THE ANTIBODY RESPONSE IN MAN FOLLOWING INFECTION WITH VIRUSES OF THE POX GROUP

II. ANTIBODY RESPONSE FOLLOWING VACCINATION

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(With 8 Figures in the Text)

The prophylactic value of vaccination against smallpox *before* exposure to the disease is not seriously disputed today. There is a vast experience, both clinical and epidemiological, to support this opinion, although there is not general agreement about the duration of immunity following vaccination. Further there are differences of opinion about the efficacy of vaccination or revaccination immediately *after* exposure to smallpox. Clinical observations suggest that both these matters are probably subject to considerable variation in different individuals (Ricketts & Byles, 1908). The presence or absence of effective immunity of vaccinated persons to smallpox can be directly determined by observations following accidental exposure to smallpox or by revaccination with a potent vaccinial lymph. It seemed to us, however, that useful information might be obtained by studying the antibody response following primary vaccination or revaccination using for this purpose the techniques of neutralization, haemagglutination-inhibition and complement fixation.

Neutralizing antibodies were demonstrated many years ago in the serum of persons who had been vaccinated or had suffered from smallpox (Béclère, Chambon & Ménard, 1899). Neutralization tests were usually made by injecting serum-virus mixtures into the skin of susceptible animals; this technique has been used more recently in the study of post-vaccination sera by Loutit & McClean (1945) and by Gispen (1953*a*). Since Keogh (1936) showed that the action of antibody to vaccinia virus could be demonstrated by the reduction of pock counts on the chorio-allantois this method has been studied by various workers. Buddingh (1943) showed that variola virus could also be used for this purpose and in a previous paper we have indicated why variola is preferable to vaccinia virus for neutralization tests on the chorio-allantois (McCarthy, Downie & Armitage, 1958). The demonstration of vaccinial haemagglutinin by Nagler (1942), and its inhibition by immune serum, provided a simple *in vitro* technique for the estimation of vaccinial antibody and has been frequently applied to the measurement of antibody in vaccinated persons in recent years (Nagler, 1944; Collier, De Cloe-Enklaar & Geiger-Koedyk, 1949; Kempe & Benenson, 1953; Gispen, 1953*b*; Elisberg, McCown & Smadel, 1956; Herrlich, Mayr & Munz, 1956; Szathmáry & Baranyai, 1957).

The haemagglutinins of variola virus appear to be immunologically identical with those of vaccinia (North, 1944) and inhibition of vaccinial haemagglutinin has been used to estimate antibody in the sera of smallpox patients by North (1944); Collier & Schönfield (1950); McCarthy & Downie (1953). Prior to the development of the haemagglutinin-inhibition technique the complement fixation test provided the most sensitive *in vitro* method for the detection of antibody in human sera. The complement-fixing, antihaemagglutinin and neutralizing antibodies are however not identical (Chu, 1948) and it is probable that the titration of neutralizing antibody gives a better estimate of immunity to infection than either of the *in vitro* tests.

In this paper we have recorded the results obtained by the use of these three methods in the study of the time of appearance and persistence of antibody following primary vaccination and the type of antibody response following revaccination.

MATERIALS AND METHODS

Sera

Normal control sera were obtained from unvaccinated young adults. Observations on antibody development following primary vaccination were mostly made on sera from young adults, either students, laboratory staff or recent recruits to H.M. Forces; 104 sera from eighty-six persons were examined. Sera following second or subsequent vaccinations were obtained from similar groups of persons and also from members of the staff of the Ministry of Health; in this group there were 120 sera from sixty-six revaccinated individuals. All sera were stored at 4° C. after collection and were inactivated at 58° C. for 20 min. before being examined for antibody by the various techniques.

Virus

The virus was the same strain from variola major that was used in the experiments described in the previous paper (McCarthy *et al.* 1958).

Neutralization tests

The technique of the neutralization test carried out by inoculating mixtures of serum and variola virus on the chorio-allantois has been described (McCarthy *et al.* 1958); volumes of 0·2 ml. of undiluted sera were added to 0·4 ml. volumes of virus suspension and the mixtures left at room temperature for 1 hr. before inoculation on five or six eggs. A reduction in mean pock count of 50 % or greater was regarded as indicating the presence of neutralizing antibody. This seemed justified on the basis of the analysis of data presented in the previous paper. In some experiments the titre of antibody was estimated by testing sera in fivefold dilutions against a constant amount of virus.

In comparative experiments human sera were tested for their ability to neutralize variola and vaccinia viruses by the chorio-allantoic technique. Twenty-one post-vaccination sera and twelve convalescent smallpox sera were tested against the two viruses. The results showed close agreement, but the reduction of the variola pock count was rather greater than that for vaccinia virus—in nine of

these thirty-three sera by more than 10 %. This is in accord with the observations of Downie & McCarthy (1950) that vaccinia virus was less easily neutralized than variola virus on the chorio-allantois by immune sera from hens or rabbits.

At an early stage in our work sera were tested for neutralization of vaccinia virus by inoculating mixtures of undiluted serum and tenfold dilutions of vaccinia virus intradermally into rabbits. Nineteen post-vaccination sera and four smallpox convalescent sera were tested by this technique and also by the variola-neutralization test in eggs. Of the post-vaccination sera five were negative by both tests and fourteen reduced the pock count by 50–90 % and neutralized 1 to 100 infective doses of vaccinia virus on the rabbit skin. The four smallpox sera neutralized 10 to 1000 infective doses of vaccinia in the rabbit and effected more than 80 % reduction in the variola pock count. These tests indicate fairly reasonable agreement between these types of neutralization test. The results to be discussed below, however, have been obtained with the variola-neutralization test on the chorio-allantois. We have found it the most suitable method for quantitative work.

Haemagglutinin inhibition tests

The technique used was similar to that of Nagler (1944) and has been previously described (McCarthy & Downie, 1953). Vaccinia haemagglutinin was obtained as a saline extract of chorio-allantoic membranes infected 2 days previously with egg-passaged vaccinia virus. The titre of haemagglutinin obtained from variola-infected membranes was generally lower than that obtained from membranes infected with vaccinia and consequently haemagglutinin from the latter has been used.

Complement fixation tests

The technique used was that described by Downie & Macdonald (1950). The vaccinial antigen was the clear supernatant fluid obtained by high-speed centrifugation of dermal pulp from rabbits inoculated by scarification 3 or 4 days previously with a rabbit-passed strain of vaccinia virus. The antigen was standardized, before being taken into routine use, by testing twofold dilutions against twofold dilutions of a rabbit hyperimmune antivaccinial serum. The dilution of the stock vaccinial antigen used to test human sera was the highest that still showed maximal serum titre with the antivaccinial rabbit serum; this was usually a dilution of 1/50 to 1/80 of the stock antigen. Comparative tests were made with a variola antigen prepared from smallpox crusts by the method of Craigie & Wishart (1936). This antigen was standardized before use in the same way as vaccinial rabbit antigen against the standard rabbit antivaccinial serum. Fourteen post-vaccination sera and fifteen smallpox sera when tested in parallel against the two antigens gave practically identical titres. However, the smallpox crust antigen, unlike the vaccinia rabbit antigen, sometimes gave non-specific results with low dilutions of sera from persons suffering from chickenpox or other febrile disturbances and so rabbit vaccinial antigen was used for the complement fixation tests referred to below. In all estimations of antibody, titres have been expressed in terms of dilutions of serum *before* the addition of antigen or virus.

RESULTS

Antibody response following primary vaccination

Time of appearance and degree of antibody response following primary vaccination

The results of tests for variola-neutralizing antibody in samples of serum taken at intervals after primary vaccination of three students is shown in Fig. 1. Significant neutralization was first demonstrated in two of them 13 days, and in the third student 16 days, after vaccination; all three individuals showed increased amounts of antibody in subsequent specimens. Complement-fixing antibody was first observed in low titre (1/5) in samples taken at 17 days in two students and in the 23-day sample in the third. Inhibition of haemagglutinin was first demonstrated after 13 days, 17 days and 23 days in the three subjects. Complement-fixing

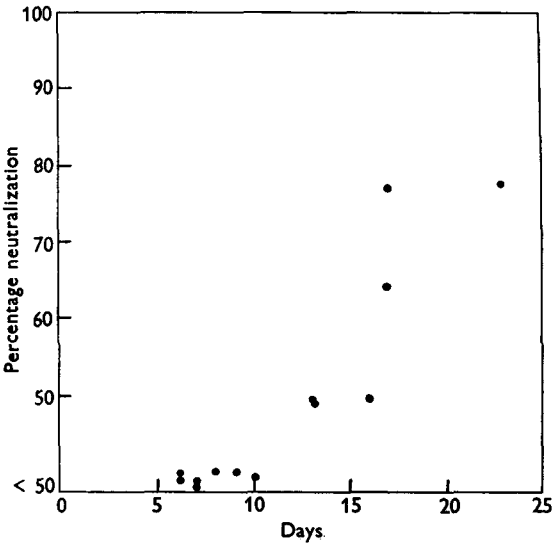

Fig. 1. Appearance in the blood of neutralizing antibody to variola virus following primary vaccination of three subjects.

antibody was not always found by our technique in the third week following primary vaccination. Of forty-three army recruits from whom blood samples were taken 17 days after successful primary vaccination only fourteen showed complement-fixing antibody. Table 1 shows the titration of the sera of sixteen of these individuals for antibody by the three techniques. In two of these (nos. 7 and 10) neutralization had not reached a significant level; all showed antihaemagglutinin but only five gave a positive complement fixation test.

There were available to us very few sera taken during the fourth week after uncomplicated primary vaccination at a time when antibody titres might be expected to be at their highest. In the majority of sera examined the reduction in pock count was less than 80 % when undiluted sera were tested. When titrated in fivefold dilutions only an occasional serum produced more than 50 % neutralization when diluted 1/5. Three sera from cases of *generalized vaccinia*, however, showed higher antibody levels. The 50 % neutralization titres of these sera were

determined by testing them in serial fivefold dilutions and plotting the dilutions against percentage reduction of pock count. The three sera collected after 26 days, 28 days and 105 days respectively, had 50 % neutralization titres of 1/70, 1/25 and 1/45. These titres more nearly resembled those sometimes found with sera of revaccinated persons (see later) and in the sera of smallpox patients as recorded in the following paper (Downie & McCarthy, 1958).

Table 1. *Antibody titres* 17 *days after successful primary vaccination*

Serum no.	Serum titre*		Neutralization, percentage reduction of variola pock count
	Complement fixation	Haemagglutinin inhibition	
1	—†	80	67
2	5	160	60
3	—	80	65
4	5	160	62
5	—	80	68
6	—	160	68
7	—	10	23
8	10	80	54
9	—	40	62
10	—	80	33
11	—	160	64
12	5	80	72
13	—	80	71
14	—	80	68
15	—	160	64
16	20	320	67

* = titre expressed as reciprocal of serum dilution.
† = negative at 1/5 serum dilution.

Persistence of antibody following primary vaccination

The results of tests on sera collected from a few days up to 50 years after vaccination are shown in Figs. 2, 3 and 4. It will be observed that no antibody was detected by any of the three techniques before the thirteenth day after vaccination but the results obtained by the three methods varied considerably in the later samples. Neutralizing antibody apparently persisted in many instances for 20 or even 40 years following primary vaccination. However, although all sera tested for antihaemagglutinins from the third week to 8 months showed antibody, most became negative thereafter although a few low titres were recorded even after 20 years. Only four sera were examined by complement fixation between 2 months and 2 years after primary vaccination but no positive results were recorded more than 4 months after vaccination.

Antibody response after revaccination

Time of appearance of antibody following revaccination

The time of antibody response was estimated by collecting samples of blood immediately before revaccination and, at intervals of a few days, up to one month thereafter. As many of the sera showed neutralizing antibody with undiluted

serum before revaccination it was again necessary to titrate sera in dilutions—usually fivefold—in order to determine an increase in titre. The mean pock count obtained with each dilution of serum was compared with that obtained with normal sera devoid of antibody. The neutralizing titre of the serum was estimated

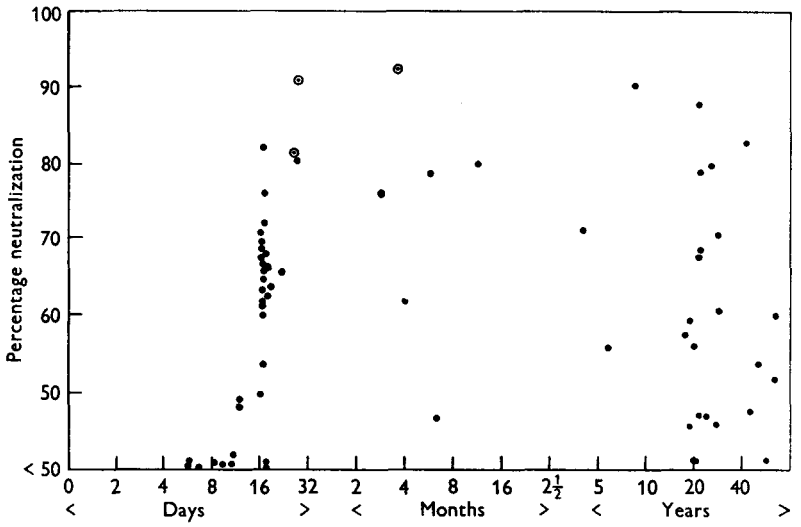

Fig. 2. Neutralizing antibody to variola virus following primary vaccination. ⊙ = cases of generalized vaccinia.

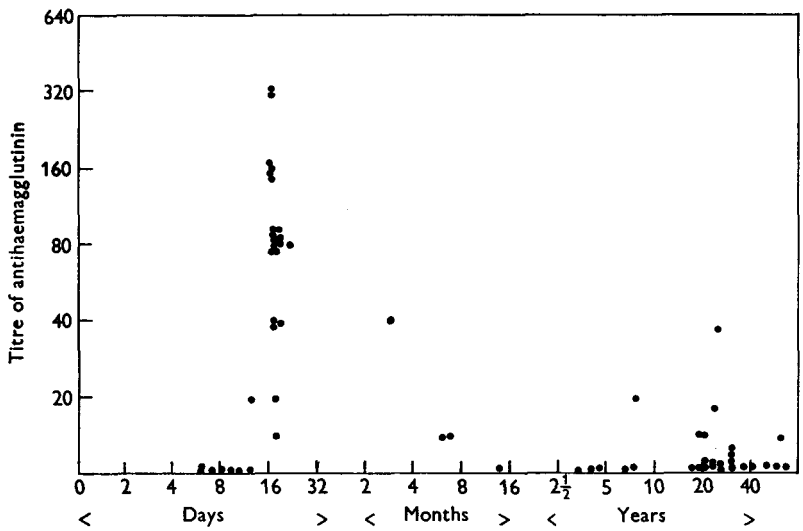

Fig. 3. Vaccinial antihaemagglutinin titres following primary vaccination.

by plotting the percentage reduction of mean pock count against serum dilution to determine the dilution corresponding to 50 % neutralization. An example of the kind of results obtained is shown for serum samples from student M (Table 2). Fig. 5 shows the neutralizing antibody response curve after revaccination of five students whose sera were tested in this way. Students B, M, K, S and F had had their primary vaccination 7, 8, 23, 27 and 22 years previously. B, M and F each

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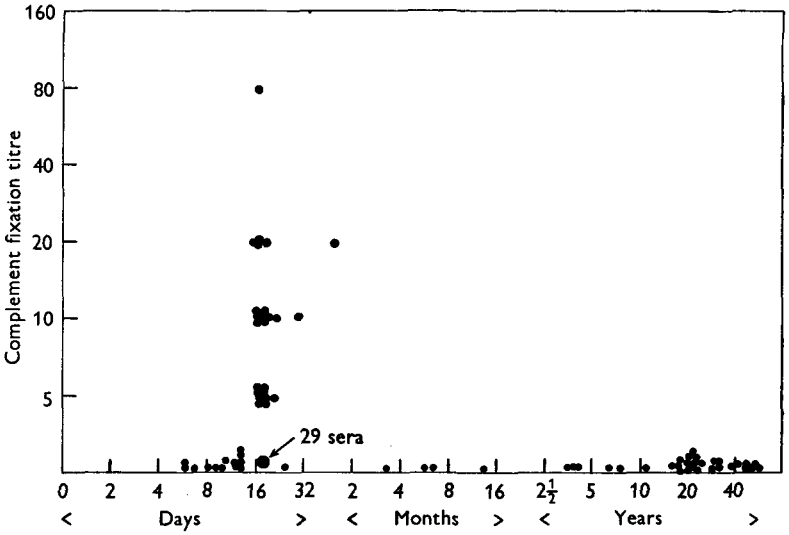

Fig. 4. Complement-fixing antibody levels following primary vaccination.

Table 2. *Neutralizing antibody in serum following revaccination. Percentage reduction of variola pock counts*

Serum dilutions	Sera from student M. Days after revaccination				
	0	4	7	10	14
Undiluted	91	92	N.T.	N.T.	N.T.
1/5	46	43	88	91	89
1/25	31	37	78	88	86
1/125	N.T.	N.T.	38	53	71

N.T. = *not tested.*

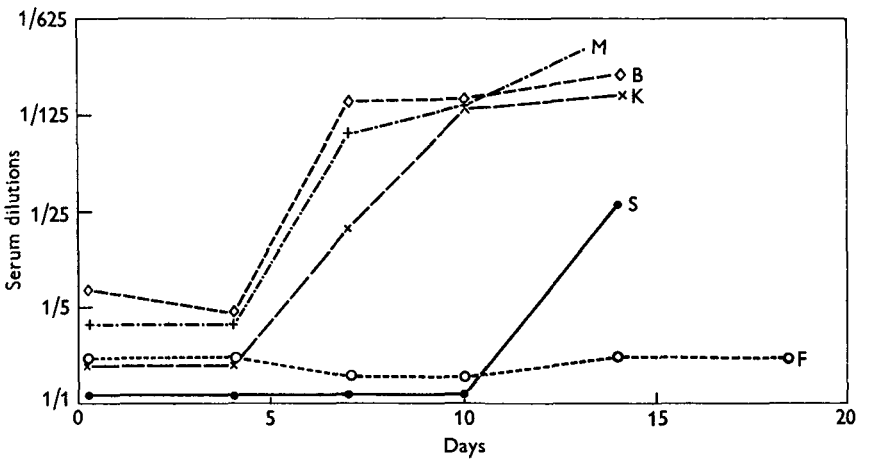

Fig. 5. Variola neutralizing antibody response following revaccination of five students (see text).

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showed an 'early' response with a papular reaction, maximal on the second or third day but no vesiculation. Student K had a good vesicular 'vaccinoid' response approaching primary in type, but maximal on the sixth–seventh day while S had a typical 'primary' type reaction maximal about the eighth–tenth days.

It is apparent from Fig. 5 that F had no antibody response to revaccination while B, M and K all showed an increase in antibody as early as the seventh day after revaccination. S, whose initial serum even undiluted failed to give significant reduction in pock count, and who had a primary-type vaccination reaction, showed an antibody response which in time of appearance resembled that seen after primary vaccination; however, the titre on the fourteenth day after revaccination was higher than that usually seen at this time after primary vaccination. Subject B failed to show an accelerated antibody response in serum samples tested for antihaemagglutinin and complement-fixing antibody. Antihaemagglutinin was first found after 28 days at a dilution of 1/20 while no complement-fixing antibody was detected. Student M had an antihaemagglutinin titre of 1/20 before revaccination and this did not increase in post-revaccination specimens. His 14-day serum gave a doubtful positive complement fixation test and other sera were negative. Student K showed antihaemagglutinin and complement-fixing antibody after 10 days, rather earlier than the rise observed after primary vaccination, and the titre for both had increased after 14 days. Student S first had antihaemagglutinin after 14 days but no complement-fixing antibody while student F showed neither antibody in any serum specimen.

It would appear from these observations that, following revaccination, a good neutralizing antibody response may appear quickly without any corresponding appearance of antihaemagglutinin or complement-fixing antibody. This point is referred to later. It should be noted that the 'takes' on students B, M and F were clinically indistinguishable. In two there was a well-marked antibody response, while in the third there was none.

The degree of response and persistence of antibody after revaccination

Neutralization

The distribution of titres of sera in relation to time since revaccination is shown in Fig. 6. The percentage neutralization recorded was obtained with undiluted sera so that the results may be compared with those obtained following primary vaccination as shown in Fig. 2. In some instances serum samples were obtained before revaccination. These were tested along with post-vaccination specimens from the same individuals. The majority, although not all, showed an increase in neutralizing antibody.

It will be observed that many of the sera after revaccination showed more than 80 %, and some more than 90 %, neutralization of variola virus, whereas of the sera tested at the corresponding period after primary vaccination (Fig. 2) very few showed such high neutralizing power. It was found that the sera showing a high percentage of neutralization when tested undiluted (Fig. 6) were those which showed high titre when titrated in fivefold dilutions, like the sera from B, M, and K (Fig. 5).

Antihaemagglutinin

The distribution of antihaemagglutinin in relation to time is shown in Fig. 7. It will be observed that the titres are no higher than those following primary vaccination and, indeed, many sera were negative between 14 and 30 days after revaccination, while all sera examined at this period following primary vaccination

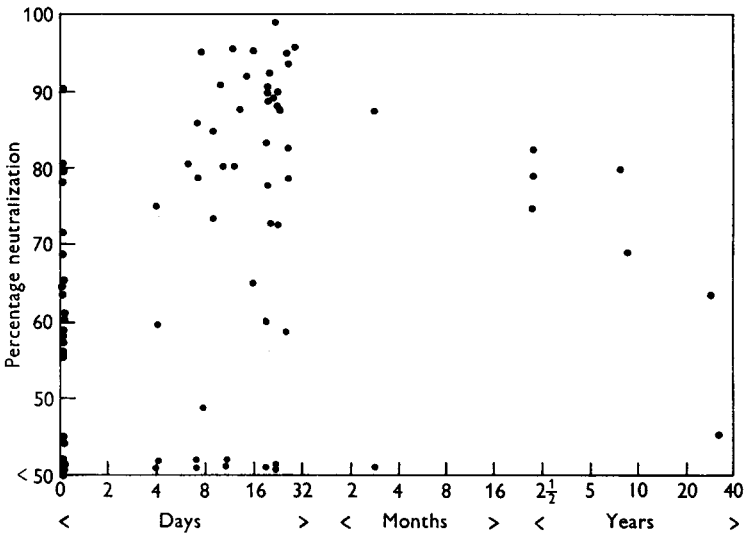

Fig. 6. Neutralizing antibody to variola virus after revaccination.

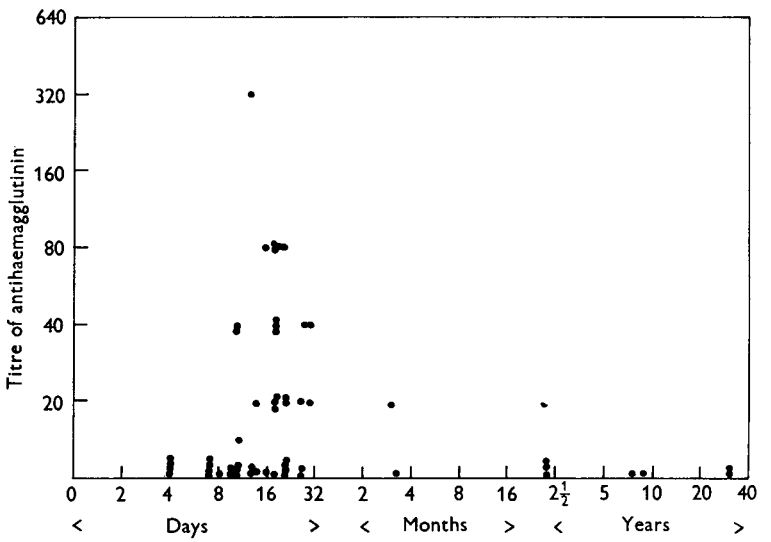

Fig. 7. Vaccinial antihaemagglutinin titres after revaccination.

showed antihaemagglutinin. A few but not all of the sera without antihaemagglutinin came from individuals who showed no increase in neutralizing antibody. Of twenty-two individuals who showed an increase in neutralizing antibody following revaccination five showed no inhibition of haemagglutination.

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Complement-fixing antibody

The distribution of complement-fixing antibody titres following revaccination is shown in Fig. 8. Previous vaccination in all instances had been done years before, so that it was unlikely that any individuals had complement-fixing antibody in their sera prior to revaccination. In fact sera from thirty-three were tested before revaccination and all were negative (Fig. 8). The distribution of titres is not very different from that following primary vaccination except that four sera were positive after 10 days; no sera were found to give a positive result as early as this after primary vaccination and these early positives after revaccination presumably indicate an accelerated antibody response. Several positive results were recorded from 2 to 8 months after revaccination but none after one year.

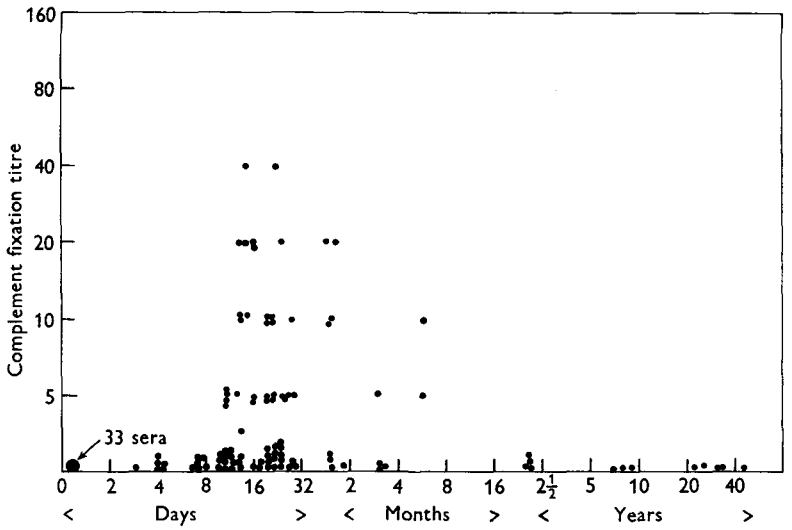

Fig. 8. Complement-fixing antibody levels after revaccination.

A neutralizing antibody response occurring after revaccination in the absence of complement-fixing antibody was found in the case of students B and S (Fig. 5) and was observed following primary vaccination (Table 1). Of twenty-one persons who showed an increase in neutralizing antibody after revaccination and whose sera were also tested by complement fixation ten failed to show complement-fixing antibody.

The early 'take' after revaccination

Recommendations for the assessment of vaccination reactions stress that the early type of take without vesiculation should not be interpreted as a successful vaccination, as such a response may be obtained with heated lymph (Hooker, 1929; Broom, 1947; Benenson, 1950); on subsequent vaccination with active lymph a successful take with vesiculation may be achieved. Amongst the subjects who formed the basis of the present study there were twenty-eight whose sera had been tested for neutralizing antibody immediately before revaccination and again afterwards and whose local reaction to vaccination was carefully recorded. In

most instances sera had been titrated for neutralizing antibody against variola virus in serial dilutions; a few had been tested against tenfold dilutions of vaccinia virus in the rabbit skin. Four persons who showed a primary type of 'take' and nine with vaccinoid reactions had a marked rise in neutralizing antibody, but of fifteen who had an early reaction without vesiculation only eight showed a subsequent increase in antibody. These results, therefore, support the view that while the early type of response to revaccination may on occasions be associated with an immune response, this is frequently not the case; moreover, in our experience and in the experience of others, it is impossible from inspection of the local reaction, to separate the so-called immune reaction from what must be considered the allergic type of response.

DISCUSSION

Following primary vaccination, antibody was not detected during the first 10 days but was present after 13 days and increased in amount for some days afterwards. The time taken for the antibody response to occur perhaps explains why primary vaccination within a day or two after exposure to smallpox may fail to protect the exposed person. In all subjects tested from 15 days to 2 months after primary vaccination antibody was detectable by the inhibition of haemagglutination test and in the majority by the neutralization technique. On the other hand, less than half were positive by the complement fixation test used. Neutralizing antibody was detectable in many sera taken years after primary vaccination when only a few showed antihaemagglutination and then only in low titre; no positives by complement fixation technique were recorded later than 6 months after vaccination.

Most observers agree with Nagler's original observation that antihaemagglutinin constantly appears after primary vaccination (Nagler, 1944). Nagler found that the titre dropped fairly rapidly but reported that half of those who had been vaccinated years before showed some residual antibody. Similar observations have been made by Szathmáry & Baranyai (1957), by Herrlich, Mayr & Munz (1956), by Elisberg *et al.* (1956) and by Kempe & Benenson (1953). Our serum titres were calculated in terms of serum dilutions before admixture with haemagglutinin. It is not always clear from the papers quoted whether serum titres were calculated in terms of final serum dilutions in reacting mixtures, as described by Collier and his colleagues (1949). The lowest serum dilutions tested by us were 1/10 or 1/20, which would correspond to 1/30 or 1/60 by Collier's method of recording. This difference in method of assessing titres and the inclusion of lower serum dilutions in the tests of other workers may account for the higher levels of antihaemagglutinin recorded by them and possibly also for the longer persistence of antihaemagglutinin in low titre following vaccination or revaccination.

Herrlich *et al.* (1956) failed to detect complement-fixing antibody after vaccination, while Kempe & Benenson (1953) in their study of mothers and their babies recorded a large number of positive complement fixation results in mothers, some of whom had been vaccinated more than 5 years previously. Most of their titres, however, were low, the majority being positive only in dilutions of 1/2 or 1/4—dilutions below the lowest used in our tests.

After revaccination the neutralization test appears to give a more reliable indication of antibody response than the test for antihaemagglutinins or complement-fixing antibody; in some subjects antihaemagglutinin and complement-fixing antibody failed to appear after revaccination even although a marked rise in neutralizing antibody occurred. The absence of antihaemagglutinin response was observed in those who showed an early or vaccinoid reaction to revaccination and was noted also by Nagler (1944) and by Elisberg, McCown & Smadel (1956). In revaccinated persons neutralizing antibody response was observed as early as the seventh day, that is several days earlier than antibody was detected after primary vaccination. This suggests that prompt revaccination of smallpox contacts may give greater protection than primary vaccination. The neutralizing antibody titres following revaccination also tended to be considerably higher than after primary vaccination. When complement-fixing antibody and antihaemagglutinins were detectable after revaccination the titres recorded were, in general, no higher than those observed after primary vaccination.

Our observations on the antibody response in relation to the nature of the local reaction following revaccination indicate that the 'early' reaction is, in many instances, not associated with a rise in antibody. In those who do show a marked rise in neutralizing antibody, tests for antihaemagglutinin and complement-fixing antibody may be negative.

By testing sera for vaccinia-neutralizing antibody in the rabbit's skin, Sato & Kuroda (1929) found this antibody in all but two of thirty-six revaccinated individuals who showed an early type of skin reaction. The fact that revaccination of their subjects was effected by three insertions ('Kreuzimpfungen') probably accounted for the greater proportion of positive antibody responses observed by them; for even two in place of a single insertion of an active vaccine lymph greatly increases the proportion of takes in revaccinated individuals (Horgan & Haseeb, 1944).

SUMMARY

Following primary vaccination no antibody was detected up to the tenth day. After this time neutralizing antibody and antihaemagglutinin were present in the majority of individuals, while, with the technique used, complement-fixing antibody was found in less than half those examined. Neutralizing antibody may be found in the blood more that 20 years after primary vaccination; by this time very few show antihaemagglutinin and then only in low titre. Complement-fixing antibody was not found more than 6 months after primary vaccination.

In revaccinated individuals the antibody response tended to be higher than after primary vaccination and, when it occurred, often appeared within a week. *This suggests that prompt revaccination of smallpox contacts may offer even greater protection than does primary vaccination.*

The neutralization test is a better measure of immunological response than are either of the other two tests, for these may give a negative result even though there may be a well-marked rise in neutralizing antibody. The 'early' type of reaction to revaccination was associated with an antibody response in only about half the

subjects examined. In the remainder, the skin reaction, although identical in appearance, must be regarded as allergic in nature and not accompanied by increase in immunity.

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