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Evaluation of the precipitation-in-gel reaction in the diagnosis of smallpox *

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Specimens of vesicular or pustular fluids and of scabs from patients with smallpox as well as emulsions of variola-infected chorioallantoic membrane (CAM) were tested for virus titres and by the precipitation-in-gel (PIG) reaction. They were also tested after exposing them directly to sunlight and after keeping them at temperatures of −20°C, 4°C, and 25°C. It was found that when extracts of fresh specimens were diluted to the point where the PIG reaction became negative there was still a titre of 10^4–10^5 infectivity in the swab extracts and 10^3–10^4 infectivity in the scab extracts. It was also found that the PIG reactions were all negative on specimens that were kept for 14 days at 25°C, and that several were negative after only 7 days; the loss in infectivity titre, however, was only slight in all the specimens tested. It is concluded that the laboratory diagnosis of smallpox by virus inoculation of CAM is more reliable than by the PIG test.

In recent years, the precipitation-in-gel (PIG) reaction has been used in the diagnosis of various diseases. Agar gel diffusion, especially microdouble diffusion, has proved to be the most effective method for the antigenic analysis of the pox group of viruses as well as for the diagnosis of smallpox. The test is convenient, does not require complicated or expensive apparatus, and its rapidity is a special advantage. However, with vesicular and pustular material or scabs from patients with smallpox, the results from different laboratories have not been consistent (Arita, personal communication, 1973). The present work investigates some of the factors influencing the PIG reaction when tested with material from patients with smallpox.

MATERIALS AND METHODS

There were three objectives in this experiment:

(1) To determine the effects on the PIG reaction of keeping the vesicular or pustular material in cotton swabs at different temperatures, and whether any changes in the reaction corresponded with the content of viable virus in the swab.

(2) To find out the relationship between the PIG reaction and the virus titres in smallpox scabs.

(3) To determine the relationship between the PIG reactions and virus titres in suspensions of chorioallantoic membranes (CAM) from embryonated eggs infected with variola virus.

Studies on swabs

The vesicular or pustular fluids from 6 patients with smallpox were collected in sterilized cotton swabs after the vesicles or pustules had been punctured. From each patient, 7 swabs were taken and each swab contained material from 3 vesicles or pustules. One swab was tested immediately. Two swabs were kept at room temperature (22–25°C), 2 in a refrigerator (4°C), and 2 in a deep-freeze (–20°C); each of these was tested after 1 week and 2 weeks. In addition, some swabs taken from 3 of the patients were tested after exposing them, while inside test-tubes, to direct sunlight (about 38.5°C) for 3 h.

In setting up tests for the PIG reaction and for estimating virus titres, the swabs were soaked in 1 ml of phosphate-buffered saline (PBS) containing antibiotics (penicillin and streptomycin), then squeezed, and the expressed fluid was lightly centrifuged. PIG tests on the supernatant emulsions were carried out on slides by the method recommended by WHO (3). Readings were taken after 4 h and 24 h. For estimating virus titres, egg inoculation and pock

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Table 1. Log titres of virus and PIG reactions (indicated by + or −) on extracts of swabs from 6 patients with smallpox. The tests were carried out on day 0 and after keeping the swabs at temperatures of −20°C, 4°C, and 25°C for periods of 7 and 14 days

Patient	Clinical type	Day 0	Day 7			Day 14		
			−20°C	4°C	25°C	−20°C	4°C	25°C
1	confluent	7.31 (+)	7.34 (+)	7.34 (+)	7.27 (+)	7.41 (+)	7.41 (+)	7.11 (−)
2	discrete	7.64 (+)	7.64 (+)	7.64 (+)	7.51 (+)	7.64 (+)	7.51 (+)	7.34 (−)
3	discrete	6.90 (+)	6.90 (+)	6.84 (+)	6.84 (+)	7.04 (+)	7.00 (+)	6.69 (−)
4	confluent	6.11 (+)	6.14 (+)	6.11 (+)	6.00 (−)	6.10 (−)	6.10 (−)	6.00 (−)
5	discrete	6.11 (+)	6.15 (+)	6.11 (+)	6.04 (−)	6.07 (+)	6.07 (+)	6.00 (−)
6	confluent	6.40 (+)	6.60 (+)	6.40 (+)	6.31 (−)	6.42 (+)	6.30 (−)	6.21 (−)

counts were performed in the standard manner as reported previously (2).

Studies on scabs

Dried scabs from patients with smallpox were collected and preserved at −20°C. Three or four crusts were weighed accurately and a 10% emulsion of them in PBS was made with the help of a pestle and mortar. The emulsion was centrifuged lightly and the supernatant was diluted ten-fold a few times: the PIG test and virus titrations were then performed on each dilution.

A few scabs in a test-tube were exposed to direct sunlight for 3 h before being prepared for testing as described above.

Studies on CAM suspensions

Infected chorioallantoic membranes were made into a 20% emulsion in a pestle and mortar with neutral sand. The supernatant of the centrifuged material was diluted ten-fold a few times before tests for virus content and PIG tests were carried out on each dilution. Two such emulsions were heated at 60°C for 10 min and then tested as above.

RESULTS

Studies on swabs

The log titres of virus and the results of the PIG reactions (indicated by + or −) on the extracts from the swabs (Table 1) show that after keeping the swabs at room temperature (25°C), or at 4°C or −20°C for up to a fortnight, the virus titres remained practically unchanged while the PIG reaction was altered more or less at all temperatures by the passage of time. With the specimens kept at room temperature the PIG reaction became negative even though the log titre of virus was 6 and over.

The effect of exposure of the swabs to sunlight was that the log titre of virus was lowered by 0.03–1.0. Any change in the PIG reaction followed the level of virus titres in the emulsions from 2 patients, but in the third patient the PIG reaction was more adversely affected than the virus titres.

Studies on scabs

The results of virus titres and PIG reactions are shown in Table 2, which also shows that the exposure of the scabs to direct sunlight affected both the virus titre and the PIG reaction, although the latter was affected more.

Studies on CAM suspensions

Four separate emulsions were made with four strains of variolavirus. Table 3 shows the log titres of virus (for different dilutions of the emulsion) with the corresponding PIG reactions, as well as the results of examination of 2 such emulsions after heating at 60°C for 10 min. It is seen that the PIG reaction was

Table 2. Log titres of virus and the corresponding PIG reactions before and after exposure to sunlight for 3 h of a 10 % emulsion of scabs and of several ten-fold dilutions of it.

Patient	Before exposure		After exposure	
	titre	PIG	titre	PIG
1	7.29	+	6.21	+
	6.29	+	5.11	—
	5.29	+	4.21	—
	4.29	+		
	3.29	—		
2	6.21	+	6.00	+
	5.21	+	5.00	—
	4.21	—	4.00	—
3	4.61	+	4.25	—
	3.61	+	3.25	—
	2.61	—	2.25	—

Table 3. Relationship of log titres of virus and PIG reactions of different dilutions of CAM emulsions of 4 virus strains, and the effect of heating 2 such emulsions at 60°C for 10 min.

CAM emulsion	Before heating		After heating	
	virus titre	PIG	virus titre	PIG
1	6.34	+	0	—
	5.34	+		
	4.34	—		
2	8.12	+	NT [a]	NT
	7.12	+		
	6.12	+		
	5.12	+		
	4.12	—		
3	5.11	+	0	—
	4.11	—		
	3.11	—		
4	8.22	+	NT	NT
	7.22	+		
	6.22	+		
	5.22	+		
	4.22	—		

[a] NT = not tested.

positive when the log titre of virus in the emulsions was over 5, and that heating at 60°C for 10 min killed the viruses and made the PIG reaction negative.

DISCUSSION

The precipitation-in-gel reaction is an antigen–antibody reaction, and in smallpox the antigen primarily responsible for it is the LS antigen complex, part of which is heat labile and part heat stable. It is therefore to be expected that the titre of antigen as well as the temperature at which the antigen is preserved prior to performance of the test will have a bearing on the reaction.

The results in Tables 1 and 3 indicate that the PIG reaction depends on the level of the virus titre. However, it should be remembered that the virus particles themselves do not diffuse in agar gel or contribute to the precipitation. The titration of virus was used here as a measure of the content of antigens in the crusts or swab extracts. With the CAM emulsions used as antigen the PIG reaction was positive only when the log titre of virus was 5 or more (Table 3). With the emulsions of scabs (Table 2), a log titre of virus of about 4 was sufficient to give a positive test. With extracts from swabs of fluid taken from vesicles or pustules (Table 1), the titre of virus was always high enough (log titre >6) to give a positive PIG reaction; on dilution, however, the minimum log titre of virus to give a positive PIG reaction was found to be just over 5.

Poxviruses have a complex antigenic constitution, numerous antigenic components being demonstrable as distinct entities both in purified preparations of viral particles and in soluble extracts of infected cells. The latter contain not a single antigen, but a large number of antigenic components and some of these are involved in the PIG reaction (1). In other words, the presence or absence of these antigens and, when present, their concentrations have a bearing on the PIG reaction. Because the amounts of these antigenic components probably varied from material to material, the minimum virus titre needed to produce a positive PIG reaction was not the same in the different materials (vesicles, pustules, scabs, or CAM emulsions). Dried scabs may have contained a higher proportion of PIG-positive antigens, so that the

minimum virus titre to produce a positive PIG test was comparatively lower.

The thermostability of the different components in the soluble antigen also varies and one has been described that is labile at 37°C (1). Table 1 shows that all the materials from vesicles or pustules that were kept at room temperature (25°C) for a fortnight became PIG-negative, although the log titre of virus in the emulsions was more than 6 per ml. Thus, even a virus concentration of log titre 6 was not sufficient to give a positive PIG reaction in the absence of certain thermolabile antigens in the suspension. Heating the CAM emulsions at 60°C for 10 min destroyed the viruses and produced negative PIG reactions (Table 3). Exposure to sunlight of extracts of vesicular or pustular fluids and of scab emulsions also affected the PIG reaction more than it did the level of virus titres (Table 2).

CONCLUSION

With specimens of vesicular or pustular fluids, or scabs, CAM inoculation appears to be a more sensitive method for diagnosis than the PIG reaction, because specimens with a low titre of virus do not give a positive PIG test. Heat or sunlight may lead to negative PIG reactions even before they destroy the viability of the viruses. For both tests to be positive, a high concentration of the material (scabs, or vesicular or pustular fluids) is required in the emulsions and the specimens should not be subjected to heat or sunlight as far as possible before performing the tests.

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RÉSUMÉ

ÉVALUATION DE LA RÉACTION DE PRÉCIPITATION EN MILIEU GÉLIFIÉ POUR LE DIAGNOSTIC DE LA VARIOLE

On a étudié le rapport entre les résultats de la réaction de précipitation en milieu gélifié (PMG) et les titres du virus variolique à l'aide de matériel pathologique provenant de varioleux. Si l'on utilise des extraits de croûtes, des titres de virus, en valeur logarithmique, égaux ou supérieurs à 4 donnent toujours une réaction PMG positive. Avec les sérosités vésiculaire et pustulaire, le titre minimal donnant une réaction positive est supérieur à 5. Il en est de même lorsque l'épreuve est pratiquée à l'aide d'une émulsion de membrane chorio-allantoïde (MCA) infectée par le virus variolique.

Si l'on expose le matériel infectieux (croûtes, sérosités) directement à la lumière solaire, à 38,5°C environ, ou si on le maintient à des températures de −20°C, +4°C ou +25°C, les résultats de la réaction PMG sont davantage affectés que les titres de virus. Le chauffage des MCA à 60°C pendant 10 minutes a pour effet d'inactiver complètement le virus et de négativer les réactions PMG.

Les auteurs concluent que l'inoculation de la MCA de l'embryon de poulet est une méthode plus sensible et plus fiable que la réaction de précipitation en milieu gélifié pour le diagnostic de la variole.

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