medium sterile liquid vaseline at a temperature of about 60° C. and to a depth of half an inch. The completed medium, therefore, consists of a thin layer of serum agar containing a fragment of sterile kidney tissue and the infective material, with a superimposed column of serum bouillon, the whole being sealed with a layer of vaseline.

In the case of blood, pleural fluid, and cerebro-spinal fluid, the cultures are put up in conical Erlenmeyer flasks of a capacity of 50 c.cm. It is sufficient to add 10 c.cm. of the blood or other fluid, the amount of agar being about 15 c.cm. In the case of blood and pleural fluid ordinary agar and bouillon are used; for cerebro-spinal fluid the serum constituent must be added.

It is essential to put up a series of controls involving all the constituents of the medium.

It may be matter of surprise that such an apparently simple method has not been universally employed in the investigation of diseases of unknown etiology, yet in practice it is one of the most difficult and disheartening things in bacteriology.

One of the chief sources of trouble is the *sterile kidney tissue*, which is not always sterile. Quite apart from the risk of contamination in removal of these organs, it sometimes happens that they are infected with *B. pseudotuberculosis*, or with a bacillus of the Gaertner group, even though they appear perfectly healthy. The most careful examination of the abdominal viscera must be made before using the kidneys. Any hyperaemia of the intestinal canal or enlargement of the mesenteric glands should be sufficient to condemn the organs for cultural purposes.

Another danger is to be found in the fact that in the making of the complete culture the tube has to be opened five times; the risk of contamination is obviously not a light one.

The *serum factor* in the medium is important; in all cases it should be inactivated and sterilized, by heating for two hours at 56° C. on three successive days. Fresh serum has an inhibitory effect on the growth of filter-passing organisms, hence the necessity of bleeding the animal whose kidneys are to be employed as thoroughly as possible.