VACCINATION WITH HEAT-KILLED AND FORMALINIZED TUBERCLE BACILLI IN EXPERIMENTAL TUBERCULOSIS

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(Received for publication, May 27, 1933)

In recent years a number of antigens and vaccines have been prepared with the use of formaldehyde. Following the work of Ramon (1, 2), who demonstrated that formaldehyde reduces the toxicity of certain animal and vegetable toxins while maintaining their antigenic properties, Wherry and Bowen (3) and subsequently Wherry *et al.* (4) showed that certain bacterial vaccines could be "detoxified" by this method. Petragnani (5) prepared a vaccine from tubercle bacilli by the use of formaldehyde, for which it was claimed that guinea pigs were made resistant to subsequent infection with tuberculosis and also that if the vaccine were allowed to settle into two portions—sediment and supernatant fluid—, the supernatant fluid could be used for diagnostic tests in a manner similar to tuberculin. While no substance resembling a true toxin has been isolated from the tubercle bacillus or from the medium upon which it has been grown, it was thought that under the action of formaldehyde certain of the undesirable reactions to tubercle bacillus vaccines might be eliminated. The following experiments were undertaken to compare the properties of a vaccine prepared by the use of formaldehyde with the properties of a heat-killed vaccine. Comparisons were made of the immunizing power, of the skin-sensitizing power, and of the rapidity of absorption of the two vaccines. A study of the lesions produced by the intravenous injection of heat-killed bacilli was made as a result of some observations on rabbits vaccinated in this manner.

Material and Methods

Rabbits of about 2 kilos in weight were selected for use in the longevity experiment. They were kept under observation for a short period of time before any

experimental procedures were started, while records of their blood cells and their weights were made. The vaccines were all made from a strain of bovine tubercle bacilli known as B-1. The formalin-killed vaccine was prepared by suspending the organisms in an aqueous solution of 0.4 per cent formaldehyde and 0.9 per cent sodium chloride, and incubating at 37°C. for 10 days. After incubation the suspensions were centrifuged and the organisms washed in distilled water three times and resuspended in 0.9 per cent sodium chloride solution. The heat-killed vaccine was prepared by suspending the organisms in 0.9 per cent sodium chloride solution and heating to 70° for 1 hour. Samples of both vaccines were planted on Petroff's egg media and also injected intravenously into normal rabbits as a control of the sterility of the preparations. The results in each case were negative for living tubercle bacilli.

The first experiment was designed to compare the longevity of tuberculous rabbits which had been vaccinated with heat-killed vaccine with that of tuberculous rabbits vaccinated with formalin-killed vaccine. Rabbits which had received no vaccine were inoculated with the same dose of tubercle bacilli at the same time. The vaccine was given intravenously in two series of injections. The first series, 4 months before inoculation, consisted of seven daily injections of 0.3 mg. each. The second series of injections, 1 month before inoculation, consisted of ten doses of 1.3 mg. each, given at 2 day intervals. Ten rabbits received the formalin-killed vaccine and five rabbits received the heat-killed vaccine, while five animals were kept as controls. Of the ten animals vaccinated with formalin-killed bacilli, two died in the 2 month interval between the two series of injections and at autopsy showed severe snuffles and a non-tuberculous pneumonia. A third animal died after the completion of the two series of injections and before inoculation, and at autopsy showed snuffles and a non-tuberculous pneumonia. Two rabbits were added before the second series of injections to replace the two that had died, so that nine rabbits altogether received intravenous formalin vaccine and were inoculated. In the case of the five rabbits which were given heat-killed bacilli, one died with severe snuffles and a non-tuberculous pneumonia, while three others died during the course of the second series of injections. These at autopsy showed a massive consolidation of both lungs with a tubercular pneumonia, without any evidence of secondary infection, and in the livers and spleens a few epithelioid cells were found on microscopic examination. As a result of the loss of seven rabbits out of the fifteen vaccinated, the intravenous route of injection was discarded in favor of the subcutaneous route, and five normal rabbits were inoculated along with the nine animals which had received formalin-killed vaccine intravenously. These were all given a series of subcutaneous injections of formalin vaccine during the first 4 months of the disease, starting with the day of inoculation and repeated at monthly intervals up to the 4th month when injections were given at weekly intervals. Eight doses of 0.1 mg. were given in all.

A second experiment was started later with two groups of ten rabbits, one vaccinated with heat-killed bacilli and the other with formalin-killed bacilli sub-

cutaneously at 10 day intervals before inoculation. Five doses of 0.1 mg. were given in total. One of the rabbits injected with formalin-killed vaccine died just before inoculation.

RESULTS

The mean longevity of each of the four groups of vaccinated rabbits is shown in Chart 1. The longevity of the unvaccinated, tuberculous

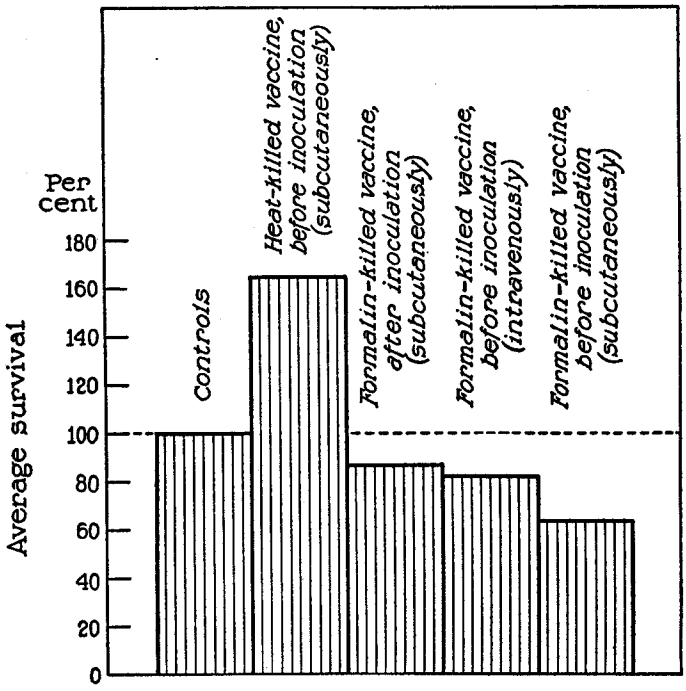

CHART 1

controls is expressed as 100 per cent and the longevity of the vaccinated animals compared with this figure. It will be seen that the rabbits which were vaccinated subcutaneously with heat-killed bacilli survived much longer than their controls, while in none of the other groups was the survival as long as that of their controls.

Chart 2 shows the distribution of deaths by months after infection in the two groups which received vaccine subcutaneously and in the

group of control rabbits which were inoculated at the same time. It will be seen that while the deaths in the group vaccinated with formalin-killed organisms were distributed in a manner similar to that of the controls, the group which was vaccinated with the heat-killed organisms showed a much greater survival. It is noteworthy that in

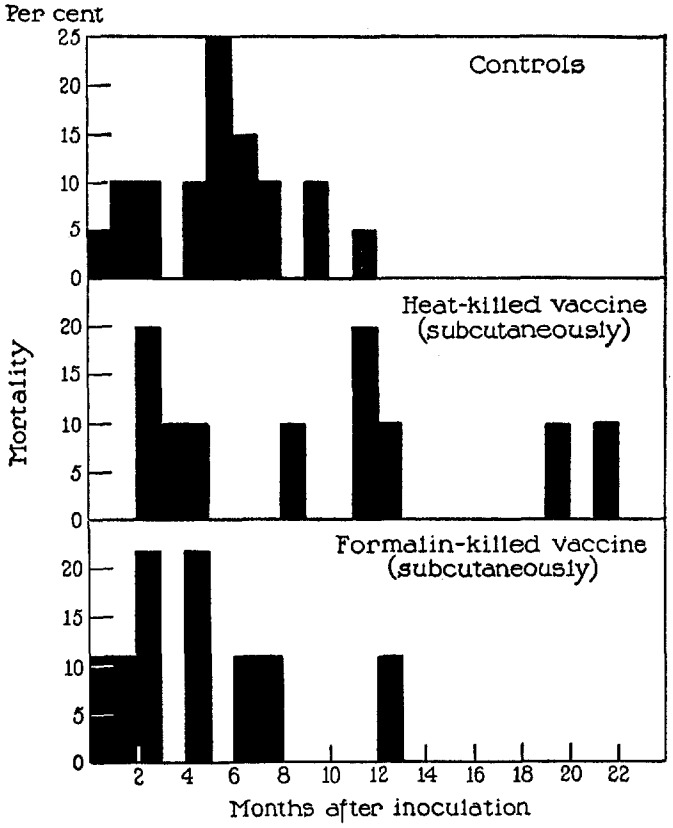

CHART 2

each case some rabbits died in the first phase of the disease, that is, during the first 3 months: in the case of the controls, 25 per cent; in the case of the formalin-killed vaccine group, 44 per cent; and in the case of the heat-killed group, 20 per cent. The effect of the vaccine was thus more apparent in slowing down the chronic phase of the disease than it was in preventing deaths from occurring in the first, acute phase of the disease.

Differences in the course of the disease between the treated and control groups were sought, but in each case the two groups were similar, in so far as direct comparison was possible. In comparing the lesions of one group of rabbits with those of another group, control animals of equal or nearly equal survival must be chosen for comparison, as the extent and the nature of the lesions found in untreated tuberculous animals during successive months after infection vary greatly. Thus, despite the increased longevity of the first group, vaccinated subcutaneously with heat-killed bacilli, the lesions found at autopsy were similar both in extent and type to those found in control animals of the same survival. The changes which occurred in the blood cells, the variations in weight, and the autopsy findings were all typical of the disease as it is seen in untreated control rabbits. Hence it would appear that acquired resistance to tuberculosis in rabbits manifests itself in the ability of the treated animals to retard the rate of progress of the disease, rather than in their ability to modify or heal the lesions.

The rate of absorption of the two vaccines when injected under the skin appeared to be similar. Both produced a soft, fluctuant mass when introduced in this manner, which required 1 or 2 weeks for absorption. Sections of these masses showed them to be typical of tubercular tissue undergoing caseation.

The skin-sensitizing ability of the two vaccines appeared to be equal. Two groups of guinea pigs were injected intraperitoneally with three doses of 2.5 mg. of heat-killed and formalin-killed vaccine respectively. 36 days later, all of the guinea pigs were found to be markedly sensitive to tuberculoprotein (MA-100), and no significant difference between the two groups could be detected.

The failure of the formalin-killed vaccine to induce any appreciable resistance to tuberculous infection in rabbits is evident from the foregoing experiments. Whether the washing to which the organisms were subjected after incubation with the formalin removed any antigenic substance, or whether the formalin specifically destroyed the antigenic substance is problematical. Since the main water-soluble constituents of the tubercle bacillus, namely tuberculopolysaccharide and tuberculoproteins, are not known to possess any immunizing properties (aside from the skin-sensitizing ability of the tuberculoprotein), it was felt that washing would have no deleterious effect.

One is forced to return to the conclusion, reached before by many workers, that the antigenic properties of a tubercle bacillus vaccine are easily destroyed by chemical manipulation and are retained when the vaccine is prepared by gentle heating.

Pathology of Lesions Caused by Heat-Killed Vaccine

The close resemblance of the lesions found in the three rabbits which died during the course of intravenous injections of heat-killed vaccine, to those lesions found in rabbits inoculated intravenously with living bacilli suggested a further study of the effects of inoculation with dead organisms.

Ten rabbits were given two series of intravenous injections of heat-killed vaccine and used for this study. One animal was sacrificed just before the beginning of the second series of injections, and subsequent rabbits were sacrificed after the second, third, fifth, sixth, and seventh doses of the second series of injections. One rabbit was allowed to live for 2 months after the second series of injections and was then sacrificed. The blood cells of two of the rabbits were followed at frequent intervals during the second series of injections.

In the lung of the animal which was sacrificed after two doses of vaccine, the alveoli and smaller bronchioles showed a reaction which consisted of polymorphonuclear leucocytes, monocytes, and epithelioid cells, and some red cells and fibrin. In the lung of the rabbit sacrificed after the third dose, the reaction of polymorphonuclear leucocytes was less marked and there were more epithelioid cells; the lung at this time showed the beginning of a tubercular pneumonia; after the fifth, sixth, and seventh doses the lungs were massively consolidated with a tubercular pneumonia, the air sacs being filled with epithelioid cells and epithelioid giant cells; no polymorphonuclear reaction or fibrinous exudate was seen. There was no congestion of the capillaries of the lung. The lungs at autopsy were increased in consistency and on cut surface showed a thick creamy exudate, which when examined by the supravital method proved to be made up of epithelioid cells and monocytes. The rabbit which was sacrificed 2 months after the last injection showed in the lung no pneumonic lesions, but a number of isolated tubercles, scattered rather uniformly throughout the entire parenchyma of the lung. These tubercles were made up of epithelioid cells which were degenerating, showing vacuolization and degeneration of their cytoplasm, and in some cases nuclear degeneration. One large, confluent tubercle showed slight caseous changes in the center with a few polymorphonuclear leucocytes. Other tubercles were heavily infiltrated with small lymphocytes and plasma cells. The spleens of these animals were relatively free from involvement. In one or two cases epithelioid cells were seen in the spleen, in the supravital technique; in the liver, however, the presence of epithelioid giant cells in small numbers was the rule.

The blood counts of the rabbits were followed carefully throughout the second series of injections. During the 1st day there was a leucopenia, the total white count going as low as 1,150 cells. On the 2nd day there was a recovery, with a leucocytosis, the total white blood count going to 13,200 cells. During the first 2 days, however, the monocytes remained at very low levels, the leucocytosis of the 2nd day consisting of a polymorphonuclear increase; the 3rd day showed in all cases a rise in monocytes, which was accompanied by the appearance of stimulated monocytes and epithelioid cells. These remained high until the 5th and 6th days after which they receded to practically normal levels. The coincidence of the appearance of stimulated monocytes and epithelioid cells in the blood stream with the production of a massive tubercular pneumonia is very clearly shown to occur in the disease produced by intravenous inoculation of living organisms, the two phenomenona occurring at about the 30th day after infection (6).

From the foregoing observations it would appear that the sequence of pathologic changes which occur after the injection of living tubercle bacilli intravenously into rabbits is simulated very closely following the injection of dead organisms. The one characteristic feature of the lesions produced by the dead organisms is their striking uniformity throughout the entire lung. The epithelioid cells are usually in the same stage of development or degeneration, depending upon the time at which the lesions are examined. In the lungs of rabbits infected with living bacilli, tubercles which are practically adjacent to one another may show widely differing characters. One may be judged as relatively recent, while another may be obviously of long standing, possibly calcified. On the other hand, if the tissues of rabbits which have been inoculated intravenously with living tubercle bacilli 2 or 3 months previously are examined, lesions in the lungs and spleen will be found which are regressing. This is seen much more prominently in the spleen than in the lung. Within these lesions the presence of polymorphonuclear leucocytes is not the rule, as in caseation; there may be no accessory cells within the lesions, while the periphery of the area may show an infiltration of lymphocytes, in contrast to an area of caseation, where the center may be infiltrated with leucocytes and the periphery ill defined.

If, as it appears, this process of regression and ultimate absorption of tuberculous lesions occurs without the intervention of caseation, the parenchyma of the involved organ should eventually be found to have returned to the normal state. That this occurs in the spleens of tuber-

culous rabbits, there is little doubt. In the lungs, where the disease regularly progresses in rabbits, there is evidence that both processes, caseation and regression, occur simultaneously. Following intravenous injection of living organisms, a widespread tuberculous pneumonia develops, which reaches its height at the end of the 1st month, while if animals are sacrified 2 or 3 months after inoculation, the disease is confined to discrete tubercles scattered throughout the lung. Sections show the parenchyma of the lung away from these tubercles to be apparently normal, while the lesions themselves show the greatest diversity in type, consisting of tubercles which are undergoing caseation, others which would be considered as recently developed, and areas of pneumonic infiltration and confluent tubercles which appear to be regressing. The extent or total mass of tuberculous lesions in the lungs is considerably reduced during the 2nd and 3rd months after inoculation, and since cavitation is relatively rare at this period it would seem likely that many of the lesions regress in a manner entirely similar to that after the injection of dead organisms. It is probable that certain parenchymal infiltrations of tuberculosis, in infants, which resolve slowly, leaving but a trace of their existence roentgenologically, undergo a similar change as result of a like process.

SUMMARY

1. Rabbits vaccinated with tubercle bacilli killed by exposure to formalin (0.4 per cent) did not show any acquired resistance to subsequent infection with bovine tubercle bacilli, while rabbits vaccinated with tubercle bacilli which had been killed by heating to 70° for 1 hour survived more than half as long again as their controls.

2. Intraperitoneal injection of either the formalin-killed vaccine or the heat-killed vaccine into guinea pigs made them skin-sensitive to tuberculoprotein MA-100.

3. The rate of absorption of the formalin-killed vaccine when introduced beneath the the skin was similar to that of the heat-killed vaccine.

4. Following the intravenous injection of heat-killed tubercle bacilli, it was found that rabbits developed a massive tubercular pneumonia. A study of the production and ultimate absorption of the cellular exudate showed that these processes were similar to those found after the

injection of living bacilli. The lesions which followed the injection of heat-killed bacilli differed from the lesions found in active tuberculosis in that in any one animal they showed a striking uniformity in appearance, while in the active disease the lungs showed a great diversity in type of lesion. Studies of the blood cells during the period of injection of dead organisms showed that the changes which are characteristic of the period during which a tuberculous pneumonia develops in rabbits (30 to 40 days after inoculation) were faithfully reproduced. It is suggested that the process of regression described may be similar to that which occurs in childhood tuberculosis, in which rather extensive pulmonary lesions resolve without leaving evidence of damage to the parenchyma of the lung.

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