

Antibody response in non-haemorrhagic smallpox patients

BY A. W. DOWNIE, L. ST VINCENT, L. GOLDSTEIN, A. R. RAO
AND C. H. KEMPE

*Department of Pediatrics, University of Colorado Medical Center, and the
Infectious Disease Hospital, Tondiarpet, Madras*

(Received 12 April 1969)

Various studies have been made on the antibody response in smallpox. In some of these haemagglutinin inhibition tests (HI) have been used for measurement of antibody (Collier & Schönfeld, 1950) in others, complement-fixation tests (CFT), precipitation tests (pt.) and HI tests (Herrlich, Mayr & Mahnel, 1959) or CF, HI and neutralization tests (Downie & McCarthy, 1958). In the present study, all four tests were used and the findings have been used for a comparative study of cases of haemorrhagic smallpox and for assessment of possible subclinical infection in contacts of smallpox patients and the antibody response in minimal infections.

MATERIALS AND METHODS

The sera were collected from patients in the Infectious Diseases Hospital in Madras during the years 1963–6 and were held frozen at -20°C . until examinations were completed. There were 130 paired sera and 33 single specimens from patients taken mostly during the first 14 days of illness. In addition, there were 53 specimens collected during convalescence or after recovery. With five exceptions these 53 samples were collected between 2 and 4 weeks after the onset of illness. In all, 216 sera from 151 individuals were studied. Fourteen of the patients died, 11 of these had not been vaccinated. In all there were 21 patients who had not been vaccinated. This is a much smaller proportion of unvaccinated patients in this series than in the general population of smallpox patients in the hospital during this period (Rao, 1968). Most unvaccinated smallpox cases were in children, whereas blood for antibody studies was collected mostly from adults. Of the 151 patients studied only two were below 17 years of age: these two were unvaccinated males aged 2 and 6 years, respectively, who died of their disease.

The techniques used in carrying out precipitation, HI, CFT and neutralization tests for antibody were described in the previous paper (Downie, St Vincent, Rao & Kempe, 1969). All sera were examined routinely for antigen by precipitation and complement-fixation tests using suitable dilutions of vaccinia immune rabbit serum. Blood specimens were examined for the presence of virus from 19 patients only.

Requests for reprints should be addressed to Dr C. H. Kempe, 4200 East Ninth Avenue, Denver, Colorado 80220.

RESULTS

Examination for virus and virus antigen in blood

Of the 19 patients whose blood was examined for virus, seven died, all between the 10th and 17th day of illness. Blood was examined for virus from one patient on the 2nd day of illness, from another on the 3rd day, from three on the 4th, from two on the 5th, from three on the 6th, from two on the 7th, from two on the 9th, from one on the 11th, from one on the 13th, from one on the 17th day of illness, and in two patients the date of blood culture was not recorded. From only two specimens was virus isolated and in both the amount of virus was small, for virus was recovered only by subinoculation of the egg membranes inoculated with the specimen. One of the positive specimens was collected on the 6th day of illness from a patient with a confluent eruption, the other on the 4th day from an unvaccinated patient showing a discrete eruption. Both these patients recovered.

All serum specimens subsequently examined for antibody, 216 in all, were examined for the presence of antigen by precipitation and CF tests with an anti-vaccinial serum prepared in rabbits. Undiluted sera were tested for antigen by precipitation, and sera in a dilution of 1/10 by CFT. None of the sera showed antigen by these methods.

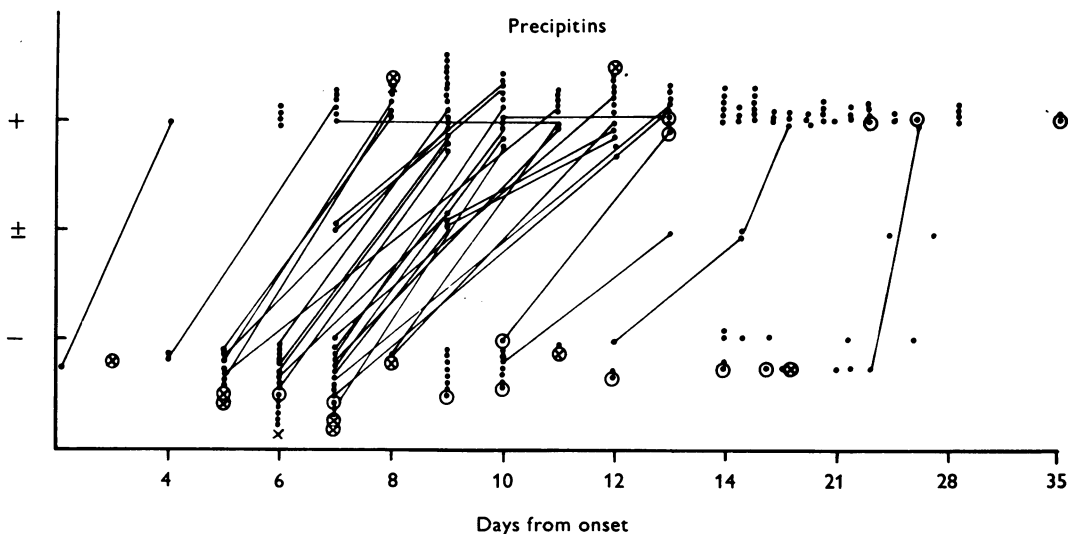


Fig. 1. Precipitating antibody in the sera of smallpox patients. O, Unvaccinated; x, fatal cases.

*Tests for antibody**Precipitation in agar gel*

The results of these tests are shown in Fig. 1. The sera positive for antibody by this test usually showed precipitation lines between the sera and antigen within 5 hr., but a few showed lines only after 20 hr. The sera recorded as giving a \pm result showed only a faint line after 48 hr.

It will be seen from the figure that the majority of sera tested before the 8th day of illness were negative, but from the 8th day onwards most of the sera showed

precipitating antibody. Sera from unvaccinated patients were less likely to be positive, there being nine negative results as against seven positives from the 8th day onwards. Three sera from patients who died showed no precipitins as against two which were positive during this period. The lines in the figure connect first and second specimens of serum. Where both specimens gave the same result in the test lines were not drawn between the two specimens; consequently the number of lines in the figure is less than the number of paired specimens examined. This also applies to Figs. 2-4.

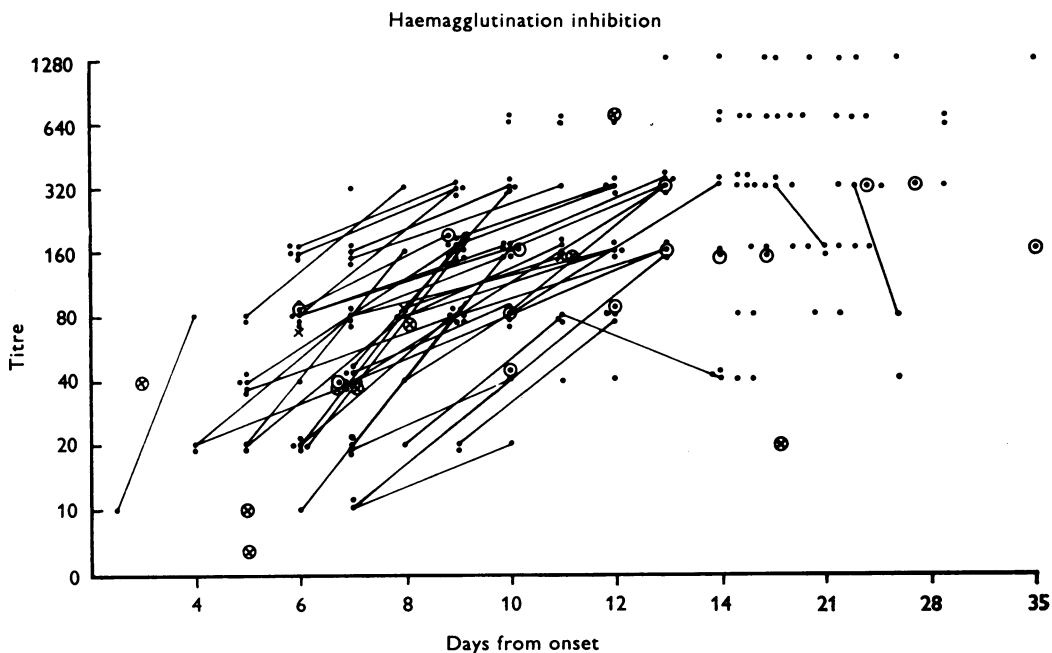


Fig. 2. Haemagglutinin-inhibiting antibody in the sera of smallpox patients.
O, Unvaccinated, x, fatal cases.

Haemagglutination inhibition tests

It is apparent from the results shown in Fig. 2 that antibody shown by this test appears rather earlier than that revealed by precipitation in agar gel. Maximum titres tend to be reached between the 12th and 21st day from onset by which time all show significant titres (1/20 or more by the technique used). Sera from unvaccinated patients showed HI antibody from the sixth day onwards although the titres tended to be somewhat lower than those attained by the sera of previously vaccinated patients.

Complement-fixation test

With one exception, no sera tested before the 6th day of illness gave a positive test for antibody by this technique, but from the 8th day most were positive (Fig. 3). Maximum titres were attained after the 14th day and the only three sera negative at this time were from unvaccinated patients. As with the precipitation

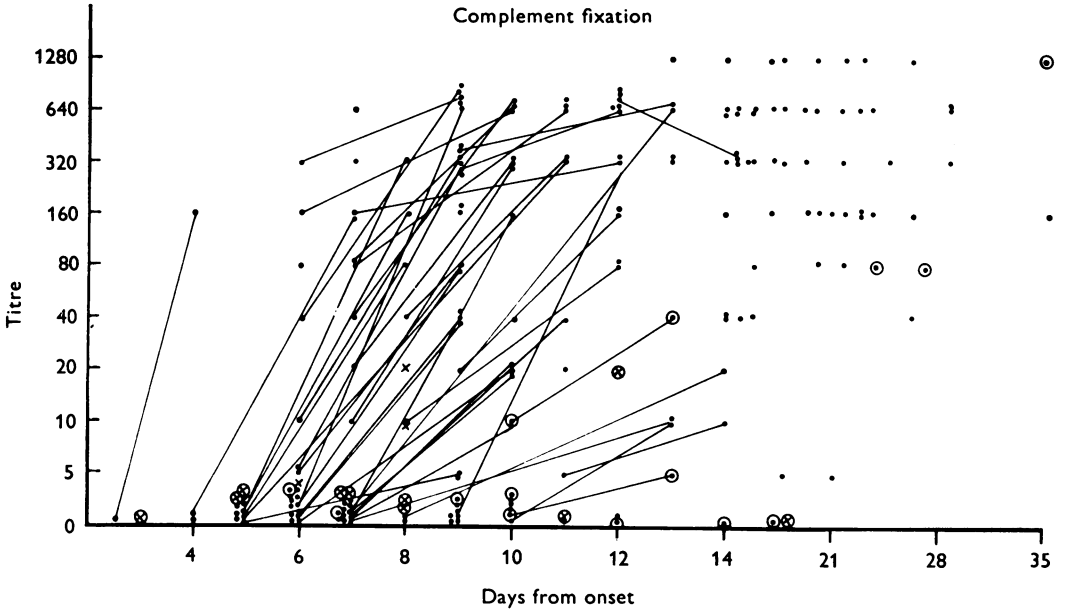


Fig. 3. Complement-fixing antibody in the sera of smallpox patients.
O, Unvaccinated, x, fatal cases.

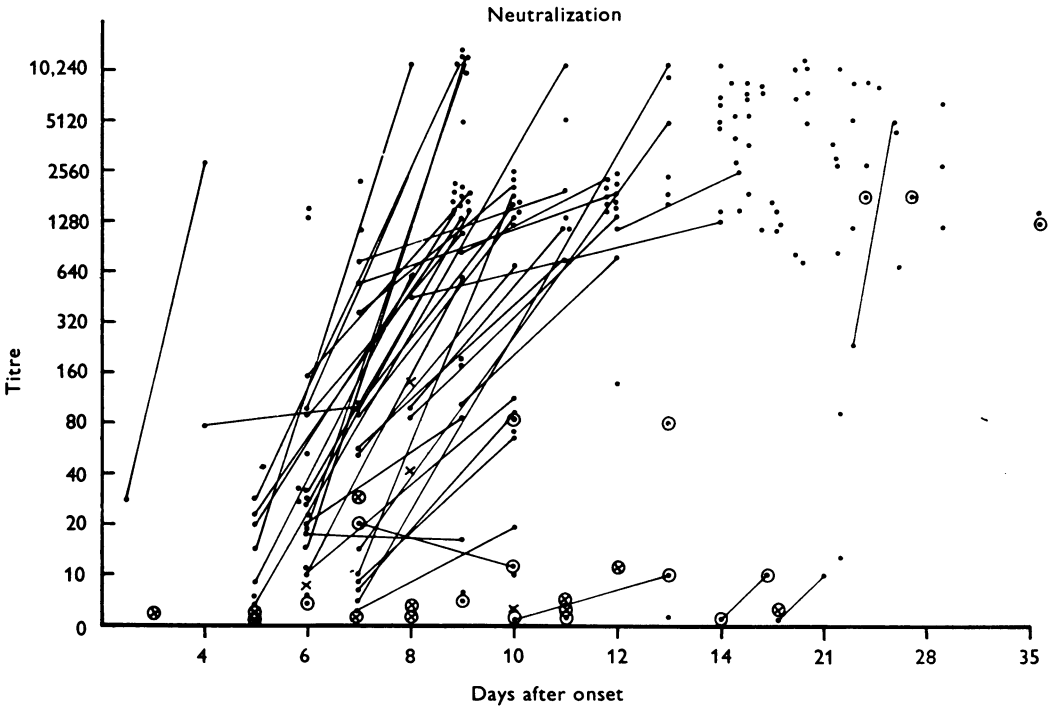


Fig. 4. Neutralizing antibody in the sera of smallpox patients.
O, Unvaccinated; x, fatal cases.

tests, more than 50% of sera from unvaccinated individuals examined after the 8th day were negative. As will be seen in Fig. 3, only three of 13 patients who died showed CF antibody and these three had titres of 1/10 or 1/20.

Neutralization tests

The results of titrations of neutralizing antibody are shown in Fig. 4. Most of the sera tested showed neutralizing activity from the 5th day of illness and maximum titres were frequently reached by the 9th day. Many of the sera shown grouped at 1280 from the 9th to the 12th day of illness were not tested in higher dilutions. The few sera showing little or no neutralizing activity from the 8th day onwards were obtained from patients who suffered severe and fatal infections, most of them lacking a history of previous vaccination.

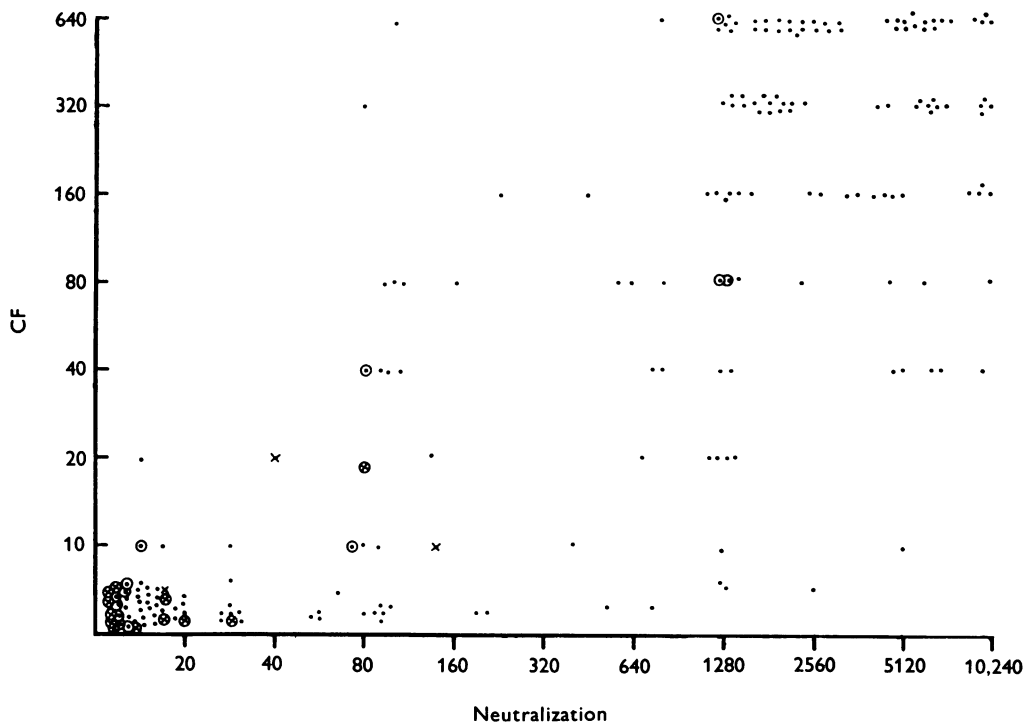


Fig. 5. Comparison of complement-fixing and neutralizing antibody titres in the sera of smallpox patients. ○, Unvaccinated; ×, fatal cases.

Correlation of results of individual tests

The titres for complement fixing and neutralizing antibody on individual sera are shown in Fig. 5. A rough correlation of the results of these two tests is apparent, although some sera showing good neutralization had low CF titres. From this figure too it can be seen that most of the sera from fatal and unvaccinated cases had low antibody titres by both tests.

There is not, however, such good correlation between the HI and the CF or neutralization tests (Figs. 6, 7). While many sera showed high titres by all three

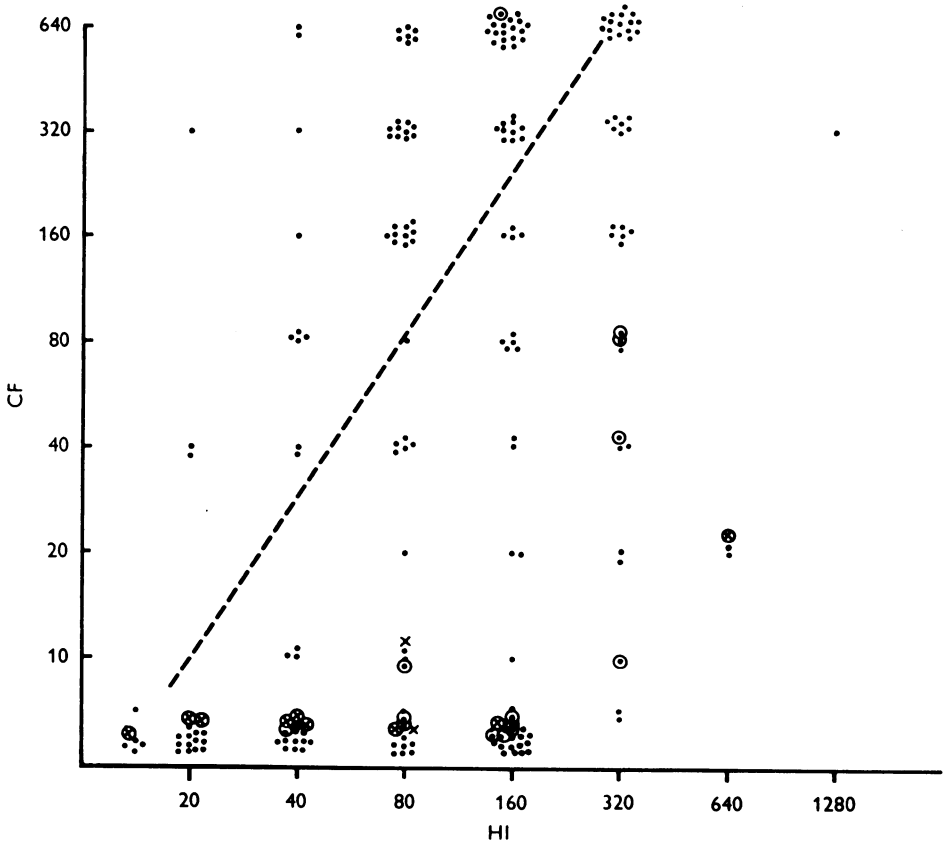


Fig. 6. Comparison of complement-fixing and haemagglutinin-inhibiting antibody titres in the sera of smallpox patients. O, Unvaccinated; x, fatal cases.

Table 1. Comparison of results of precipitation in agar gel and complement-fixation tests for antibody in the sera of smallpox patients

Complement-fixation titre	Precipitation		
	-	+	++
640	1	3	39
320	1	3	27
160	3	2	18
80	2	2	11
40	2	5	7
20	3	1	4
10	3	1	4
5	6	2	1
0	57	0	3
Totals	78	19	114

tests, other sera, including those from unvaccinated patients, had reasonably good titres by the HI test but little antibody when tested by the CF and neutralization techniques.

The comparison of the results of precipitation-in-agar-gel tests with those of CF

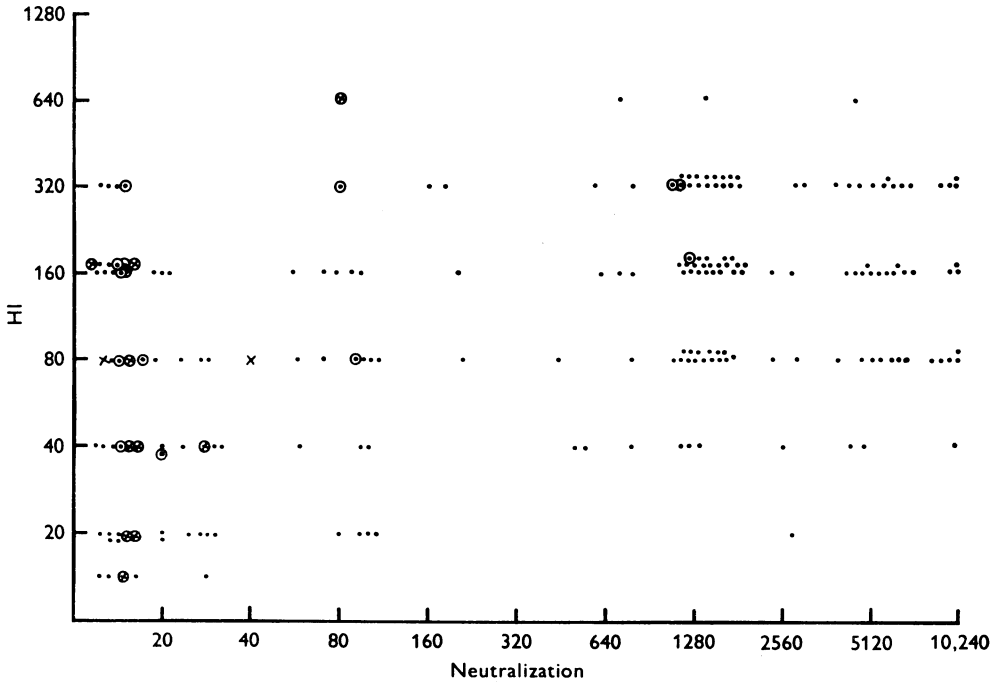


Fig. 7. Comparison of haemagglutinin-inhibiting and neutralizing antibody titres in the sera of smallpox patients. O, Unvaccinated; x, fatal cases.

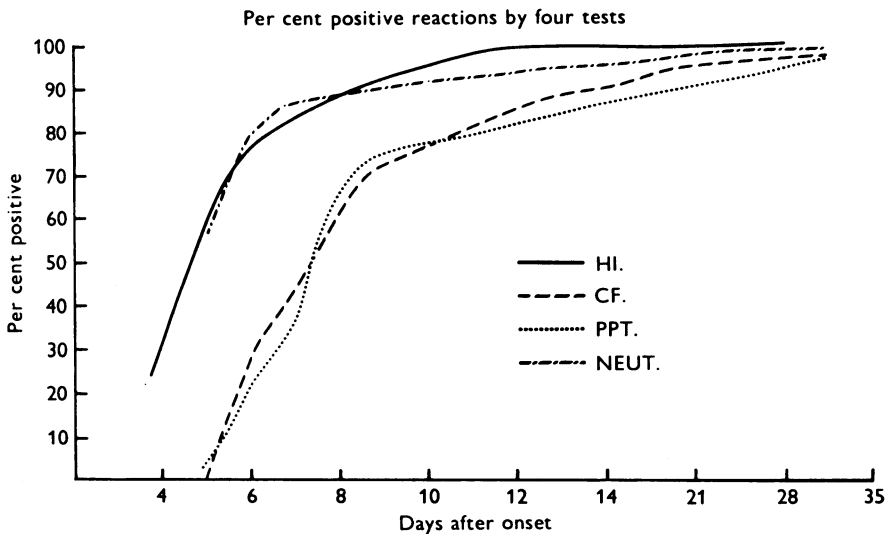


Fig. 8. Time of appearance of antibody as determined by four techniques, in relation to day of disease.

and neutralization tests are shown in Tables 1 and 2. Reasonable correlation is shown by the results of the three tests in that the majority of sera with high CF and neutralization titres gave rapid precipitation with vaccinal antigen in agar gel. Most of the sera showing low or absent titres by CF and neutralization were also negative by precipitation. However, there were a few sera which, although showing little or no antibody by CF and neutralization techniques, gave good precipitation with a vaccinal antigen. (A few sera examined by the precipitation and CF tests were not tested for neutralizing antibody and this accounts for the different totals of sera shown in the two tables.)

Table 2. *Comparison of results of precipitation in agar gel and neutralization tests for antibody in the sera of smallpox patients*

Neutralization titre	Precipitation		
	-	+	++
10,000	0	1	12
1000 to < 10,000	0	8	79
100 to < 1000	9	4	13
10 to < 100	37	1	7
< 10	31	0	4
Totals	77	14	115

DISCUSSION

Only a relatively small number of examinations were made for the presence of virus in the blood of these non-haemorrhagic smallpox patients. However, the finding of virus, even in low concentration on the 4th and 6th day of illness in two patients who recovered, is contrary to the experience of Downie *et al.* (1953) who found no virus after the 2nd day in 25 patients who recovered. It appears that the presence of small amounts of virus in blood as late as the sixth day of illness is not incompatible with recovery. On the other hand, earlier findings were confirmed in that virus antigen was not detected in the serum of any of the patients in this series, including those who died.

It is apparent from Figs. 1-4 that antibody response is detected earlier in the disease by HI and neutralization tests than by precipitation and CF techniques. This is illustrated in Fig. 8, in which the percentage of positive results by the four techniques is shown in relation to the day of disease. These findings are in agreement with those of Downie & McCarthy (1958), who tested sera for HI, CF, and neutralizing antibody and with those of Herrlich *et al.* (1959) who used precipitation, HI and CF tests. Although there were fewer unvaccinated patients in the present series, the low antibody titres in these patients by precipitation, CF and neutralization tests are in accord with the findings in the two previous studies quoted. On the other hand, relatively high titres of HI antibodies were frequently observed in unvaccinated and fatal infections in all three series. This and the lack of correlation of HI titres with CF and neutralization titres shown in Figs. 6 and 7 suggest that HI antibodies do not provide a good indication of immune response

and are of low prognostic value—a point which is also illustrated by the study of haemorrhagic cases. The CF antibody titres recorded by Herrlich *et al.* (1959) in various types of smallpox were considerably lower than those recorded by Downie & McCarthy (1958) and in the present series—a result presumably attributable to the less sensitive technique which they employed. This is reflected in their failure to demonstrate CF antibodies in eight cases of alastrim examined after the 12th day of illness, whereas such antibody was found in all cases of alastrim examined after the ninth day by McCarthy & Downie (1953).

The data of Downie & McCarthy (1958) indicate the decline in HI and CF titres with passage of time after smallpox infection. We have no information on the persistence of precipitin except that positive reactions are obtained up to 35 days after onset of illness and are not found a few years after recovery from smallpox. Tests for precipitins were negative in the sera of six cases of generalized vaccinia observed in Denver.

In the retrospective diagnosis of missed cases of smallpox and in minimal or subclinical infections, the above findings, together with those in the previous paper, suggest that with the techniques used, the presence, in a single specimen of serum, of a positive precipitin-in-agar-gel test, a CF titre of 1/20 or greater, an HI titre of 1/80 or higher and a neutralizing titre of 1/500 or greater are indicative of recent smallpox infection. The majority of smallpox sera examined gave a positive precipitation test in agar gel and titres in the other tests greater than those mentioned. Where sera taken early and late in the course of illness are available rises in titre will strengthen the evidence of smallpox infection. But in missed cases such paired samples of sera will rarely be available. In unvaccinated patients the antibody levels mentioned above may not be reached, but in these the disease is likely to be severe and unlikely to be overlooked. Minimal infections generally occur in vaccinated persons in whom antibody titres reach a high level.

SUMMARY

Two hundred and sixteen sera from 151 patients suffering from smallpox (non-haemorrhagic) were examined for antibody by precipitation in agar gel, by haemagglutinin inhibition (HI), complement fixation (CF) and neutralization tests. Most of the patients were adults and the majority had been vaccinated earlier in life. HI and neutralizing antibodies showed rising titres from the 6th day of illness while the majority showed precipitins and CF antibodies from the 8th day. The results of the precipitation-in-agar-gel tests are in marked contrast to the findings in healthy vaccinated and revaccinated individuals, none of whose sera gave a positive result for antibody by this technique. In unvaccinated patients the antibody response was frequently delayed and the titres lower than those attained by the previously vaccinated patients. There was no exact correlation in antibody titres obtained by the four methods of measurement, HI antibody, in particular, reaching in some cases relatively high titres when other tests showed low titres. It is suggested that with the methods and materials used, a positive precipitation test in agar gel, a CF titre of 1/20 or more and an HI titre of 1/80 or higher in a single specimen of serum would be suggestive of recent

smallpox infections. Such a result might be of special value in the retrospective diagnosis of missed cases and in the detection of minimal or subclinical infections.

This investigation was supported in part by Public Health Service Grant AI-1632-16 VR from the National Institute of Allergy and Infectious Diseases, by the World Health Organization and by the Marcus T. Reynolds III Fund.

REFERENCES

- COLLIER, W. A. & SCHÖNFELD, J. K. (1950). Studies on the serological diagnosis of smallpox. *Med. J. Aust.* ii, 363-6.
- DOWNIE, A. W. & MCCARTHY, K. (1958). The antibody response in man following infection with viruses of the pox group III. Antibody response in smallpox. *J. Hyg., Camb.* 56, 479-87.
- DOWNIE, A. W., MCCARTHY, K., MACDONALD, A., MACCALLUM, F. O. & MACRAE, A. D. (1953). Virus and virus antigens in the blood of smallpox patients. Their significance in early diagnosis and prognosis. *Lancet* ii, 164-6.
- DOWNIE, A. W., ST VINCENT, L., RAO, A. R. & KEMPE, C. H. (1969). Antibody response following smallpox vaccination and revaccination. *J. Hyg., Camb.* 67, 603-8.
- HERRLICH, A., MAYR, A. & MAHNEL, H. (1959). Das Antikörperbild der Variola-Vaccineinfektion. 2. Serologische Untersuchungen an Variolapatienten. *Zentbl. Bakt. ParasitKde* (I. Abt. Orig.) 175, 163-82.
- MCCARTHY, K. & DOWNIE, A. W. (1953). The serum antibody response in alastrim. *Lancet* i, 257-60.
- RAO, A. R. (1968). In *Smallpox Eradication. Tech. Rep. Ser. Wld Hlth Org.* no. 393.