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RECENT STUDIES ON THE IMMUNITY RESPONSE TO ADMINISTRATION OF DIFFERENT PLAGUE VACCINES *

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SYNOPSIS

Evaluation of the immunity produced by plague vaccines was made by measurement of the reactions in the body fluids of man and different animals to demonstrate altered refractoriness. Four serological methods were used : the agglutination test, the complement-fixation test, the passive mouse-protection test, and the haemagglutination test. Results showed that the majority of animal and human hosts, in response to injections of *Pasteurella pestis*, vigorously develop antibodies which may persist in the peripheral blood serum for several months.

Critical tests to establish the correlation between antibody response following inoculation with plague antigens or vaccines and resistance to infection showed that inoculation of 30μg of Fraction I antigen consistently protected mice against *P. pestis* infection and that there is a definite correlation between the appearance of serum antibodies and the immunity state of the mice. There is a similar correlation in guinea-pigs, although the immunogenicity of antigens and vaccines for these animals is very variable. Primates are rendered resistant to massive infection with 1,000,000,000 to 2,000,000,000 viable *P. pestis* by suspensions of formalin-killed bacilli and by heat- or chemically-killed broth vaccine administered in the customary human dose ; basic immunization with Fraction I renders approximately 60% of monkeys immune.

Evidence from experiments carried out on groups of human volunteers points to the conclusion that any plague vaccine containing 2-3 mg of Fraction I or capable of producing that amount when inoculated into the body will favourably alter the susceptibility of 50% of inoculated persons. But, to alter favourably the responsiveness of a greater percentage and to enhance the moderately effective immunity, re-inoculation at intervals of 3-6 months is essential.

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The purpose of the various methods which, since the days of Haffkine, have been tried in the immunobiological prophylaxis of plague has been : (*a*) to eliminate the manifest symptoms of the infectious processes; or (*b*) to reduce the case-fatality rate. Prophylactic immunization against plague is based on the observation that a non-fatal clinical infection protects against a second attack or at least transforms the reinfection into a milder disease. Animal experiments and epidemiological records leave no doubt that every vaccine or antigen used to any extent has exerted its inhibitory effect on the pathogenic, and primarily on the fatal, effect of the infection, rather than on the infection itself. Through experiments on animals it has been established that immunity of varying completeness can be conferred by inoculation with suitably killed broth cultures, with whole agar-grown suspensions of plague organisms (antigens), with certain fractions of such bacilli, or with suspensions of living plague strains of low virulence (the vaccines are still toxic in high concentrations). The available data are impressive, but in evaluating them, one must know that the animal experiments used to prove the efficacy of a chosen antigen or vaccine have not always been planned to give complete information. Quantitative studies must be made on large series of animals of uniform susceptibility.

The choice of the experimental animal is most important. The claim that the kind of test animal, rather than the kind of immunogenic preparation, is decisive in the results of immunity experiments requires careful appraisal. The impossibility of immunizing guinea-pigs by means of killed plague bacilli in saline suspensions or broth cultures actuated research with living plague strains of relatively low virulence. It is now known that soluble antigens of low immunogenic activity, particulated with alum or mixed with adjuvants, may be as active as living organisms. Furthermore, certain residue fractions of the plague bacillus are highly immunogenic for guinea-pigs, but are of very low activity for white mice or rats. The two latter rodents are readily protected against fatal plague infections with any plague antigen or vaccine, provided an adequate amount of the preparation is inoculated, preferably in a two-step injection procedure. The knowledge that the antigen that is immunogenic in the mouse, rat, and monkey apparently differs from the antigen that immunizes guinea-pigs has raised many important problems. The pertinent question is whether man in his immunological response to a plague vaccine or antigen reacts like the mouse, rat, and monkey or like the guinea-pig. There is increasing evidence that the antigen which stimulates immunity in the mouse and monkey is equally immunogenic in man.

But the mere selection of an experimental animal suitable for the evaluation of the immunogenicity of a vaccine or antigen by no means solves the majority of the problems. In many ways the animal experiment does not reflect natural conditions of infection, because it is as a rule impossible to reproduce the infectious disease exactly with regard to the portal of entry

and the dose of the disease agent. Furthermore, it furnishes little or no information about the duration of the protection created. Through the years, experimentation on immunization against plague has generally been guided by the prevailing concept that if preparations present very good presumptive evidence of their efficacy in animals and do not induce severe local and systemic reactions, they may be used in the effort to immunize human beings. But the opportunities afforded by such trials must be more fully realized; selecting and administering the agent alone is not enough. The complete trial must include a second step : the determination of the refractory or immunity state of the inoculated.

The evaluation of the immunity produced is most important, and the possibilities are four : (1) intentional challenge; (2) intentional exposure to a natural infection; (3) natural exposure, permitting the prevailing epidemic chances to act freely; and (4) measurement of the reactions in the body fluids to indicate altered refractoriness of the inoculated or immunized. Obviously the first and second procedures are not justifiable and will never be used by morally responsible investigators. The third method is used only under special conditions of urgency, primarily dictated by the quantity of the agent available. It will give dependable information provided the chance of exposure is great, and uniform in the inoculated and in the non-inoculated group. Since these premises can rarely be fulfilled in the face of a plague epidemic, the information thus secured is of quite uncertain analytical value. Despite the fact that the number of persons inoculated may be large, and thus the effect of the procedure can be expressed statistically, this method has many inherent difficulties. It has been used mainly among indigenous population-groups in regions where the faults in the reporting and diagnostic systems are obviously quite difficult to combat. By inoculating every other member of the same family, the results may be more dependable.[7] The statistics from India,[8] Indonesia,[10] and Madagascar [3, 4] amply attest to the fact that prophylactic plague inoculations in bubonic plague definitely do reduce the mortality rate, the only measurable response to immunization used until recently. But every investigator stresses the facts that the immunity probably does not last long, that a single subcutaneous vaccination does not protect against the pneumonic form of plague, and that persons who have been repeatedly re-inoculated or revaccinated may die of plague. In addition to these limitations, the two types of plague vaccines —broth cultures or bacillary suspensions and antigens extracted from dead bacilli, and vaccines using living attenuated strains—have not been compared in field trials. The Joint OIHP/WHO Study-Group on plague recommended at its first session that investigations of this kind be undertaken.[12]

The fourth method is, to all intents and purposes, based on analogical deductions and has therefore never been tried as an evaluation of prophylactic plague inoculations. In order to ascertain its value, it is first imperative to answer the following questions : (1) What methods—in vitro

serological tests or passive protection tests—may be used in the appraisal of immunity to plague ? (2) Are the reactions merely auxiliary or ancillary phenomena ? (3) Are they absolute and essential conditions of favourably altered susceptibility ? These questions suggested a series of preliminary studies for the purpose of developing several serological methods to determine immunity to plague and of ascertaining their accuracy in reflecting such immunity.

Serological Techniques

Agglutination test

Formalin-killed, repeatedly washed suspensions of a virulent strain of *Pasteurella pestis* which produces more than 15 mg of Fraction I per 100 ml of suspension are used as antigen in the customary macroscopic tube-test.

Complement-fixation test

According to the procedure developed by Chen et al.,[2] Fraction I is dissolved in physiological saline in a proportion of 2 mg per 1 ml (stock solution), and for routine tests is further diluted 1 : 500. In a series of tubes are placed 0.2 ml of twofold dilutions of inactivated sera to be tested. To this series of tubes are then added 0.2 ml of 1 : 500 dilution of stock Fraction I antigen solution and 0.2 ml of complement (2 units). The tubes are shaken, incubated for 4 hours at 4°C (refrigerator), and then kept for one hour at 37°C (water-bath). Blood cells sensitized by appropriate amounts of haemolysin 15 minutes before use, in the amount of 0.4 ml, are added to each tube. After incubation at 37°C for 30 minutes, the reactions are read.

Passive mouse-protection test

The procedure developed by the Hooper Foundation laboratory [5] was adopted. At least 10 mice are given intravenous injections of 0.5 ml of the undiluted serum to be tested (or diluted 1 : 2 if the protection is strong), and then immediately 100 MLD (minimum lethal dose) of a highly virulent culture of *P. pestis* are given subcutaneously. The mice are observed for a time adequate to allow complete development of infection in these animals, about 14 days. Sera collected after recovery from a plague infection confer considerable protection on the mice, as indicated by lower fatality rates and the extension of the average death times over a longer period. The differences, first, in percentage of animals that die and, secondly, in the length of survival time, are not strikingly significant when considered separately, but have some significance when considered together. In order to facilitate comparison by expressing these two factors in one figure, the

percentage of mice that die is divided by the average day of death. The quotient is designated the " mouse protection index " (MPI).

Haemagglutination test

The technique recently developed by Chen,[1] using erythrocytes saturated with the haemagglutination antigen present in old hormone-broth cultures, has been used as a confirmation test. It serves to distinguish differences between plague antigens prepared by different methods. To tubes containing 0.4 ml of serial dilutions of serum is added 0.4 ml of a 0.5% suspension of sensitized red cells. The tubes are shaken and incubated in a 37°C water-bath for 75 minutes. The results are read immediately after incubation and again after holding at room temperature overnight.

Plague Antibodies in Sera Derived from Plague-Infected and Recovered Human Beings and Animals

The use of the four tests mentioned above, chosen to evaluate the immunogenic response to plague infection or to inoculation of antigens or vaccines, yielded the following results.

The sera of all patients who had recovered from plague, although limited to six specimens, invariably gave complement-fixation titres varying from 1 : 32 to 1 : 512. The agglutination titres rarely exceeded 1 : 160, while the MPI was invariably below 10 (below 5 in two; average : 5.6). Haemagglutinins were rarely present.

The human host, in response to bubonic or pneumonic plague, develops antibodies which persist for at least 44 days, although complement-fixing and protective antibodies sometimes decline during convalescence. It is imperative that at the earliest possible moment these preliminary observations be extended to larger series of patients in order to secure more informative patterns of the serum antibodies that follow plague infections.

The sera collected from primates (*Macaca mulatta*) on the 28th day after they had recovered from plague invariably agglutinated the plague bacillus in dilutions of from 1 : 32 to 1 : 320. The complement-fixation titres were positive in dilutions of 1 : 16 to 1 : 32, and the MPIs were below 5. The sera of some individual monkeys protected all the mice and thus yielded an index of 0; the majority protected from 7 to 9 of the 10 mice and thus furnished indices of 3.3 to 1.3. In fact, the observations on 30 monkeys emphasize that the individual response to plague infections, expressed in the reactions of the sera collected on an arbitrarily chosen day of convalescence, varies. However, it is clearly demonstrated that the relationship between the protective power and the antibodies, measured by in vitro tests, is definitive. If the complement-fixation titre is high, the

MPI is low, and vice versa. If no complement-fixing antibodies are demonstrable, the index is rarely below 10. Both the protective and the complement-fixing antibodies may drop to low levels—in fact, may disappear from the peripheral blood—within 60 to 90 days, while the agglutinins may persist.

The antibody response of mice, guinea-pigs, and rabbits to plague infection is quite similar to that of man and primates. For example, a serum pool from a group of mice immunized, infected, and recovered yielded, on the 28th day after challenge, agglutinins, complement-fixing antibodies, and an MPI of 2.6.

The agglutination and complement-fixation titres in sera of nearly every guinea-pig one month after recovery from infection reacted in the complement-fixation and agglutination tests in dilutions of 1 : 8 to 1 : 256. As might be expected, the titres obtained with in vitro tests were very high, and the protective power was of the highest order when rabbits were subjected to repeated massive infection with virulent or avirulent strains of *P. pestis*. For example, a rabbit serum containing 1.32 mouse protective units may have an agglutination titre of 1 : 10,240 and fix complement in a dilution of 1 : 2,560. On the other hand, sera of rabbits and guinea-pigs devoid of in-vitro-reacting antibodies have high MPIs, varying from 15 to 24, and no mouse-protection units.

These data establish the basic facts on which the subsequent conclusions are based. The majority of animal and human hosts, in response to a single or repeated infection with *P. pestis*, vigorously develops specific in-vitro-demonstrable and protective antibodies, and these persist in the peripheral blood serum for several months.

In order to decide whether or not these antibodies are essential in the immunity against plague, a series of 40 monkeys that had survived infection, either because they had been immunized and then infected or because their infection had been cured with antibiotics, was reinfected. Their antibody response to the first infection, as measured by serum tests, had been excellent; complement-fixing antibody titres were 1 : 16 to 1 : 128 on the 20th-30th day after infection. All MPIs were below 10, and some were as low as 1 or even 0. Despite this indirect evidence of immunity, reinfection with as few as 472,000 *P. pestis* on the 55th day after the primary infection proved fatal to one monkey. Of the total series of 40 monkeys, 5 (12.5%) succumbed to the reinfection with relatively large infective doses (2,300,000,000 to 4,800,000,000) of virulent *P. pestis*. No significant correlation between the local and general reaction to the primary infection, the antibody level on the 30th day, and the resistance to reinfection could be found in the histories of the five monkeys that died. At the time of reinfection, the serum antibodies had reached the level characteristic for normal monkeys. But in this respect they behaved in no way differently from the other animals of the series infected at the same time. However,

there was a striking exception at the site of the cutaneous infection : the monkeys that succumbed to the reinfection had a diffuse, oedematous, ulcerating inflammatory area with local buboes, accompanied by fever, while in the resistant monkeys no visible or palpable local reaction and no fever were noted. Finally, in a series of 16 surviving monkeys with serological histories quite similar to those of the subcutaneously challenged, two succumbed rapidly to an intratracheal infection. These two belonged to a group of four that received 100,000,000 viable *P. pestis*. Those infected with 1,000, 10,000, or 1,000,000 organisms survived. No significant correlation between complement-fixing and protective antibodies and resistance to pulmonary reinfection was evident. The level was the same in the non-resistant as in the resistant animals six months after the challenge infection.

There is no need to stress the significance of these observations. A single attack of plague on the primate leaves only a *relative* immunity in approximately 90% of the animals. Circulating protective and complement-fixing antibodies are not an absolute prerequisite of immunity to plague. Since histogenetic factors do influence the reactivity of the host, it must be realized that this individual genetic disposition will guide the ultimate outcome of an infection. However, the importance of antibodies in plague infections is fully documented in the reports on passive immune-serum therapy. This passive anti-infectious immunity depends on humoral antibodies in high concentrations.

Altering susceptibility therefore depends on the rapidity and intensity with which antibodies may be mobilized by the host. Since a single attack of plague apparently rarely engenders this state in every individual, it must be reluctantly admitted that artificial immunization, even with the most efficient prophylactic, will create a merely relative immunity. The published records on prophylactic mass inoculations throughout the world furnish ample documentary evidence in support of this conclusion.

Correlation between Antibody Response following Inoculation with Plague Antigens and Vaccines and Resistance to Infection

It is recognized as a fundamental principle of immunity that a plague prophylactic which fails to stimulate antibodies is, a priori, non-immunogenic. It is equally reasonable to expect that the response expressed in in-vitro-demonstrable and in protective antibodies in some measure reflects altered susceptibility. As far as one can see in the published records, this has never been subjected to experimental evaluation. A correlation between the antibody levels and degree of immunity to plague has not hitherto been tested. There may be certain reasons why this has never been attempted.

Two species of animals are usually chosen for the evaluation of plague vaccines. The white mouse, which is very susceptible to plague and is readily immunized with certain plague antigens, is unsuited. Serum samples adequate for the various tests, particularly for the passive protection test, cannot be collected from individual animals to be subjected to challenge infections. The guinea-pig has been used, but reservations should be made concerning it. This rodent, although quite susceptible to plague, is subject to seasonal and individual innate resistance; but, more important, it cannot be adequately immunized with suspensions of killed plague bacilli or heated broth-cultures or with the highly immunogenic Fraction I antigen. For rapid orientation and to spare expense, however, preliminary experiments may be executed on guinea-pigs.

In critical tests, the primates have proved useful. *P. pestis* infections in monkeys resemble in many ways those in man. The overall response to injections, by the intracutaneous, subcutaneous, or intratracheal route, of small or large doses of *P. pestis* is quite constant. Local lesions and buboes develop readily after the plague bacilli are injected in doses equivalent to those transferred through the bite of an infected flea (11,000 to 24,000 organisms). Sustained fever, profound lethargy, tachypnea, and hypotension resemble the clinical manifestations observed in man. The resistance of individual monkeys is pronounced and recovery of small young monkeys despite extensive local lesions and even positive blood cultures may be observed in 50%-70% of the infected. In order regularly to cause rapidly fatal, and usually septicaemic, infections, at least 5,000,000 to 1,000,000,000 living virulent plague bacilli must be inoculated. Even under the impact of such massive infections, 5% of the rhesus monkeys may occasionally recover. In view of these facts, it is obviously necessary to include not less than 10 monkeys in an experimental series.

From a rather extensive series of tests, some illustrative experiments have been selected.

Appraisal of plague antigen in mice

The experiments were limited to evaluation of Fraction I. Series of 10-20 mice were inoculated with ascending doses of the antigen and were exsanguinated on the 8th day after the last injection. Two in vitro tests disclosed that at least 5-20 μg of the antigen are required to stimulate the appearance of agglutinins in 1:32 dilutions of the serum. Complement-fixing antibodies did not appear at this stage of the immunization. No antibodies appeared when the antigen was inoculated in doses of less than 5 μg and none of the mice inoculated in a parallel series with the same antigen dilutions survived a challenge infection with 1,200 *P. pestis*. However, the inoculation of 30 μg of antigen Fraction I consistently protected all the inoculated mice against such an infection.

Thus a definite correlation between the appearance of serum antibodies and the immunity state of mice following the inoculation of plague antigens has been established. Development of both antibodies and immunity depends on the antigen mass. In fact, the antibody level and the immunity expressed in the percentage of mice surviving challenge infections parallel the amount of Fraction I inoculated. This observation is significant and deserves further discussion in connexion with the prophylactic immunization of man.

Appraisal of plague antigens in guinea-pigs

It has been repeatedly demonstrated [6, 7] that suspensions of killed bacilli or broth cultures of *P. pestis* confer little protection on guinea-pigs. This defect may in part be compensated by increasing the antigenic substance and by using synergists (alum and oil-water emulsion).

Whenever the immunizatory mass contained residue antigen (plague bacilli repeatedly extracted with saline) in the amount of 1μg in an oil-water emulsion, the protection afforded to guinea-pigs was 100%. By contrast, the injection of purified Fraction I in an aqueous or in an oil-water menstruum in the massive dose of 180 μg failed to immunize.

In the light of these facts, it seemed desirable to determine whether there was any parallel between the proved resistance or the lack of immunity and the presence or absence of antibodies. Such tests were readily combined with an appraisal of different vaccines containing varying amounts of Fraction I or residue. Large series of guinea-pigs were injected, in two-step inoculations, with the preparation to be studied. A random sample of 4 or 5 inoculated guinea-pigs was bled, and their sera were pooled and tested as usual. The immunity of not less than 10 animals was challenged subcutaneously with 100,000 MLD (approximately 1,000,000 organisms) of virulent *P. pestis* (strain 195/P) on the 21st day after the second and last injection of the antigen or vaccine.

The experiments pointed out in a conclusive manner that serum antibodies are related to the immunity of guinea-pigs given massive plague infections. A high MPI accompanied by a lack of agglutinins and particularly of complement-fixing antibodies accompanies a low survival rate. Guinea-pigs cannot be protected against a massive plague infection with suspensions of heat- or chemically-killed *P. pestis*. Suspensions of bacilli killed with formalin or acetone or detoxified with alcohol are equally inefficient. An acid casein-hydrolysate liquid culture vaccine is also devoid of immunogenicity, since the active antigen consists largely of the lyophilic Fraction I component. However, all these prophylactics became powerfully efficient when they were injected as oil-water emulsions (Bayol-Arlacel mixture used according to the procedure of Salk et al.[9]). Provided the plague prophylactic contains 1.3 mg of nitrogen and is administered in two doses,

antibodies demonstrable in in-vitro tests and low MPIs will predict the 60%-80% survival in the inoculated guinea-pigs subjected to infection. Even non-immunogenic broth vaccines which contain relatively little residue antigen when mixed with adjuvants protect 40% of the animals.

Two further facts deserve mention. (1) Neither Fraction I nor residue antigen in aqueous solution stimulates the production of antibodies or a noteworthy degree of immunity. (2) The residue antigen in the low dose of 0.85 mg in oil-water emulsion immunizes 100% of the inoculated guinea-pigs; the immune state is in part shown by the low MPI and the relatively high complement-fixation titres. Since the refractoriness of individual guinea-pigs to immunization is quite variable, even 22.5 mg of residue antigen washed ten times and nearly free from Fraction I fail to protect every animal.

Equally conclusive are the experiments with the well-known strain EV 76 (obtained in 1951 from Dr. G. Girard), which is of relatively low virulence. Although a single inoculation of 2,500 viable bacilli stimulates antibody production quite irregularly, the immunity is quite strong. When 250,000 viable, relatively avirulent *P. pestis* are injected, the MPI drops to 8.8; when 2,500,000 organisms are used, it drops further to 4.9, and nearly 100% of the guinea-pigs are then fully protected. Lyophilization in some manner as yet unknown affects the immunogenic power of the EV 76 strain. At least 4,870,000 viable, lyophilized organisms are required to reduce the MPI to 10 and to immunize 100% of the guinea-pigs. The inability of the lyophilized strains to stimulate the production of complement-fixing antibodies is worthy of record.

These and many similar experiments attest to the variable immunogenicity of antigens and vaccines for the guinea-pig. For reasons already discussed, the results, significant though they appear, are not applicable to the problem of prophylactic inoculation against plague in man.

Appraisal of plague antigens and vaccines in primates

Primates are rendered resistant to exceedingly massive plague infections (average challenge dose 1,000,000,000-2,000,000,000 viable *P. pestis*) by suspensions of formalin-killed bacilli and by heat- or chemically-killed broth vaccine inoculated in the customary human dose. Smaller doses confer little or no immunity.

Basic immunization with Fraction I, irrespective of the dose, within a range of 0.1-5 mg, has rendered approximately 60% of the monkeys immune to plague.

The potency of vaccines consisting of bacillary suspensions or of Fraction I is strikingly enhanced by synergists, such as alum, or by adjuvants. such as Pendill, Bristol, or Bayol-Arlacel. In fact, the oil-water emulsion preparations containing merely 1 mg of Fraction I, tested on an adequate sample of primates, appear to be the most efficient prophylactic.

Antibody production as a whole is much less extensive in primates than in other species of animals. Complement-fixing antibodies appear quite irregularly, and only in primates inoculated with Fraction I. Although the MPIs were lower than those of normal monkeys, the averages rarely fell below 10, and then only when Fraction I was the immunizing agent. The complement-fixation titre, using Fraction IA as antigen, was of negative significance; the absence of this antibody in a group of monkeys injected with an antigen indicated a relatively weak immunogenicity of the preparation or an inadequate dosage. Prolonged stimulation through slow absorption of the antigen or repeated inoculations improved the serological response.

The strain EV 76, of relatively low virulence, in the customary dose for man—1,000,000,000 in total count, or 565,000,000 live bacilli—is an excellent immunizing agent. Though complement-fixing antibodies were not demonstrable, the average MPI on the 28th day after the single protective inoculation was within the range characteristic for monkeys inoculated with antigens. Within three months the MPI had risen, and in at least one observation the immunity of some individual primates had apparently declined.

Just as with antigens, so with the living EV 76, the actual dose required to create resistance to a fatal infection is of importance. Experiments on mice and guinea-pigs have shown that multiplication of the injected avirulent plague bacilli takes place over a period of 5-10 days and thus produces the amount of Fraction I needed to immunize.[10] The inoculum of 1,000,000,000 organisms fulfils the requirements, while the smaller dose of 4,500,000 is rapidly disposed of by phagocytosis and a correspondingly smaller amount of antigen is formed. Fewer primates become immunized.

The results of the work on primates warrant the tentative conclusion that in all probability any antigen or vaccine, provided it is administered in adequate dosage, would be equally efficient in man. It was these conclusions that prompted tests on human volunteers.

Appraisal of plague antigens and vaccines in human volunteers

Guided by the results obtained on primates, but influenced by the desire to reduce the number of inoculations, the first large-scale field trial on human volunteers with plague prophylactics was executed with seven preparations. Only the suspension of formalin-killed bacilli was inoculated in the recommended dose and in two steps. The remaining preparations were inoculated in a single dose. The mixtures of two avirulent strains, 1122 and EV 76, as living (known as Otten-type vaccine) or as killed agents were compared. In order to prove the theory that multiplication of the inoculated avirulent strain is essential to the immunogenicity of the vaccine, an attempt was made to check the growth by treatment of some of

the inoculated volunteers with a small dose of chloramphenicol. A highly purified Fraction I preparation in aqueous solution or incorporated in a peanut-aluminium-stearate preparation (Bristol) was used.

The relevant clinical and serological reactions, classified as severe lymphangitis, developed in 2 cases after the suspension prophylactic was administered, and in 2 others after use of the same prophylactic with aluminium hydroxide. These reactions disappeared within 72 hours. Mild local reactions were slightly more frequent after the second inoculation. The Otten-type vaccine, containing live avirulent *P. pestis*, induced only 6 very mild local reactions; in a group that received antigens in Bristol adjuvant, 8 had severe local reactions followed by sterile abscess formation. These reactions appeared in the morning of the 5th day after inoculation. The preparations responsible for the local reactions in the 8 men were basically different. The acetone-killed avirulent plague bacilli contain unchanged plague toxin which itself is capable of producing local reactions in varying percentages of men inoculated subcutaneously. On the other hand, Fraction I is atoxic. Consequently, the oily Bristol, initially toxic, preparation must be regarded as the factor responsible for the severe local reactions and the cause of the abscess formation.

The serological reactions, although disappointing, amply confirmed previous observations, with one exception. In the group of volunteers, 5 sera of the total of 92 with MPIs of 0-9.5 fixed complement in dilutions of from 1:2 to 1:32. In this group, the agglutination titres were significantly more frequent and higher (1:2-1:64) than in the other groups. Similar correlations were noted in the other groups. For example, in the group inoculated with Otten-type vaccine, one serum had a protection index below 10 and the complement-fixation titre was 1:4. Complement-fixation titres were not demonstrable in the sera of the group in which the growth of the avirulent live vaccine was in part depressed or completely prevented; correspondingly, the mouse-protective antibodies in this group were not significant. As a whole, the immunogenic response to the various plague preparations expressed in the passive MPIs was disappointingly slight. This must be attributed not so much to the antigens as to the deliberate choice of single-dose inoculations. Previous tests had shown that Fraction I antigen in a total dose of 2.5 mg (given in three injections) with an average MPI ratio of normal to inoculated of 15.4:6.2 is contrasted in this field trial by the average ratio of 20.7:15 in a group of men who received twice the amount of Fraction I, but in only one inoculation.

This exploratory study of plague prophylaxis in man shows that following the injection of antigens or vaccines poor in Fraction I content a variable, relatively small percentage of individuals produces protective antibodies by the 21st day after inoculation. The percentage of failures in immunity response becomes smaller when more efficient antigens are used. This is particularly true when they are inoculated repeatedly at properly

chosen intervals. Random studies on groups of men receiving stimulating booster inoculations weeks or months after primary prophylactic inoculations strikingly illustrated this important fact. Certainly, many individuals who did not produce antibodies after the basic inoculation promptly did form them in response to a booster inoculation. The formalin-killed suspensions in the usual dose apparently establish a basal immunity, even though they fail to stimulate antibodies.

These deductions prompted two additional field tests on active immunization against plague. Fraction I antigen was chosen in view of its nontoxicity, superior immunogenic power, and stability. The tests attempted to determine: (1) the actual dose of Fraction I required to immunize; (2) the most effective route of inoculation required to stimulate protective antibodies of a high order; (3) the local and systemic reactions to the inoculations; (4) the duration of the immunity, reflected in the MPIs 3 and 6 months after basic immunization; and (5) the response to booster inoculations of small doses of the same antigen by the intracutaneous and subcutaneous routes.

It is not necessary to detail the clinical studies here. Suffice it to note that the reaction rate in a group of 56 volunteers frequently inoculated with vaccines and immunogenic agents was high following a course of three inoculations. Over 60% of the reactions did not occur until the third inoculation. They were marked local oedema, usually involving the entire arm (in many instances extending onto the forearm), accompanied by erythema and an increase in skin temperature. Local discomfort was not marked, nor was the area of injection markedly tender. Systemic reactions were never severe. One reaction reached a peak within 48 hours after the offending injection and subsided within 72-96 hours. The reactions are considered to be allergic responses to the protein Fraction I. Re-inoculation of the same antigen three months later caused few local and only one systemic reaction when a fairly large dose of 1 mg was inoculated subcutaneously. However, marked local, erythematous, indurated, slightly painful reactions followed intradermal injections in a group classed as highly allergic to plague antigens. In the third group, equally sensitive, an intramuscular booster inoculation was well tolerated; only one had a tender local swelling and a temperature of 100.2°F (37.9°C). It is probably not mere coincidence that whenever the re-inoculation caused local and systemic reactions, the antibody response was excellent.

In a second group of 67 volunteers inoculated with the same amount of Fraction I and in the same dosage as the first group, no severe local or systemic reactions were recorded; only 9 had temporary erythema with oedema of the same intensity as that provoked by lyophilized typhoid vaccine alone. Re-inoculation with Fraction I by two routes several months after the last inoculation of Fraction I produced severe local and systemic reactions in only one individual. A temperature of 101°F (38.3°C) with

local erythema and slightly tender induration followed the subcutaneous inoculation.

Indirect evidence of immunity reflected in serological reactions and mouse-protection tests, conducted on sera collected on the 30th day after the last injection of the Fraction I preparation, was shown in the titres and indices. Only an average of from 30% to 64% of the individuals in the different groups had significant concentrations of antibodies. Irrespective of the size of the dose, the immunity reflected in antibody levels is considered inadequate in the light of the comparative tests made on animals. However, it is very significant that the immunity reaction expressed in average MPIs in response to injection of 3 mg of Fraction I in three doses in four entirely independent groups of volunteers, inoculated at different localities and at different times and of a wide age-range, was nearly identical (average MPIs : 8, 36; 7, 82; 8, 5; 8, 5). Use of smaller doses of antigen (2 mg) or intramuscular injection of the immunogenic substance in a non-particulate form in a dose equivalent to 18,000,000,000 virulent, formalin-killed plague bacilli yielded sera with an average MPI of 15.1.

Detailed quantitative tests on mice have repeatedly proved that at least 20,000,000 dead plague bacilli with an adequate Fraction I content are required to ensure 100% survival after infection. To immunize a man weighing 60 kg, it was calculated that, on a weight basis, at least 60,000,000,000 plague bacilli, equivalent to 10 mg of Fraction I, would be required. In trial tests, it was noted that local and general reactions are rather severe to smaller inoculations of 15,000,000,000 killed plague bacilli. To meet the requirements, Fraction I (12 mg) served in place of the poorly tolerated bacillary suspension. Contrary to the results anticipated through comparative reasoning, the immunity response expressed by serological in vitro tests, and particularly the average MPIs to the large dose of Fraction I, was only slightly better than the response to the 3-mg dose. A fourfold increase in antigenic mass failed to alter the percentage of individuals (64%) apparently capable of reacting to basic plague inoculations.

Equally important is the recognition that the acquired immunity indicated by the MPI after an interval of 3 months was just as high in the group of men who had received the smaller dose (3 mg) of antigen. The decline in the immunity by the third month is of equal magnitude in the group having received the maximum amount of Fraction I (12 mg). Hence it must be concluded that the weak responsiveness of certain individuals cannot be overcome by large doses of antigen during the basic immunization. However, irrespective of the antigen dose, the antibody-producing mechanism continues to function in 75%-90% of human beings for 3 months and in 50%-70% for at least 7 months. When the initial stimulation is less vigorous, as for example in response to intramuscular inoculation, the persistence of antibodies is equally less marked (only 33% of the men had significant antibodies 28 and 180 days after basic inoculation).

Re-inoculation with a booster dose of 0.2 mg intradermally, or 1 mg subcutaneously, of Fraction I raises the level of antibodies demonstrable by in vitro tests and the MPI test in nearly 90%-100% of the previously immunized individuals to a point rarely, if ever, encountered in previous studies along similar lines. The principle of revaccination is thus an essential part of the immunization of man against plague. A fairly effective immunity against plague cannot be achieved with a one-dose prophylactic inoculation in the face of a plague epidemic except in those individuals who have received a basic immunization several months before the re-inoculation. Frequent re-inoculations are required (1) to enhance the low immunity level induced in 40% of those individuals who cannot respond effectively to the basic prophylactic inoculation; (2) to keep the dose of immunogenic antigen within a range in which the percentage of allergic reactions is low; and (3) to avoid an ebb of the antibody level in those adequately immunized. The method of choice for re-inoculation is to inject 1 mg of Fraction I subcutaneously.

Judged by the appearance of antibodies, man is apparently more readily immunized than primates and guinea-pigs. Since comparative protection tests have shown that nearly 100% of these animals are fully capable of overcoming artificially produced infections when the MPI is below 5, it is reasonable to conclude that men rarely exposed to such severe invasions by *P. pestis* will prove quite resistant. Indeed, if the serum reactions are valid criteria of altered susceptibility against plague, then a basic prophylactic inoculation followed by suitably spaced re-inoculations should confer on man a much better immunity than the experiments have demonstrated in animals. That on re-inoculation the sera of at least 20%-50% of the men previously injected with plague antigens protect 9-10 of the 10 mice used in the passive protection test lends strong support to this conclusion. Similarly low MPIs (0-1) have been invariably recorded in guinea-pigs that resist a severe infection with 100,000 MLD of infection. A correlation between the high level of serum antibodies and the acquired active immunity to plague infection, already accepted with considerable confidence in the case of experimental animals, may with equal assurance be applied to the data for the first time established in connexion with the immunization of man. A plea is made to extend these limited tests to a series of comparative field trials on various population-groups. An opportunity would thus be afforded of comparing the immunogenic power of the different antigens and vaccines now in use.

These observations just analysed should help to dispel pessimistic views concerning the immunogenic power of vaccines prepared from killed organisms or their antigens. On the other hand, there is equally encouraging evidence that a vaccine prepared from living *P. pestis*, strain EV 76, of low virulence in a one-dose administration is suitable for basic inoculations. However, in the customary dose of 1,000,000,000 organisms, the systemic

reactions—general malaise, generalized aching, and anorexia—have been unduly severe. The temperatures varied from 98.6° to 103.6°F (37°-39.8°C) at the end of 24 hours and remained at 100°F (37.8°C) in 2 of the 12 men for 120 hours. At least one half of the group was incapacitated for as long as 72 hours. Notwithstanding these reactions, an antibody response compatible with some degree of immunity was recorded in 4 (33%) of the vaccinated. The average MPI (12.3) was within the range characteristic for a group of men of the same age inoculated with one dose of 2.5 mg of Fraction I in Bristol adjuvant. There is no reason to doubt that re-inoculation will be followed by the same enhanced immune response as recognized for men previously inoculated with vaccines of killed organisms. There is no convincing proof that living plague vaccines may prove superior to Fraction I antigen. Since the living organisms produce this antigen in the host, they are more immunogenic than killed vaccines of low Fraction I content.

The evidence forcibly leads to the conclusion that any plague vaccine, whether killed or living, provided it either inherently contains 2-3 mg of Fraction I or is capable of producing this amount when inoculated into the body of man, will favourably alter the susceptibility of 50% of the inoculated. This basic immunity may even improve after the first month and may persist for at least 6 months. To alter favourably the responsiveness of a larger percentage of human beings and to enhance the moderately effective immunity, re-inoculations at intervals of 3-6 months are essential to the immunization of man against plague. The experimental evidence further warrants the conclusion that prophylactic antiplague inoculation, if applied in the face of an epidemic, will rarely protect more than 40%-50% of the inoculated during the first month after the institution of the procedure. Re-inoculation within 3 months greatly increases the percentage of those who acquire immunity. Annual revaccination progressively improves the immune status of the population and thus creates the impressive results reported from the countries in which this principle has been adopted as an aid in the control of plague in endemic regions.

RÉSUMÉ

Les observations épidémiologiques et les expériences effectuées depuis Haffkine ont montré que diverses préparations peuvent conférer une immunité antipesteuse plus ou moins élevée à l'homme et aux animaux : cultures tuées de *Pasteurella pestis* sur bouillon ou sur gélose, fractions antigéniques de bacilles ou suspensions de souches de faible virulence. Ces recherches n'ont pas toujours eu l'ampleur souhaitable, car de nombreux facteurs sont à considérer avant que des conclusions puissent être atteintes. Le choix de l'animal d'expérience est de la plus haute importance ; en effet, les souris, les cobayes et les singes ne réagissent pas pareillement aux divers vaccins. On s'est demandé aux réactions de quel animal celles de l'homme étaient comparables. Il paraît acquis désormais que l'antigène qui stimule les réactions d'immunité chez la souris et le singe est aussi

immunogène pour l'homme. Dans les efforts faits pour étendre à l'homme les résultats des expériences sur l'animal, un aspect de la question n'a pas été approfondi : le rapport entre la réponse sérologique et le degré d'immunité acquis par le sujet.

Pour attaquer ce problème, l'auteur a institué une série d'expériences, sur des sérums provenant d'une part d'hommes et d'animaux infectés de peste ou guéris et d'autre part de sujets vaccinés, afin d'évaluer la teneur en anticorps et d'établir, à la suite d'une infection d'épreuve, le rapport entre la réponse sérologique et l'immunité. Pour ce faire, il a soumis les sérums à quatre épreuves de laboratoire, qu'il décrit : l'agglutination, la fixation du complément, la protection passive de la souris et l'hémagglutination. Les résultats ont montré qu'en réponse à l'infection, bubonique et pneumonique, l'être humain produit des anticorps qui persistent un certain temps dans le sérum. Chez les primates, les souris, les cobayes et les lapins, la réponse a été du même ordre que chez l'homme. Il est ainsi démontré qu'en réponse à une ou plusieurs infections par *P. pestis*, la plupart des animaux et l'homme produisent des anticorps spécifiques qui persistent dans le sang périphérique, parfois pendant plusieurs mois. Une réinfection de singes guéris d'une première atteinte de peste a révélé que les anticorps circulants — protecteurs et fixateurs du complément — ne déterminent pas l'immunité de façon absolue. Des facteurs histogénétiques individuels interviennent dans la réactivité du sujet et décident en partie de l'issue d'une infection. (Cette restriction ne diminue du reste pas la valeur des anticorps humoraux dans la défense de l'organisme contre la peste, prouvée par les expériences d'immunisation passive.) La réceptivité à l'infection est modifiée par le vaccin selon la rapidité et l'intensité avec lesquelles les anticorps sont mobilisés par l'organisme. Il faut admettre, d'après l'expérience, que l'immunisation artificielle, même avec des antigènes puissants, ne crée qu'une immunité relative, ce que confirment les observations faites au cours des campagnes de vaccination antipesteuse préventive dans le monde.

On a toujours implicitement reconnu que la teneur en anticorps décelables in vitro reflétait plus ou moins le degré d'immunité du sujet. Mais jusqu'à maintenant on n'avait pas établi de rapport expérimental entre le taux des anticorps et le degré d'immunité. Le choix de l'animal d'expérience pour l'évaluation des vaccins n'est pas facile : la souris blanche et le cobaye présentent tous deux des inconvénients, mais le cobaye peut être utilisé pour les examens préliminaires. Pour les expériences plus précises, les primates sont préférables.

L'auteur décrit les essais effectués avec divers types d'antigènes sur la souris, le cobaye, les primates et sur des volontaires humains.

Chez la souris immunisée par la Fraction I, un rapport évident a pu être établi entre le taux des anticorps et le degré d'immunité. Les résultats chez le cobaye, très variables selon l'antigène employé, ne peuvent être applicables à la prophylaxie humaine. Les primates, par contre, sont rendus résistants à des doses massives de bacilles pesteux (1-2 milliards de germes viables) par des suspensions de bacilles tués par la formaline, du vaccin sur bouillon — tué par la chaleur ou par des substances chimiques — et inoculé à la dose humaine habituelle. 1-1,5 mg de Fraction I ont immunisé 60% des primates. La souche EV 76 est un excellent agent immunisant, à raison de 560.000.000 de bacilles vivants. En se multipliant durant 5-10 jours, ces bacilles produisent la quantité de Fraction I nécessaire à l'immunisation. Les résultats prometteurs obtenus sur les singes ont motivé les expériences faites sur des volontaires humains. Les expériences préliminaires permirent de serrer le problème de plus près en essayant l'immunisation au moyen de la Fraction I, qui est atoxique. Il s'agissait d'établir la quantité nécessaire à l'immunisation, la voie d'inoculation la plus adéquate, les réactions locales à l'inoculation et la durée de l'immunité, la réponse à des injections de rappel.

Les conclusions auxquelles l'auteur aboutit, après avoir relaté en détail les expériences faites pour répondre à ces diverses questions, sont les suivantes :

Tout vaccin antipesteux qui contient 2-3 mg de Fraction I (vaccin tué) ou est capable de susciter dans l'organisme la production d'une telle quantité (vaccin vivant) modifiera

là résistance à l'infection chez 50% des sujets vaccinés. Cette immunité de base peut s'accentuer au bout du premier mois et durer au moins 6 mois. Pour augmenter le pourcentage d'immunisés et intensifier l'immunité modérée créée par l'injection de base, il est nécessaire d'effectuer des injections de rappel à intervalles de 3-6 mois. L'expérience montre qu'une vaccination antipesteuse, face à une épidémie, ne protégera guère plus que 40-50% des sujets au cours du premier mois. Une nouvelle inoculation au cours des trois premiers mois augmente fortement le pourcentage d'immunisés. Des revaccinations annuelles élèvent peu à peu le niveau de l'immunité au sein de la population, ce qui conduit aux résultats remarquables obtenus dans les régions d'endémicité où ces principes ont été appliqués comme méthode d'appoint dans la lutte contre la peste.

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