Clinical and Immunological Study of Percutaneous Revaccination in Children Who Originally Received Smallpox Vaccine Subcutaneously

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In a large multicenter smallpox vaccination study carried out from 1970 to 1973, it was found that 39% of children who were initially immunized by the subcutaneous route and then challenged percutaneously with a standard vaccination did not have measurable neutralizing antibody upon follow-up. Because of this finding, a percutaneous revaccination study was conducted at the St. Louis study center in 1975 and 1976. There were four study groups, which were composed on the basis of route of primary immunization (subcutaneous or percutaneous) and whether neutralizing antibody was detectable following the original percutaneous challenge immunization. Of 52 children revaccinated, all but four had accelerated reactions. There was no difference in size of lesions or day of maximum erythema among the four study groups. Only 66% of children originally vaccinated subcutaneously who did not have postchallenge neutralizing antibody had measurable neutralizing antibody following revaccination. Transformation studies with vaccinia viral antigen before and after revaccination were performed on lymphocytes from 50 children. There were no appreciable differences in responses either before or after revaccination when the four groups were compared. However, the mean stimulation ratio for the total group increased from 2.4 before revaccination to 4.6 3 weeks later. In primary subcutaneous vaccine recipients without pre-revaccination neutralizing antibody, lymphocyte transformation correlated directly with the neutralizing antibody response.

In 1970, because of the consideration that the morbidity and mortality from smallpox vaccination was greater than the potential risk of smallpox in the United States, the Infectious Diseases Branch of the National Institute of Allergy and Infectious Diseases sponsored a multicenter study in an effort to find safer smallpox vaccination procedures (13). This study, the results of which have recently been published (2, 6-8, 14, 18), was designed to evaluate the effect of four different vaccines administered in varied concentrations and by two routes on vaccine-related morbidity and antibody response (2). Immunity was assessed by the measurement of serological responses following primary vaccination and following challenge immunization with a standard smallpox vaccination 6 months later.

The studies were carried out at the following centers: University of California at San Diego; University of Colorado, Denver; University of Kentucky, Lexington; and St. Louis University,

St. Louis. Primary vaccination of study participants was carried out with four vaccines by two routes (percutaneous or subcutaneous) and with three possible doses per route. The vaccine strains employed were: New York City calf lymph (NYC-CL), New York City chorioallantoic membrane, CV-1, and Lister. Concentrations of vaccines administered percutaneously were 10^6 , 10^7 , and 10^8 pock-forming units (pkfu)/ml and those of the subcutaneously administered vaccines were 10^3 , 10^4 , and 10^5 pkfu/ml.

Primary vaccinations were performed on 1,746 children between September 1970 and November 1972. Of the primary vaccinations, 49.6% were administered percutaneously and 50.4% subcutaneously. Approximately 6 months after primary immunization, challenge vaccination was carried out on 1,285 of the children with standard NYC-CL vaccine (10^8 pkfu/ml) by percutaneous administration. Later analysis of the serological data revealed that 91% of those chil-

dren who were initially immunized percutaneously with a resulting cutaneous lesion (clinical "take") at the vaccination site and/or serological response had a measurable level of serum-neutralizing antibody following percutaneous revaccination (6). In contrast, only 61% of the children who were initially immunized subcutaneously and then challenged with standard NYC-CL vaccine percutaneously had a measurable level of neutralizing antibody on follow-up. This failure to detect neutralizing antibody in such a high percentage of children who were initially immunized subcutaneously was an unexpected finding. It was particularly alarming and intriguing since over one-half of these children without detectable neutralizing antibodies had a clinical take at the revaccination site. Because these findings suggested that the children without neutralizing antibody might be unprotected against smallpox but perhaps abnormally sensitized to vaccinia virus, the presently reported percutaneous revaccination study was undertaken.

MATERIALS AND METHODS

Study population. In St. Louis, there were 319 children who were vaccinated subcutaneously in whom results of serum antibody studies were available. Of this group, 132 (41%) had a final neutralizing antibody titer <10 . The planned study population was to include as many of the 132 children without detectable neutralizing antibody as could be located. In addition, for control purposes, a small number of children in the following categories were also to be revaccinated: children initially vaccinated subcutaneously who, following revaccination, had measurable neutralizing antibody and children who received primary immunization percutaneously and who, after challenge vaccination, did or did not demonstrate neutralizing antibody. Informed consent was obtained from the parents of all children in the study.

Study plan. Following the collection of a blood sample, all participants were vaccinated with one lot of standard NYC-CL vaccine (Wyeth Dryvax), 10^8 pkfu/ml, using a bifurcated needle with five insertions (10 punctures) at the deltoid insertion. Parents were given forms on which they were asked to record daily their child's temperature as well as measurements of the central lesion and diameter of the area of erythema at the vaccination site. On day 4 to 7, a member of the study team saw the child, photographed the vaccination site, and recorded the child's temperature and the size and character of any lesion present. Occasionally, needle aspirates from the lesions were obtained for viral culture. Approximately 3 weeks after vaccination, a sample of venous blood was drawn, and a measurement of the residual vaccination scar was obtained.

Serological studies. The hemagglutination-inhibition (HAI) test was performed as previously described (2). The hemagglutination antigen was provided by James Nakano of the Vesicular Disease Laboratory, Center for Disease Control, Atlanta, Georgia.

The neutralization test employed has also been previously described (2). However, the present study method varied slightly from that originally used in St. Louis. The viral antigen was from the same lot of freeze-dried Dryvax calf lymph smallpox vaccine that was used for vaccination. This was reconstituted and diluted in Eagle basal medium containing 0.5% bovine serum albumin. The concentration of virus was that which yielded approximately 30 plaque-forming units per 0.1 ml in LLC-MK2 tissue culture. Fourfold dilutions of serum from 1:10 to 1:2,560 were made in Eagle basal medium. Virus and serum dilutions were incubated for 24 h at 37°C in a 5% CO₂ atmosphere. Tissue cultures were grown in the wells of plastic trays and were incubated in a 5% CO₂ atmosphere at 37°C; maintenance medium contained no serum. In the original neutralization tests performed in St. Louis, 20% sterile skimmed milk rather than 0.5% bovine serum albumin was used, incubation was conducted in stoppered tubes rather than in a 5% CO₂ atmosphere, and tissue culture tubes rather than plastic trays were employed.

All titers are expressed as reciprocals of serum dilutions. Throughout this communication, a titer of ≥ 10 indicates the presence of neutralizing antibody, and a titer of <10 indicates the absence of antibody.

Lymphocyte transformation studies. Vaccinia viral antigen was prepared from Dryvax vaccine which was propagated in LLC-MK₂ tissue cultures maintained on Eagle basal medium without serum. The antigen (titer, 5.0×10^5 50% tissue culture infective doses per 0.1 ml) was inactivated by exposure to ultraviolet light (wave length, 2,537 nm) in a flat-bottom tray at 15 cm for 4 min; the depth of the medium-virus mixture in the tray was 0.5 cm. The antigen was quick frozen and thawed and centrifuged at 1,000 *g* for 10 min. The antigen was checked for residual infectivity in LLC-MK₂ cells, and no cytopathic effect was noted.

Lymphocytes were separated using the Ficoll-Hypaque method of Boyum (5) and resuspended (5.0×10^6 lymphocytes per ml) in RPMI 1640 medium with 10% heat-inactivated fetal calf serum. Incubations were performed in flat-bottom microtiter wells. Six wells were used for each of four groups—control, control antigen, antigen, and phytohemagglutinin. Lymphocytes (0.2 ml) were placed in each well, and to the appropriate wells were added: 0.02 ml of phytohemagglutinin (1:4 dilution), 0.02 ml of vaccinia control antigen (noninfected LLC-MK₂ tissue culture treated in a manner identical to the antigen preparation), and 0.02 ml of vaccinia antigen. The plates were incubated in 5% CO₂ at 37°C for 5 days, following which 0.02 ml of [³H]thymidine (100 μ Ci/ml; specific activity, 20 Ci/ μ M) was added to the wells. The cultures were incubated for another 18 h and then harvested on filters with a multiple automated sample harvester. The dried filter disks were placed in scintillation fluid and counted on a beta scintillation counter. Results are expressed as geometric means of the replicates and as a ratio of stimulated to unstimulated counts.

Viral cultures. In selected instances, samples from papular or vesicular lesions were obtained by needle aspiration of the lesion. With papular lesions, 0.01 ml of saline was injected prior to aspiration. Aspirated

material was placed in 2 ml of Hanks balanced salt solution which contained 2% bovine albumin. Specimens were stored at -70°C , and at a later date were inoculated into LLC-MK₂ tissue cultures.

RESULTS

General. A vigorous attempt was made to locate all 132 children who had received primary immunization by the subcutaneous route and who, following standard percutaneous revaccination, had neutralizing antibody titers of <10 . Forty-three children could not be located, and the parents of an additional 28 children were not interested in further study participation. Of the remaining 51 possible participants, 41 were enrolled, and the remaining 10 did not participate for a variety of reasons (did not keep appointment, medical contraindications, etc.).

By random selection, 20 children who received primary percutaneous vaccination and who, following standard revaccination, had neutralizing antibody titers of <10 and 40 children (20 with primary percutaneous and 20 with primary subcutaneous vaccination) who, following standard revaccination, had neutralizing antibody titers of ≥ 10 were also asked to participate in the study. Of this group of 60, 18 children participated.

Of the 59 children enrolled in the study, 9 did not complete the program, 3 were not revaccinated because of medical contraindications, and the remaining 6 either withdrew during the program or were lost to follow-up. The clinical phase of the present study was initiated in October 1975, and completed in February 1976.

Local response to revaccination. Observations of cutaneous manifestations following revaccination were available for 52 children. Primary-type responses (Jennerian central vesicle with surrounding erythema which reached its maximum on or after day 7 after vaccination) occurred in only three instances (6%), and in only one child was a cutaneous response not observed.

All five subjects who were originally vaccinated percutaneously and who had neutralizing antibody following original challenge had accelerated reactions (development of maximal erythema between day 3 and 7) with revaccination.

Of the seven children with percutaneous primaries but without neutralizing antibody after the original challenge, one had a major reaction, five had accelerated reactions, and one no reaction. The child with the major reaction had failed to have a cutaneous take after his primary vaccination, but did have a typical major reaction following the original percutaneous challenge, which occurred almost 5 years before the present revaccination. The child who had no

cutaneous response had also not responded to the two previous percutaneous vaccination attempts.

All five subjects who were originally vaccinated subcutaneously and who had neutralizing antibody following challenge had accelerated reactions with revaccination. Of the 35 children with subcutaneous primaries without postchallenge neutralizing antibody, 33 had accelerated reactions, and 2 had major reactions following revaccination. Twenty-five of this group of 35 children had cutaneous takes following original percutaneous challenge vaccination, and 10 had no cutaneous responses. All 10 with no cutaneous responses following original challenge had had HAI antibody titers ≥ 10 following primary subcutaneous vaccination. Both children who had major reactions with revaccination did not have a cutaneous response post-percutaneous challenge vaccination.

There were no appreciable differences in cutaneous responses among the four study groups, and there were no unusual cutaneous lesions, associated lymphadenitis, or arm edema or swelling.

Virological studies. Needle aspirations were obtained from the lesions of 13 subjects, and vaccinia virus was recovered on four occasions. At the time of culture, a vesicle was present in four cases, and the remaining nine lesions were more papular in nature. Positive cultures were obtained from three of the four vesicular lesions and only one of the nine papular lesions. Two of the positive cultures were obtained on days 4 and 8 from major reaction lesions. Cultures obtained on day 4 from the other major reaction failed to yield virus; in this case, the lesion at the time of culture was papular, but proceeded on to vesiculation at a later date.

Serological responses. (i) **HAI antibody responses.** Of the 49 children with complete serological study, only 12 (24%) had pre-revaccination HAI antibody titers of <5 . Following immunization, the number with HAI antibody titers <5 was reduced to six (12%). The prevalence of post-revaccination and geometric mean HAI antibody titers are presented by the method of primary vaccination and the presence or absence of neutralizing antibody following original challenge immunization in Table 1. Although there were no significant differences among the groups, the geometric mean HAI antibody titers were lower in primary recipients of subcutaneous vaccine than in those originally immunized percutaneously.

Only 84% of primary subcutaneous vaccinees who were without postchallenge neutralizing antibody had HAI antibody titers ≥ 5 .

(ii) **Neutralizing antibody responses.** The

prevalence of post-revaccination serum-neutralizing antibody and geometric mean titers are analyzed by method of primary vaccination and the presence or absence of neutralizing antibody following original challenge in Table 2. Only 66% of the 32 children originally vaccinated subcutaneously who did not have postchallenge neutralizing antibody had measurable neutralizing antibody (titer ≥ 10) following revaccination. Although there are too few subjects in the percutaneous primary without postchallenge antibody group for meaningful comparison, the geometric mean titer is considerably higher than that of the subcutaneous primary group without postchallenge antibody.

Lymphocyte transformation studies. In Table 3, the results of lymphocyte transformation studies before and after revaccination are analyzed by method of primary vaccination and the presence or absence of neutralizing antibody following original percutaneous challenge immunization. Of the total group, 36% had a stim-

ulation ratio which was ≥ 2 prior to reimmunization, and this increased slightly (46%) but not significantly after revaccination. Except for the finding that none of the percutaneous primary group without postchallenge antibody had stimulation ratios ≥ 2 prior to revaccination, there would appear to be little qualitative difference among the groups. The mean stimulation ratio of the total group prior to reimmunization was 2.4; post-revaccination, the mean stimulation ratio increased to 4.6 ($P = 0.17$). There were no significant differences in mean stimulation ratios among the four study categories.

In Table 4, lymphocyte transformation study results before and after revaccination in primary subcutaneous vaccines without pre-revaccination neutralizing antibody are examined by neu-

TABLE 1. *Prevalence of post-revaccination serum HAI antibody and geometric mean titers (GMT) presented by method of primary vaccination and the presence or absence of neutralizing antibody following percutaneous challenge immunization*

Category	No. of children	% with HAI antibody titer ≥ 5	GMT (90% confidence interval)
Percutaneous primary with postchallenge neutralizing antibody	5	100	15 (8-27)
Percutaneous primary without postchallenge neutralizing antibody	7	86	20 (8-51)
Subcutaneous primary with postchallenge neutralizing antibody	5	100	12 (7-20)
Subcutaneous primary without postchallenge neutralizing antibody	32	84	12 (9-17)

TABLE 2. *Prevalence of post-revaccination serum-neutralizing antibody and geometric mean titers (GMT) analyzed by method of primary vaccination and the presence or absence of neutralizing antibody following percutaneous challenge immunization*

Category	No. of children	% with neutralizing antibody titer ≥ 10	GMT (90% confidence interval)
Percutaneous primary with postchallenge neutralizing antibody	5	100	1,114 (231-5,387)
Percutaneous primary without postchallenge neutralizing antibody	7	71	238 (9-2,017)
Subcutaneous primary with postchallenge neutralizing antibody	5	100	640 (225-1,820)
Subcutaneous primary without postchallenge neutralizing antibody	32	66	53 (28-110)

TABLE 3. *Lymphocyte transformation studies before and after revaccination analyzed by method of primary vaccination and the presence or absence of neutralizing antibody following original percutaneous challenge immunization*

Category	No. of children	% with stimulation ratio (antigen/control) ≥ 2		Mean stimulation ratio (antigen/control)	
		Pre-revaccination	Post-revaccination	Pre-revaccination	Post-revaccination
Percutaneous primary with postchallenge neutralizing antibody	5	40	40	2.1	5.1
Percutaneous primary without postchallenge neutralizing antibody	7	0	43	1.1	1.9
Subcutaneous primary with postchallenge neutralizing antibody	5	60	75	3.3	4.5
Subcutaneous primary without postchallenge neutralizing antibody	33	39	48	2.5	4.9
Totals	50	36	46	2.4	4.6

TABLE 4. *Lymphocyte transformation studies before and after revaccination in subcutaneous primary vaccinees without pre-revaccination neutralizing antibody analyzed by neutralizing antibody response to revaccination*

Category			No. of children	% with stimulation ratio (antigen/control) ≥ 2		Mean stimulation ratio (antigen/control)	
				Pre-revaccination	Post-revaccination	Pre-revaccination	Post-revaccination
Post-revaccination	neutralizing	anti-body ≥ 10	11	18	64	1.7	7.6
Post-revaccination	neutralizing	anti-body < 10	10	30	20	1.8	1.3

^a Of the 33 original subcutaneous primary group without postchallenge neutralizing antibody, 12 were found to have pre-revaccination neutralizing antibody. (This was probably due to an increased sensitivity of the neutralizing antibody test used in the Los Angeles laboratory.) Only the 21 children without pre-revaccination neutralizing antibody are included.

tralizing antibody response to revaccination. There were no significant differences in either the percentage of stimulation ratios which were ≥ 2 or the mean stimulation ratios between the two groups prior to revaccination. However, the mean stimulation ratio increased significantly ($P < 0.02$) from 1.7 to 7.6 in association with a neutralization antibody response. In contrast, there was no change in the mean stimulation ratio in lymphocytes from children who did not develop neutralizing antibody. This difference in lymphocyte response between the two groups is significant ($P < 0.03$).

The mean sizes of the central lesion and erythema of the 11 children with post-revaccination neutralizing antibody were 0.43 ± 0.07 mm and 1.46 ± 0.40 mm, respectively. In contrast, the central lesion and erythema sizes of the children without post-revaccination neutralizing antibody were only 0.26 ± 0.02 mm and 0.43 ± 0.09 mm, respectively ($P = 0.05$ and 0.02).

In individual vaccinees, neither fever nor magnitude of cutaneous response could be correlated with either pre- or post-revaccination lymphocyte transformation responses. The presence of lymphocyte reactivity to the vaccinia virus antigen prior to revaccination did not appear to influence either the clinical cutaneous response, the neutralizing antibody response, or further lymphocyte stimulation response to the test antigen.

DISCUSSION

The present study was undertaken because of the finding that a surprising number of children who were initially vaccinated by the subcutaneous route and challenged by a conventional percutaneous immunization were found on follow-up not to have measurable neutralizing antibody titers. Concern would seem to be justified, because protective immunity to smallpox has been correlated with neutralizing antibody to

vaccinia virus (10, 17), although it has also been noted that previously vaccinated individuals without neutralizing antibody have apparently been protected when exposed to natural disease (19).

One measure for the assessment of smallpox immunity is the type of cutaneous response following revaccination. An accelerated local skin response indicates immunity to infection with vaccinia virus and also presumably smallpox virus (1, 3, 9, 16). By this parameter, it would appear that 94% of those children initially vaccinated subcutaneously who did not have measurable neutralizing antibody after original percutaneous challenge immunization were actually protected.

None of the cutaneous manifestations associated with revaccination of subjects originally immunized by the subcutaneous route were abnormal in any way, and excessive febrile reactions did not occur.

It is possible that cutaneous manifestations associated with revaccination in children originally vaccinated by the subcutaneous route could be primarily due to a cell-mediated response rather than to a more direct effect of virus multiplication. With this in mind, cultures were obtained from several lesions. Except in one instance, virus isolation was associated with vesiculation and was not related to method of original vaccination. It would seem that there is little virus associated with accelerated reactions that are not vesicular. Interestingly, one must raise the possibility that the one positive culture from a nonvesicular lesion was an error. The sibling of the child from whom the positive culture was obtained was also a study participant. This child had a significant vesicular reaction which was cultured at the same visit but failed to yield virus. Since this is the only negative vesicular culture, and the sibling yielded the only nonvesicular positive culture, it seems pos-

sible that the specimens may have been reversed.

There was little difference among the study groups in regard to post-revaccination HAI antibody prevalence or geometric mean titers. Of the whole group, 88% had HAI titers ≥ 5 following revaccination; this is similar to the antibody prevalence following percutaneous challenge of all vaccinees and following primary subcutaneous vaccination, but lower than that following primary percutaneous immunization (6–8, 14, 17). Revaccination had only a modest effect on the geometric mean HAI antibody titer, and this did not vary by primary vaccination route or by postchallenge neutralizing antibody.

Although all initial subcutaneous vaccine recipients had some evidence of a cutaneous response following revaccination (an accelerated reaction in all but two children), only 66% of the group without postchallenge neutralizing antibody had neutralizing antibody (titer ≥ 10) following revaccination. These findings are surprising and suggest that the "blocking" factor noted following original percutaneous challenge had persisted.

There is an important difference in the results of the present revaccination study as compared with the findings associated with the original challenge procedure (6). Of the original subcutaneous vaccine recipients, 36% had major cutaneous reactions following challenge immunization. In contrast, only 2 of the 37 original subcutaneous vaccine recipients had major cutaneous reactions following the second percutaneous standard immunization. It would appear that even without apparent neutralizing antibody, all but two children in the present group had protection against vaccinia virus infection.

As noted in Table 3, there was no appreciable difference in lymphocyte responsiveness to vaccinia virus antigen among the four study groups. Of most interest is the finding of a direct relationship between the neutralizing antibody response and the lymphocyte responsiveness to the vaccinia virus antigen (Table 4).

The possible factors related to the blocking effect of subcutaneous primary immunization on subsequent neutralizing antibody response to conventional percutaneous vaccination could either be humoral or cellular (6). Antibody arising after primary subcutaneous immunization could be less avid, at least as measured in our neutralizing system, than that produced after percutaneous vaccination with its associated cutaneous virus multiplication. Later percutaneous challenge would elicit early recall of the less avid antibody, and this could act as a blocking factor against the development of normal complete antibody. This apparent blocking factor could

also be related to a disproportion between antibody to extracellular and intracellular virus (4). The neutralization test employed measures mainly antibody to cell-associated antigen. Perhaps subcutaneous primary inoculation results in antibody to extracellular virus, and upon percutaneous revaccination, recall antibody is similar to the primary response rather than the typical antibody to intracellular virus. If this were the case, the vaccinee would probably be clinically protected, but the plaque-reduction neutralization test would give a falsely negative result.

It is also possible that cell-mediated mechanisms contributed in some way to the lack of a neutralizing antibody response. However, since normal cutaneous responses occurred after both percutaneous challenge immunization and the present revaccination experience, it seems unlikely that cell-mediated factors are contributory. With other vaccinees, what appear to be aberrant cell-mediated responses have resulted in exaggerated clinical problems (11, 12, 15).

The positive correlation between vaccinia antigen lymphocyte transformation responses and neutralizing antibody development following revaccination and the normal cutaneous responses associated with revaccination are reassuring. Although cell-mediated immunity at a level of sensitivity not detectable by our test could still have a blocking role in relation to neutralizing antibody development, it does not appear that it can be expected to be associated with unusual clinical manifestations with further immunization or possible natural challenge.

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