THE IMMUNE RESPONSE OF MICE TO CHOLERA VACCINE

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SUMMARY: Adult male National Institute of Health (NIH) mice were immunised with cholera vaccine grown either on liquid or solid media. Splenocytes obtained from sacrificed animals were tested by a vibriolytic plaque forming cell assay (VPFCA) using Vibrio cholerae as the target antigen to measure both IgM and IgG secreting cells. Antibody levels in serum were also measured using a vibriocidal test. No difference was detected between the two vaccines using these methods.

Introduction

Fields trials1 2, have indicated that immunisation with killed V. cholerae gives increased resistance to cholera infections. This acquired immunity however, has been found to be of short duration and incomplete3. Currently available V. cholerae vaccines are grown in quantity either on solid agar media or in broth cultures. The present study employs an animal model to compare the immunising properties of agar grown vaccine with that of vaccines grown in broth using the vibriolytic plaque forming cell assay (VPFCA) and a vibriocidal assay. The object was to test whether antigens which may be lost in the agar from the organisms, but which are presumably present in the broth vaccine, made any difference to the antigenicity of the preparation.

Materials and methods

Preparation of vaccines

Smooth colonies biochemically and serologically tested, of V. cholerae Inaba 35A3 were sown on to Cholera-Citrate Agar in Roux bottles and incubated at 37°C for 18 hours. The resultant growth was washed off and harvested with normal saline and the harvest volume measured. For the broth grown vaccine, the same organism was cultured in a Polyferm laboratory fermenter (LKB Instruments Ltd) using a non-antigenic medium of casaminoacids (DIFCO Ltd).

The fermenter was incubated at 28°C for 18 hours at a constant rate of 600 rpm and aerated at 50 l/hour. An aliquot from the agar grown and broth grown suspension was removed for purity testing and for the turbimetric estimation of bacterial numbers using a previously calibrated nephelometer. The remaining bulk of each suspension was treated with 80% phenol to produce a final phenol concentration of 1%.

Both the agar grown and broth grown vaccines were left for four days at room temperature with occasional shaking to enable the killing of the organisms.

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to be successfully accomplished. The vaccines were then diluted to a working strength of $8 \times 10^9$ organisms ml$^{-1}$ with 0.9% saline and phenol concentrations were measured spectrophotometrically at 270 nm and adjusted to 0.5 — 1% with 80% phenol. Sterility and animal toxicity tests were carried out on the finished vaccine.

Animals

Adult male NIH mice, approximately 6-12 weeks of age and weighing 20-25 g (Allington Farm, Porton) were used throughout. The mice were kept in groups of four to eight and fed with PMD pellets and allowed water ad libitum.

Immunisation

To determine the approximate optimal immunising potential of the vaccine, tenfold dilutions of agar grown vaccine from neat to $1 \times 10^{-8}$ were made using 0.9% isotonic saline. Then 500 µl of each dilution was inoculated intraperitoneally into groups of four mice. Studies done by Friedman, indicated that the peak VPFCA occurred during days 10-14, so the mice were sacrificed and tested on day 12 after immunisation. Splenic VPFCA using live V. cholera Inaba as antigen, indicated that the stronger the immunising dose, the better was the immune response. Neat vaccine gave a higher VPFCA than any of the ten-fold dilutions. It was decided not to test a stronger vaccine preparation as the neat vaccine used, containing $8 \times 10^9$ organisms ml$^{-1}$ was the standard strength of vaccines in general use.

Two batches of 36 mice were inoculated intraperitoneally with 500 µl of neat vaccine, either agar or broth grown, to achieve a primary stimulation. No further immunisation was carried out and the mice were kept for intervals ranging between 2-28 days before being sacrificed.

Vibriolytic plaque forming cell assay for IgM secreting cells

Splenocyte from an immunised animal are set in agar gel, together with live vibrios and on the addition of complement, discrete plaques are formed in the otherwise confluent growth of vibrios. The VPFCA using viable vibrios was used to detect IgM antibody plaque forming cells using the technique of McAlack, Gerny and Friedman. Immunised mice splenocytes were plated in agar with living V. cholera vibrios. After incubation and addition of complement, confluent bacterial growth occurred, with clear plaques of no growth where immunocytes were secreting antibodies to the vibrios. The zones of no growth were enumerated and reported as plaques per spleen.

Facilitation of VPFCA for IgG secreting cells

The facilitation plaque assay was used as described by Friedman. In this technique a broad spectrum rabbit anti-mouse IgG serum (Miles Laboratories) at a dilution of 1/60, was used to cause cross linking with mouse IgG antibody, thus activating the complement pathway so causing lysis of the vibrios. A plaque increase of more than 10% over the original plate indicating low efficiency IgG.
Inhibition test to determine specificity of vibriolytic plaque forming cells

Inhibition experiments were conducted on day 24 after primary inoculation to assess the degree of inhibition on the VPFCA by both specific and unrelated substances to the Inaba vibrio. Duplicate agar plates were treated with Inaba vaccine, Ogawa vaccine, bivalent cholera vaccine, Choleragen, E. coli 01:19, normal rabbit serum, neat rabbit anti-mouse IgG serum, 1/10 rabbit anti-mouse IgG serum before the addition of splenocytes. All the bacterial suspensions were used at a concentration of $8 \times 10^9$ organisms ml$^{-1}$. The degree of inhibition, as judged by the number of plaque forming cells compared to untreated plates was recorded.

Vibriocidal test for somatic cholera antibodies

The method described by Benenson, Saad and Mosley$^a$ was used, with Microtitre disposable sterile plastic plates with U wells. Samples of immunised mouse serum were taken at the time of death, pooled and frozen at $-20^\circ$C. All the antibody tests were carried out in one batch.

Results

Direct plaque assay for IgM secreting cells

A typical plaque is shown in Fig. 1. The results of the direct plaque assay for splenocytes secreting IgM antibody using agar grown vaccine (Fig 2) and broth grown vaccine (Fig 3) are shown. The agar vaccine shows a primary peak at day 12 (8870 plaques/spleen) and a secondary peak at day 20 (7210 plaques/spleen). The broth vaccine also peaks at 12 days (13,010 plaques/spleen) but with this vaccine a second peak occurs at 17 days. Unimmunised mice showed negative splenic plaque results.

![Fig 1. Illustration of an individual plaque, showing surrounding microcolonies.](image-url)
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**Fig 2.** Splenic plaque forming response to agar grown vaccine. Each • represents the average plaque forming cell response in spleens of four mice on day indicated when tested with Inaba vibrios in agar plates. O are results from individual mice.

**Fig 3.** Splenic plaque forming cell response to broth grown vaccine. Each • represents the average plaque forming cell response in spleens of four mice on day indicated when tested with Inaba vibrios in agar plates. O are results from individual mice.

**Indirect plaque assay for IgG secreting cells**

The highest IgG responses were evident at day 20 after immunisation in both agar and broth vaccines (Table I). These responses have been shown to be
Table I
Results of facilitation assay for indirect vibriolytic PFCs against Inaba 35A3

<table>
<thead>
<tr>
<th>Days after immunisation</th>
<th>Agar vaccine</th>
<th>Broth vaccine</th>
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<tbody>
<tr>
<td></td>
<td>IgM ± s.e</td>
<td>IgG ± s.e</td>
</tr>
<tr>
<td>5</td>
<td>65 ± 27</td>
<td>70 ± 33</td>
</tr>
<tr>
<td>8</td>
<td>3500 ± 1799</td>
<td>220 ± 155</td>
</tr>
<tr>
<td>12</td>
<td>8870 ± 1882</td>
<td>1270 ± 189</td>
</tr>
<tr>
<td>15</td>
<td>4340 ± 825</td>
<td>620 ± 248</td>
</tr>
<tr>
<td>17</td>
<td>3610 ± 276</td>
<td>2130 ± 646</td>
</tr>
<tr>
<td>20</td>
<td>7210 ± 924</td>
<td>5500 ± 741</td>
</tr>
<tr>
<td>22</td>
<td>4570 ± 821</td>
<td>3500 ± 908</td>
</tr>
<tr>
<td>27</td>
<td>1542 ± 520</td>
<td>5100 ± 226</td>
</tr>
</tbody>
</table>

essentially specific in that the Inaba vaccine inhibits plaque formation far more than the heterologous Ogawa vaccine. The bivalent Inaba/Ogawa vaccine is even more successful at inhibiting the plaques (Table II). Choleragen shows slight

Table II
Inhibition of vibriolytic PFC by specific and unrelated substances

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>PFC/Spleen</th>
<th>Percentage inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inhibitor</td>
<td>4840</td>
<td>None</td>
</tr>
<tr>
<td>Inaba vaccine</td>
<td>1760</td>
<td>64</td>
</tr>
<tr>
<td>Ogawa vaccine</td>
<td>3120</td>
<td>36</td>
</tr>
<tr>
<td>Bivalent cholera vaccine</td>
<td>840</td>
<td>83</td>
</tr>
<tr>
<td>Choleragen 10</td>
<td>4040</td>
<td>17</td>
</tr>
<tr>
<td>E. coli 0119 suspension</td>
<td>4840</td>
<td>None</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>4400</td>
<td>10</td>
</tr>
<tr>
<td>Neat rabbit anti-mouse IgG serum</td>
<td>6800</td>
<td>40% enhancement</td>
</tr>
<tr>
<td>1/10 rabbit anti-mouse IgG serum</td>
<td>9360</td>
<td>93% enhancement</td>
</tr>
<tr>
<td>Neat anti-human globulin serum</td>
<td>3840</td>
<td>21</td>
</tr>
</tbody>
</table>

inhibiting properties but the unrelated E. coli organism has no effect on the vibriolytic plaques. Both normal rabbit serum and anti-human globulin serum show some inhibiting activity but the former has been previously reported⁸ and the reason for the latter remains unclear.

Results of vibricidal test
The vibriolytic assay was included as it was thought to offer better correlation with the vibriolytic plaque assay as both methods rely on complement medi-
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ated lysis. From the results (Table III) it will be seen that both vaccines elicited a good response.

### Table III

**Serum antibody response of mice immunised with monovalent Inaba vaccine**

<table>
<thead>
<tr>
<th>Days after immunisation</th>
<th>Vibriolytic assay for total antibody (reciprocal of titre)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Agar grown vaccine</td>
</tr>
<tr>
<td>15</td>
<td>1024</td>
</tr>
<tr>
<td>20</td>
<td>2048</td>
</tr>
<tr>
<td>22</td>
<td>1024</td>
</tr>
<tr>
<td>27</td>
<td>2048</td>
</tr>
</tbody>
</table>

**Discussion**

A monovalent agar grown vaccine was compared with a monovalent broth grown vaccine, by an antibody plaque forming technique, after stimulation in mice. The original plaque technique described by Jernie and Nordin used sheep red blood cells as the target cell, with the appropriate antigen tagged on. This has the advantage of non-immunised animals showing a background plaque count to the sheep cells. Using vibrios as the target antigen however, no background response is present and this has the considerable advantage of allowing VPFCAs to be carried out without non-specific elements interfering.

As far as the somatic antigens are concerned the two vaccines tested appear to elicit a similar antibody response. There was no difference in the magnitude of response although the broth vaccine gives a slightly better response as judged by the VPFCA.

The bimodal curve found on both VPFCAs and the vibriolytic antibody assays are a slightly unusual finding but has been reported in responses to sheep erythrocytes and also to vibrio cholera. The double peaks could be caused by a slowly diffusing antigen or possibly be due to separate clones of cells responding to different levels of antigen stimulus thus producing a primary and a secondary peak.

The facilitation assays for IgG producing splenocytes also showed a bimodal curve. The inhibition studies showed Inaba/Ogawa whole cell vaccine to be most effective in inhibiting the VPFCA.

The vibriolytic VPFCA is particularly useful for testing the response of mice to cholera vaccines due to its specificity and lack of background response. In this series of experiments broth grown vaccines showed similar immunising properties to agar grown vaccine. Future studies should compare the VPFCA with the presently used Feeley and Pitman test of active mouse protection for assaying the potency of cholera vaccine.
Acknowledgements

This work was carried out at the David Bruce Laboratories, East Everleigh, Wiltshire, and I wish to thank Lt Col R Donalson for his help. I would also like to thank Lt Col E S Parry and Dr J M Mishler for their helpful advice.

REFERENCES


Honorary Consultants

To The Army

Dr R N T Thin, MD, FRCP(Edin), has been appointed Honorary Consultant in Genito-urinary Medicine to the Army, with effect from 1 February 1981, in succession to Dr R D Catterall, FRCP(Edin), FRCP.

Professor J R Tighe, MB, BCh, MD, FRCP, FRCPath, will be appointed Honorary Consultant in Histopathology to the Army, with effect from 1 October 1981, in succession to Professor D H Mackenzie, MBE, MRCS, LRCP, FRCPath.

Dr. Geoffrey Bennett, MA, BM, BCh, MFOM, has been appointed Honorary Consultant in Aviation Medicine to the Army, with effect from 1 February 1981. This is a new appointment.

Mr D Henderson, MB, BS, FDS, RCS, has been appointed Honorary Consultant in Dental Surgery to the Army, with effect from 16 December 1980, in succession to Mr. N. L. Rowe, CBE, FRCS, FDS RCS.

To the Cambridge Military Hospital

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