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ANTIBODY RESPONSE IN HAEMORRHAGIC SMALLPOX.

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HAEMORRHAGIC smallpox is undoubtedly the most severe form of smallpox. Recently, it has been shown by Sarkar and Mitra (1967) that the virus strains isolated from such cases were more virulent than those isolated from non-haemorrhagic (i.e. confluent and discrete) cases of smallpox, as per the parameters used for testing virulence by the authors. Antibody response in any disease process is likely to have bearing on the clinical picture, and the purpose of the present work is to study the antibody response of the haemorrhagic smallpox patients who usually die during the early stage of the disease.

MATERIALS AND METHODS.

All the cases included in this work were indoor patients from the Infectious Disease Hospital, Beliaghata, Calcutta. The criteria of diagnosing a case as 'haemorrhagic smallpox' were : extreme toxicity with generalized flush and visible haemorrhages from the skin or mucus surfaces as well as from internal organs as manifested by haematemesis, melaena, haematuria, etc. Six of the haemorrhagic cases were, however, comparatively less toxic and could be designated as 'variola pustulosa haemorrhagica' (v.p.h.). Out of nine varieties of smallpox described by Dixon (1962), his 'fulminating', 'malignant confluent', and 'malignant semi-confluent' cases fall in our 'haemorrhagic' group.

Samples of venous blood (and vesicular or pustular fluid) were collected from patients soon after admission into the hospital. Only one sample of blood was collected from each case. Relevant history, including that of previous vaccination, if available, as well as the presence or absence of a vaccination scar were recorded. Vaccination scar was not discernible in some patients due to inflammatory oedema or haemorrhage into the skin, and in a few others, the scar could not be found although the patients (or their relatives) stated that they had been vaccinated before. Vaccination history in these latter cases was recorded as 'not known'.

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Blood was allowed to clot, brought back to the laboratory in ice, and the separated sera were preserved at —20°C. Virus isolation was attempted by inoculating the sera (or vesicular fluid) on the chorio-allantoic membrane of eggs using the standard techniques used before (Sarkar and Mitra, *loc. cit.*).

HI test was carried out in MRC (plastic) trays using a previously standardized technique (Chatterjee and Sarkar, 1963). Sera inactivated at 60°C. for 20 minutes, were diluted in doubling dilutions in the cups of the trays in 0·2 ml. volumes, the starting dilution being 1 : 10. Four haemagglutinating (HA) units of virus in 0·2 ml. volume were added and the serum-virus mixtures were incubated at 37°C. for one hour, after which 0·2 ml. of 0·5 per cent red blood cells in normal saline from previously tested roosters (Hirst, 1952) was added. The trays were then incubated for one hour at 37°C. before the reading was made. Absence of button formation and scattered deposition of clumped red cells constituted positive haemagglutination. The antigen of HI test was prepared according to the techniques of Downie *et al.* (1961), but the virus strain used in this work was vaccinia virus isolated from Russian freeze dried vaccine, in its 3rd and 4th egg passage levels. This strain was used satisfactorily in a previous work (Sarkar *et al.*, 1964). The haemagglutination titre of the antigen suspensions generally varied between 1 : 128 and 1 : 256. Suitable positive and negative serum controls and antigen titrations were set up simultaneously along with each test.

Neutralization tests were carried out according to Downie *et al.* (*loc. cit.*) with the following modifications : (*a*) Vaccinia virus was used for preparation of stock antigen, (*b*) sera were diluted in doubling dilutions starting from 1 : 10, (*c*) four eggs were used for each dilution of serum, and (*d*) the titre of each serum was estimated, as was suggested by McCarthy and Downie (1953). Controls using virus alone was set up along with each test. The highest dilution of serum showing 50 per cent or more reduction in the number of pocks as compared to the number produced in the control represented the titre.

For the sake of comparison, sera of other smallpox cases, which were not haemorrhagic and were admitted in the early stage of the disease, were collected and their titres of antibody determined in a similar manner.

Results.

The present series consists of 45 haemorrhagic cases (including six of v.p.h.) and 22 non-haemorrhagic cases. All these cases were virologically positive, variola virus having been isolated from the sera of 39 haemorrhagic cases and from the skin lesions of the six v.p.h. and 22 non-haemorrhagic cases. Because of the severe nature of the presenting features, the haemorrhagic cases sought early admission into the hospital. The position was not the same with the non-haemorrhagic cases who came to the hospital comparatively late in the disease. Therefore, the number of the latter cases who could be investigated during the first few days of illness was smaller. The HI and neutralizing titre of the sera of the haemorrhagic cases, together with age, sex and vaccination status of the patients are presented in Table I. It can be seen from Table I that if the six cases of v.p.h. are excluded, 28 out of 39 or 71·8 per cent of the

TABLE I.

Showing HI and neutralizing titre of haemorrhagic smallpox cases.

Case number.	Age/Sex.	Day of illness.	Previous vaccination.	HI titre.	Neutralizing titre.	Results.
1	8/M	2	No	<10*	<10†	Died
2	18/M	2	No	<10	<10	Died
3	18/M	2	No	<10	<10	Died
4	20/M	2	No	<10	<10	Died
5	21/M	2	No	<10	<10	Died
6	22/F	2	No	<10	<10	Died
7	22/M	2	No	<10	<10	Died
8	26/M	2	No	<10	<10	Died
9	27/M	2	No	<10	<10	Died
10	28/M	2	No	<10	<10	Died
11	29/F	2	Not known	<10	<10	Died
12	35/F	2	No	<10	<10	Died
13	35/F	2	Not known	<10	<10	Died
14	14/M	3	Not known	10	<10	Died
15	14/M	3	No	10	<10	Died
16	18/M	3	Not known	20	<10	Died
17	19/M	3	No	<10	<10	Died
18	20/M	3	Not known	<10	<10	Died
19	20/M	3	No	<10	<10	Died
20	21/M	3	Not known	<10	<10	Died
21	21/M	3	No	<10	<10	Died
22 (+)	22/M	3	Not known	80	<10	Died
23	23/F	3	No	<10	<10	Died
24	23/M	3	No	<10	<10	Died
25	26/F	3	Not known	20	<10	Died
26	30/M	3	No	<10	<10	Died
27	31/M	3	No	23	<10	Died
28	32/M	3	No	<10	<10	Died
29	32/M	3	No	20	<10	Died
30	35/M	3	No	<10	<10	Died
31	35/M	3	No	<10	<10	Died
32	42/F	3	Not known	<10	<10	Died
33	22/F	4	No	20	<10	Died
34	22/M	4	No	<10	<10	Died
35	23/M	4	Not known	<10	<10	Died
36	30/M	4	No	<10	<10	Died
37	37/M	4	Not known	20	<10	Died
38	40/F	4	No	40	10	Died
39	40/F	4	No	40	10	Died
40 (+)	49/M	4	Not known	80	10	Died
41	34/F	5	Not known	40	10	Died
42 (+)	20/M	6	Not known	320	40	Survived
43 (+)	23/M	6	Not known	80	10	Died
44 (+)	30/M	6	Not known	320	40	Survived
45 (+)	35/M	6	No	80	10	Survived

* Indicates highest dilution of sera inhibiting haemagglutination.

† Indicates highest dilution of sera giving 50 per cent or more reduction of pocks in comparison to those of the control.

(+) Indicates cases of 'variola pustulosa haemorrhagica'.

cases on the 2nd to 4th (average 2·3) day of illness did not show any detectable HI antibody and 36 out of 39 or 92·3 per cent on the 2nd to 4th (average 2·5) day of illness did not show any detectable neutralizing antibody in their sera diluted 1 in 10. Even in those whose sera were positive by HI or neutralization tests, the titres were

extremely low. The v.p.h. patients whose sera were collected between 3rd to 6th (average 5·2) day of illness showed average HI titre of 160 and average neutralizing titre of 20 in their sera. Age of the haemorrhagic patients varied from 8 years to 49 years, average being 22·5 years. There were 40 males and 5 females in this series. In 29 patients reliable vaccination history could be obtained, and all of them were unvaccinated. Vaccination status could not be definitely ascertained in the other sixteen. Except three cases of v.p.h. all others died.

Table II shows the HI and neutralizing titres of the sera of the non-haemorrhagic patients, who were available for investigation between 3rd and 5th day of illness. No case was found on the 2nd day of the disease. 95·5 per cent of the 22 cases had HI antibody titre ranging from 10 to 320 and 59 per cent had detectable neutralizing titre in sera diluted in 1 in 10. Fifty per cent of the patients whose vaccination history was reliable were unvaccinated.

TABLE II.

Showing HI and neutralizing titre of smallpox cases who were not haemorrhagic.

Case number.	Age/Sex.	Day of illness.	Previous vaccination.	HI titre.	Neutralizing titre.	Result.
1	47/F	3	Yes	20*	<10†	Survived
2	20/M	3	Yes	10	<10	Survived
3	43/M	3	No	<10	<10	Died
4	20/M	3	Yes	320	40	Survived
5	60/F	3	No	10	<10	Survived
6	22/M	3	No	20	<10	Survived
7	25/M	3	No	20	<10	Survived
8	28/M	4	No	80	10	Survived
9	22/M	4	Not known	160	20	Survived
10	31/F	4	Not known	40	<10	Survived
11	17/F	4	Yes	80	10	Survived
12	42/M	4	No	10	10	Survived
13	26/M	4	Yes	40	<10	Survived
14	70/M	4	Yes	20	10	Survived
15	25/M	4	Yes	80	10	Survived
16	12/F	4	No	40	<10	Survived
17	19/M	4	Not known	40	10	Died
18	25/M	5	No	160	40	Survived
19	20/M	5	No	80	10	Died
20	20/M	5	Yes	160	20	Survived
21	20/M	5	Yes	80	10	Survived
22	18/M	5	Not known	160	20	Died

* Indicates highest dilution of sera inhibiting haemagglutination.

† Indicates highest dilution of sera giving 50 per cent or more reduction of pocks in comparison to those of the control.

DISCUSSION.

In smallpox, the incubation period of 12 days is a fairly long period, but even then, high concentration of different antibodies in the blood of the patients during the early stage of the disease is not expected (Downie and McCarthy, 1958). There do not seem to be many publications on the study of HI and neutralizing antibodies on the sera of haemorrhagic smallpox cases who usually die in the early stage of the

disease. HI antibody is expected to appear early. In 2 out of 7 haemorrhagic cases of Paniker and Kalra (1962), HI antibodies in titres of 1 in 32 and 1 in 64 were detected on the 2nd and 3rd day of illness. In the series of haemorrhagic cases presented here 28 out of 39, i.e. 71·8 per cent of the cases did not show HI antibody detectable in 1 in 10 dilution of their sera. Neutralizing antibody was not tested in any of the 7 cases of Paniker and Kalra (*loc. cit.*). In 2 out of the 7 fatal (non-haemorrhagic) smallpox cases of Downie and McCarthy (*loc. cit.*), this antibody was present in the blood on the 2nd and 3rd day of illness. In the present series, 36 out of 39 or 92·3 per cent of the haemorrhagic cases did not show neutralizing antibody in the sera in dilutions of 1 in 10. This failure of antibody formation might have relation to the prolonged high viraemia (Mitra *et al.*, 1966) found in haemorrhagic smallpox cases, and this might as well contribute to the clinical severity of the disease, although it does not explain the haemorrhagic manifestations in the patients. Whether the virulent virus strains of haemorrhagic smallpox cases (Sarkar and Mitra, *loc. cit.*) had also exerted depressive action on the antibody forming mechanism in these cases cannot be said with certainty. However, as expected, the antibody titres were higher in the sera collected late in the disease than in those collected earlier.

The six 'variola pustulosa haemorrhagica' patients were clinically much less severe and the titres of antibody in their sera much higher than in the case of other 39 haemorrhagic patients. It seems that the v.p.h. cases belong to a category different from that of other haemorrhagic smallpox cases presented in Table I.

In the series of haemorrhagic smallpox cases of Rao (1964), many of the patients were pregnant females and quite a number of them had been vaccinated previously. Titres of antibodies in the sera of none of the cases were determined. This point is relevant, because vaccination carried out many years preceding infection and not followed by revaccination, may leave no or negligible amount of antibodies in the sera of these subjects, thus rendering them almost to the status of unvaccinated persons (Downie *et al.*, *loc. cit.*). Twenty-nine out of 45 haemorrhagic cases of the present series were unvaccinated. In the other 16 patients, vaccination status could not be established satisfactorily due to reasons mentioned before, but the chances are that they were not vaccinated, because in some of them whose skin over the arms was not very oedematous or haemorrhagic, vaccination scar could not be found. None of the 5 females in the present series were pregnant.

When we come to the non-haemorrhagic cases (Table II), the position about the antibody response is quite different from that of the haemorrhagic cases (Table I). Both HI and neutralizing antibody titres were higher in the non-haemorrhagic group. In Table III, a comparison has been made between the antibody titres of haemorrhagic and non-haemorrhagic smallpox cases during the same period of illness, although the number of cases in the two groups, due to reasons mentioned previously, are not identical. The v.p.h. cases have been excluded from the haemorrhagic cases in Table III. For calculating the average value of antibody titres, value of less than ten has been taken as zero. It is apparent from Table III that the antibody response of the haemorrhagic cases was much lower (undetectable in many) than in the

TABLE III.

Average HI and neutralizing titres of haemorrhagic and non-haemorrhagic smallpox cases on the same day of illness.

Day of illness.	HAEMORRHAGIC :			NON-HAEMORRHAGIC :		
	Number of cases.	HI.	Neutralizing.	Number of cases.	HI.	Neutralizing.
2	13	0	0	*Nil*	—	—
3	18	5*	0	7	57	5·7
4	7	17·1	2·8†	10	59	7
5	1	40	10	5	128	20

* Indicates highest dilution of sera inhibiting haemagglutination.

† Indicates highest dilution of sera giving 50 per cent or more reduction of pocks in comparison to those of the control.

N.B. In calculating the average titre, value < 10 has been taken as zero.

non-haemorrhagic cases and this is true even if the sera collected on the 3rd, 4th and 5th days of illness (excluding those of the 2nd day, on which sera of non-haemorrhagic cases were not available) are considered. Out of 26 sera of the haemorrhagic cases of this period, 61·5 per cent had no detectable HI antibody and 88·5 per cent had no neutralizing antibody, whereas the same figures for the 22 sera of non-haemorrhagic cases during the same period of illness was 4·5 per cent and 41 per cent respectively. Previous vaccination in some of the non-haemorrhagic cases might have bearing on this difference in the antibody response, although some of the vaccinated persons were found to have very low titres of antibody, and a number of patients without any previous vaccination had fairly good amount of antibody response (Table II). Whatever might have been the reason, the facts that have come out are, that both from the point of veiw of presence or absence of antibodies or their amounts in the sera, the non-haemorrhagic cases had better antibody response than the haemorrhagic smallpox cases.

In all the sera tested, titres of neutralizing antibody were lower than the titres of HI antibody and this was the experience of Downie and McCarthy (*loc. cit.*).

SUMMARY.

1. Forty-five virologically positive cases of haemorrhagic smallpox (including six of 'variola *Pustulosa haemorrhagica*') were studied mainly in respect of haemagglutination-inhibiting and neutralizing antibodies in their sera. Excepting three cases of 'variola *Pustulosa haemorrhagica*' all cases died. Most of the cases from whom reliable vaccination history could be obtained were unvaccinated.

2. The six cases of 'variola *Pustulosa haemorrhagica*' whose sera were collected on 3rd to 6th day of illness, had much higher titres of HI and neutralizing antibodies in their sera than in the case of other 39 haemorrhagic smallpox cases.

3. Excluding the cases of 'variola *Pustulosa haemorrhagica*', 71·8 per cent of the other 39 cases had no detectable HI antibody in their sera diluted 1 in 10 and

collected between 2nd and 5th day of illness. 92·3 per cent of these 39 sera diluted 1 in 10 had no neutralizing antibody. In comparison to this, 95·5 per cent of the 22 smallpox cases who were not haemorrhagic and whose blood could be collected between the 3rd and 5th day of illness, had HI antibody titre ranging from 10 to 320, and 59 per cent of them had neutralizing titre of 10 or above. Thus, the antibody response of the haemorrhagic cases was very poor in comparison to that of non-haemorrhagic cases. Fifty per cent of these 22 cases whose vaccination history was reliable were unvaccinated.

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