EXPERIMENTS ON IMMUNIZATION AGAINST *Bacillus paratyphosus* A.

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Since the publication of our preliminary note on immunization against *Bacillus paratyphosus* A, in the *Journal of the Royal Army Medical Corps* for October, 1912, we have desired to carry out investigations on similar lines on groups of human beings, as we realised that experiments on animals, though useful in affording a basis for dosage and in giving a general idea of the results following inoculation, could never justify conclusions as to the desirability or otherwise of issuing a paratyphoid vaccine for the use of soldiers proceeding to India. The opportunity was recently given us of doing some further work on the subject when six privates of the Royal Army Medical Corps offered themselves as volunteers and enabled us to inoculate two groups of three men each, the one with a vaccine of *B. paratyphosus* A alone and the other with a mixed emulsion of that organism and *B. typhosus*. We were able to assure the men that no harm would follow the inoculation as one of us had previously administered doses of 500 and 1,000 millions of *B. paratyphosus* A to a chronic carrier of that germ with no untoward results, and we had also the advantage of access to the work of Major R. W. Clements and Captain W. R. Galwey, R.A.M.C. ("Notes on a Case of a Paratyphoid 'A' Carrier Treated with a Specific Vaccine," *Journal of the Royal Army Medical Corps* February, 1913), who gave a series of doses rising from 20 to 800 millions to their patient without causing any inconvenience. Still, we wish to express our warm thanks to the volunteers who came forward with the knowledge that the larger doses necessitated by the administration of a mixed emulsion might well cause them several days of pyrexia and malaise. For the purpose of this experiment, a vaccine was prepared from an Indian strain of *B. paratyphosus* A (James), all the steps followed in the case of the routine preparation of the antityphoid vaccine being repeated to make the two vaccines comparable in every way. After killing at 53° C. and preservation through the addition of 0.4 per cent. of lysol, the preparation was allowed to stand for some days before use, and was employed in combination with an antityphoid vaccine of the same date. None of the men forming the "groups" had ever had
typhoid fever, with one exception none of them had ever been out of England, and all were in good health. The one man who had been abroad had been in Malta as a child when his father was stationed there, and he had then suffered from Mediterranean fever. Each man of "Group I" received a first dose of 500 million followed by a second dose of 1,000 million twelve days later, only B. paratyphosus A being given. Each man of "Group II" received 500 million each of B. paratyphosus A and B. typhosus as a first dose and 1,000 million of each germ as a second dose on the twelfth day after the first. With regard to reaction, the only man who suffered from any general reaction after the first dose was a member of Group II, and, indeed, was the man who had been in Malta in his childhood. He had a very sore arm for some days after the inoculation and had to retire to bed with nausea and malaise within an hour of the dose. His temperature rose to 101.8°F. on the evening of the inoculation. He was much better next morning, though still feeling poorly, and was not quite himself until the third morning after the dose. The other two men in this group had congested areas at the site of inoculation for about twenty-four hours, such as usually follow injections of antityphoid vaccine, but beyond this had no symptoms. The three men forming Group I were quite free from reaction beyond a slight redness at the site of injection. The paratyphoid A vaccine alone appeared to give rise to a degree of reaction decidedly less than that usually following antityphoid vaccine. After the second dose all the men in Group II had a fairly smart reaction, though not such as to be regarded as exceptional after a dose of antityphoid vaccine alone. The man who had suffered from a severe reaction on the first occasion escaped with a decidedly less troublesome result on the administration of the second dose. All the members of Group I again got off very lightly, none of them having anything more serious than a patch of redness at the point of inoculation.

Technique of Opsonin Estimation.—The serum of all the men under examination was collected just before the first dose, and twice weekly during the period of the experiment. The serum from each group was pooled and the possibility of small personal idiosyncrasies becoming manifest thus diminished. The blood was allowed to stand for twenty hours in all cases before the serum was drawn off from the clot. The pooled serum of each group was then heated to 58°C. for twenty-five minutes to remove complement and to allow only the specific opsonins to remain demonstrable. For the opsonic tests the bacterial emulsions were invariably made

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from twenty-hour growths on agar at 37° C. subcultured from an agar slope kept at room temperature, the same slope being used throughout the experiment to avoid loss of virulence in subculture. For the opsonic estimations, the emulsion was counted by means of the hæmocytometer and standardized to contain 1,000 million bacilli per 1 c.c. before each observation, so as to make successive "counts" as far as possible comparable with each other. The phagocytes used were derived from the same individual throughout.

The opsonic mixtures were kept in contact for ten minutes only at 37° C. in order to avoid intracellular digestion of the bacilli. It will be noted that the bacillary emulsion used was much stronger than usual in opsonic estimations by the method of Wright. In employing the dilution method of Klien a rather thick emulsion is necessary. Klien, however, worked with unheated serum, the object of dilution being to eliminate the influence of bacteriolysis on the "counts," whereas we used heated serum, successive dilutions being employed in order to obtain a more complete picture of the development of specific opsonins than is afforded by the examination of the serum in one concentration only. The highly important work of J. C. G. Ledingham and H. R. Dean on the action of the complement-fractions on a Tropin-B. typhosus system, has opened up the whole question of the action of complement on phagocytosis and has shown that the relative concentration of "mid-piece" and "end-piece" in any given serum may lead either to inhibition or increase of the phagocytic action of the specific opsonin present. In view of these facts we decided to adhere to the method employed in our preliminary experiments on rabbits and to use heated serum only. This must be borne in mind when comparing our results with those of other observers, as the use of heated serum, not the production of a less complete immunity, explains the fact that in our experiments the end-point reached is lower, in the case of B. typhosus, than that recorded by Klien or Grattan. The opsonic films were stained by Leishman's stain and fifty phagocytes were counted in each dilution.

Technique of Agglutinin Estimation.—The bacterial emulsions used were not standardized by counting, but by the addition of a known volume of saline to a 20-hour growth on an agar slope, this giving a sufficient approximation to constancy for tests not involving the delicate method of counting the bacilli in phagocytes. The serum used was heated, as, even in agglutinin experiments, we

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believe that the results of successive estimations are more comparable when complement has been eliminated. The results were read macroscopically, the serum dilution and the bacillary emulsion being kept in contact, not in the capillary portion of the pipette, but at the junction of the shoulder with the capillary stem. The greater volume of fluid thus made available enables a better idea
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of the agglutination process to be obtained than where only the capillary limb of the pipette is filled with fluid. The mixtures were kept at 37°C for three hours before reading the results.

**TABLE I.—THERMOSTABLE OPSONINS.**

<table>
<thead>
<tr>
<th>Day of observation</th>
<th>1</th>
<th>4</th>
<th>8</th>
<th>11</th>
<th>13</th>
<th>15</th>
<th>19</th>
<th>22</th>
<th>26</th>
<th>29</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilutions of serum</td>
<td>1/10</td>
<td>1/50</td>
<td>1/250</td>
<td>1/1250</td>
<td>1/6250</td>
<td>1/31250</td>
<td>1/156250</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I, B. para- typhosus A</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>16</td>
<td>32</td>
<td>64</td>
<td>128</td>
<td>256</td>
<td>512</td>
</tr>
</tbody>
</table>

No attempt was made to calculate the bactericidins, as our previous work on this subject had not given satisfactory results. This is to be regretted, as the curious divergence between the development of agglutinins and opsonins in the case of B. para-
typhosus A. raises the question whether the bactericidal powers of the serum follow the one or the other. Further experiments will be necessary to decide this point.

Opsonins.—Chart I gives the end-points of the opsonins, calculated by Klien’s method, for B. paratyphosus A in Group I, and for both organisms in Group II. It will be at once apparent that both

The average number of bacilli ingested by each phagocyte, under the influence of each dilution of serum, is represented, for each day of observation, by the number of small squares shaded grey in each large square. Each large square contains twenty-five small ones, a number equivalent to the maximum bacillary content countable in each phagocyte, and each small square therefore corresponds to one bacillus per phagocyte. Half a small square, being equivalent to 0.5 bacilli per phagocyte, represents the opsonic end-point of Klien.

in Group I and Group II the production of opsonins for B. paratyphosus A is very small. An examination of the charts will show further that a slight rise took place after each injection, to be followed by a drop when the agglutinins rose to their highest point. A similar phenomenon has already been noticed by us in our experiments with rabbits. The opsonins, in both groups, appeared to rise slightly towards the end of the experiment. The production
of opsonins for *B. typhosus* in Group II affords a marked contrast to that for *B. paratyphosus* A, rising steadily to a maximum and then beginning to fall gradually. The contrast between the two organisms in this matter of opsonin-production is very apparent in Table I, where the actual counts are recorded, up to an end-point and one dilution above it, in a series of columns. In Chart II an attempt has been made to give a graphic representation of the formation of opsonins for *B. typhosus* in Group II, the average number of bacilli per phagocyte being used as a basis. It should be said that, as in previous experiments, we had decided that no count of more than twenty-five bacilli in one phagocyte could be reliable, and we accordingly recorded all uncountable phagocytes as containing twenty-five bacilli. The chart consists of a series of squares, each containing twenty-five smaller squares. Each large square may be taken to represent the average phagocyte in each dilution on each day of observation. Each small square represents one bacillus per phagocyte, and half a small square is equivalent to 0·5 bacilli, the opsonic end-point of Klien, while the whole large square, consisting of twenty-five small ones, represents the maximum opsonic capacity demonstrable for a phagocyte. It is at once apparent on examining the chart that the maximum of concentration of the serum does not go hand in hand with the maximum of phagocytosis. As the end-point rises, the degree of dilution leading to the maximum of phagocytosis also rises, both end-point and degree of phagocytosis appearing to fall together as the wave of immunization recedes. It might be possible to explain this by assuming that there was more rapid intracellular solution of bacilli in the higher concentrations, this being less marked in the dilution that gave the greatest phagocytic index. We are, however, inclined to think that the optimum concentration of serum for phagocytosis varies with the rise in antibody and the concentration of bacteria in the mixture, and that, where the latter is constant, as in our experiments, the optimum conditions for phagocytosis will be found in higher dilutions as the titre in specific opsonin rises. We called attention to a similar phenomenon in our preliminary note on immunization against *B. paratyphosus* A, already quoted.

Returning to the difficulty of producing opsonins for *B. paratyphosus* A, it occurred to us that, in heating the serum, we might conceivably have brought about conditions unfavourable to the demonstration of phagocytosis in the case of this organism. We decided to test this by repeating an observation with the addition
of normal guinea-pig serum in a dilution of 1 in 10 to each phagocytic mixture. The result was to bring about a slight increase of phagocytosis, both as to end-point and number of bacilli per phagocyte in the higher concentrations of serum, but

this was no more in the case of the anti-paratyphosus A serum than in heated normal serum used as a control, and, therefore, must be attributed merely to the normal opsonins present in the guinea-pig serum itself.
The fact that the failure to induce phagocytosis for *B. paratyphosus* A was common to the sera of both Group I and Group II, and that it was consistently demonstrable throughout the investigation leads us to think that the phenomenon is a genuine one, and not dependent upon any inaccuracy of technique, though further work will be necessary to settle this point. We do not attempt to explain it, but merely record the results of our experiments for what they are worth.

Agglutinins.—Turning to the production of agglutinins, the result of our observations is shown in Chart III. This chart brings to light a great difference between the results of inoculation with *B. paratyphosus* A in men and in rabbits. In our previous work, we failed to produce any satisfactory agglutinin titre for this organism in rabbits with doses at all equivalent to those used in the prophylactic inoculation of human beings against *B. typhosus*. In men, however, similar doses led to high production of agglutinins in both Group I and Group II. This success in producing agglutinins contrasts markedly with the failure to evoke opsonin-production in the same groups. The agglutination of *B. typhosus* by the serum of Group II, though giving an end-point lower than for *B. paratyphosus* A, was much more complete and rapid than in the case of the latter organism in all positive dilutions. Still the fact remains that the inoculation of *B. paratyphosus* A led to good formation of agglutinin in men, though failing to do so in rabbits. We anticipated some such result, as Clements and Galwey, already quoted, had recorded high agglutination titres in the serum of their carrier case as the result of inoculation with a vaccine of *B. paratyphosus* A. The salient feature of our research is, then, the curious divergence between the production of opsonin and agglutinin as a result of inoculation with *B. paratyphosus* A vaccine. Whether this observation may have any bearing on the question of the advisability or otherwise of employing this organism for the preparation of a prophylactic vaccine we are as yet unable to say. It appears certain that no harm can result from such inoculations, and we anticipate that the use of a vaccine made from *B. paratyphosus* A alone will be found to give rise to even less reaction than a similar dose of antityphoid vaccine. We are also of opinion that a mixed emulsion containing both organisms can be given with safety, the doses being such as we employed in the above experiments, and it seems that the immunity produced for each organism will be no less than when a single vaccine is given. But the fall in specific opsonins just when the agglutinins reach their
height, in the case of *B. paratyphosus A*, is a point that may require further consideration. Some careful observations on the opsonins formed during an attack of the disease would be of the highest value in elucidating this question, and such observations would yield the most valuable information if carried out by Klien's method. We realize the difficulties that confront the medical officer in India when it is a question of carrying out elaborate and lengthy bacteriological procedures, but possibly some worker in a well-equipped laboratory may find time to investigate this question. We may summarize the result of our work as follows:—

1. *Bacillus paratyphosus* A vaccine, given in two doses of 500 and 1,000 millions respectively, at an interval of twelve days, gave rise to no reaction of importance.

2. A mixed emulsion of *B. paratyphosus A* and *B. typhosus* in similar doses gave rise to a reaction not appreciably more severe than that following antityphoid inoculation. In saying this we make the reservation that individuals may differ in their reaction, and that a final conclusion cannot be reached without a more extended trial.

3. The serum of persons inoculated with a vaccine of *B. paratyphosus A* was found to develop very little specific opsonin.

4. Inoculations with *B. paratyphosus A* led, in human beings, to a considerable production of agglutinin in the serum, thus differing from similar inoculations in rabbits.

In conclusion, we desire to express our thanks to the following for their kindness in volunteering to undergo the experimental inoculations: Privates W. B. Symington, J. C. Taylor, B. Querney, W. G. Cocks, A. S. Tilbury and G. Harper, of the Royal Army Medical Corps.