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Skin tests with influenza virus

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SUMMARY

Skin reactions have been produced in normal subjects by the injection of highly purified influenza A and B viruses. The reactions reached a maximum at 24–48 hr. and the histological pattern was compatible with a delayed hypersensitivity reaction. There was no close relation between skin test results and circulating antibodies. Twenty-seven subjects were challenged intranasally with attenuated influenza A (H3N2): 5 of 14 skin-test-negative subjects were infected, but none of 13 skin-test-positive subjects.

INTRODUCTION

The presence of haemagglutination-inhibiting antibody in serum has been known for a long time to indicate resistance to infection with the influenza virus (Hobson, Beare & Gardner, 1971). However, in recent studies it has not proved possible to account completely for the resistance of volunteers to infection with live influenza virus vaccines by the presence of antibody against either of the surface components of the virus, i.e. haemagglutinin (HA) or neuraminidase (N), whether this was measured in sera or nasal secretions (Freestone *et al.* 1972) It therefore seemed reasonable to ask whether cell-mediated immunity might play a part, and we decided to start by investigating the skin reactions to the virus, which were reported many years ago (Beveridge & Burnet, 1944; Beveridge, 1952).

MATERIALS AND METHODS

Volunteers

The subjects were 41 male and 52 female members of the staff of this institute, aged between 18 and 66 years, who volunteered to take part after the nature of the procedures and the objectives of the investigation had been explained to them by one of us. The whole project had been previously reviewed and approved by the Ethical Committee of the Northwick Park Hospital.

Antigens

Influenza viruses were propagated in the allantoic cavity of chick embryos and purified by zonal ultracentrifugation. They were prepared and safety-tested as for influenza vaccine production, being inactivated with formaldehyde and stored at 4° C. in 1·2% sucrose. Immediately before use these antigens were diluted in

isotonic saline. Three batches of antigens were used, two being prepared from A2/Aichi/68 (H3N2) and B/Mass/3/66 by Richardson Merrell Laboratories, and one from A/Hong Kong/1/68X (H3N2) by Evans Medical Ltd. A control fluid was prepared from allantoic fluid from uninoculated eggs. This was diluted to the same protein concentration as the skin test antigen.

Antibody measurements

Haemagglutination inhibition (HI) tests were performed by a standard method (Tyrrell, Peto & King, 1967).

Skin tests

In all cases the materials were inoculated in 0·1 ml. volumes into the skin of the volar aspect of the forearm. The skin was examined and any erythema or swelling was measured, usually after 18–24 hr. but in early experiments at shorter and longer intervals than this.

Histology

Typical lesions were excised under local anaesthesia after 24 hr. The tissue was divided into two portions. One part was snap-frozen with liquid nitrogen and cryostat sections cut. These were stained by immunofluorescent techniques for viral antigen, immunoglobulins and complement. The remaining tissue was fixed in formalin and paraffin sections examined after staining with haematoxylin and eosin, azur A and methyl green–pyronin.

RESULTS

The injection site was examined repeatedly during the first 5 days. Reactions only became apparent after some hours and usually reached a peak about 24 hr. after inoculation. They declined somewhat at 48 hr. and then continued for several further days. They seemed to be specific. For example, in a series of tests using 4 CCA units of antigen, reactions were seen in 12 of 42 subjects against influenza A, and 17 of 42 subjects against influenza B; there were no reactions to the control fluid apart from one mild immediate type reaction. Eleven subjects reacted to only one antigen (Table 1), but there was an excess of subjects reacting to both, perhaps because some developed skin reactivity more readily than others. Repeated tests were fairly reproducible in size; the smallest significant reaction was arbitrarily taken as one over 5 mm. in diameter and this is referred to as 'positive' later in this paper. There was a relation between the amount of antigen administered and the reaction observed; thus subjects who failed to react to 4 units might react to a larger dose, while those who did react to 4 usually had a larger reaction to a larger dose (Fig. 1).

Histology

Biopsies of typical lesions due to both influenza A and B were examined. They showed considerable infiltration with lymphocytes and mononuclear cells aggregated mainly around small blood vessels but with a lesser infiltrate about sweat glands and skin appendages (Plate 1). Only scanty mast cells were noted. Cryostat

Table 1. *Results of skin tests in 42 subjects each tested with both A and B antigens using 4CCA units*

		Tested with A antigen	
		No. skin-test positive	No. skin-test negative
Tested with B antigen	No. skin-test positive	9	8
	No. skin-test negative	3	22

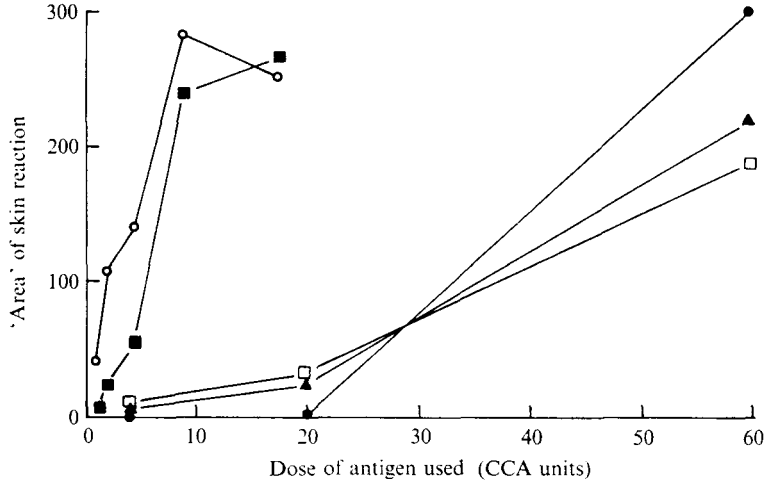

Fig. 1. Relation between amount of antigen injected and size of skin lesion at 24 hr., expressed as the product of the greatest and least diameters ('area'). Five subjects tested with A2/Aichi/68.

sections examined for immunoglobulin and complement components gave negative results. We consider that the histological and immunological features indicated a delayed type reaction and were not compatible with an Arthus reaction.

Relationship to circulating antibodies

Blood collected from volunteers at the time of testing was titrated for antihaemagglutinin antibodies by the HI test, and these results are shown in Fig. 2. It can be seen that there were few volunteers with low titres of antibody who also had positive skin reactions, although there were substantial numbers of subjects with high titres of antibody who nevertheless had negative skin reactions.

The tests were repeated using radial immunodiffusion (Schild, Henry-Aymard & Pereira, 1972) with an H3N2 virus as antigen. This technique measures antibodies against both neuraminidase and haemagglutinin. The test was not quite so sensitive, but again (Fig. 3) there was no close relationship with the results of skin tests. Eleven of 27 subjects seronegative by this test had positive skin tests, while 9 of 23 seropositive subjects had positive skin tests. These data again discount the possibility that the skin lesions are those of an Arthus reaction.

Relation to infection

We assumed that skin sensitivity must arise as a result of exposure to an antigen related to the one used in the test and tried to find out what type of stimulus would induce skin sensitivity.

Table 2. *Response to vaccine challenge*

	Initial reciprocal HI titre	No. infected	Total
Skin-test positive	<20	0/0	0/13
	$\geqslant 20$	0/13	
Skin-test negative	<20	2/3	5/14
	$\geqslant 20$	3/11	

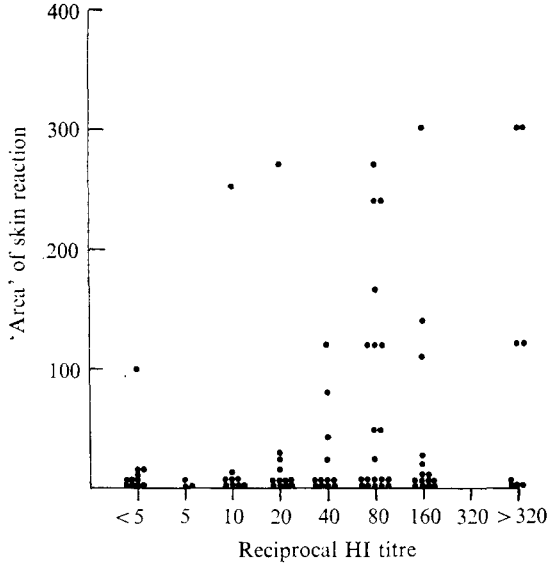

Fig. 2. Relation between the 'area' of skin reactions to H3N2 strain and the serum haemagglutination inhibiting antibody titre. Results in 83 subjects. Those with lower titres have less reactions, but there are reactions in some with very low titres.

Seven volunteers were inoculated intramuscularly with a standard dose of killed vaccine prepared from A2/Hong Kong/68 (H3N2) virus, and three developed a substantial rise in circulating HI antibodies. Nevertheless there was no significant change in skin reactivity. Thinking that infection of the respiratory tract might be needed, other volunteers were given as an intranasal spray 10^6 infectious doses of an attenuated live influenza virus vaccine. Of 27 volunteers five became infected as judged by an HI antibody response, but again there was no significant increase or decrease in skin reactivity when they were tested 2 weeks after inoculation. Thinking that the infection might have been too mild, we also tested five patients who had just recovered from clinical influenza due to laboratory-confirmed infection with an H3N2 virus of serotype A2/Eng/42/72; none had positive skin tests.

We also considered that a positive skin test might be associated with resistance to infection. We therefore analysed the outcome of giving live vaccine to volunteers according to the skin test results and antibody titres. Table 2 shows that whether the volunteer had antibody or not, the infection always occurred in subjects who were skin-test-negative; and the overall differences reach statistical significance.

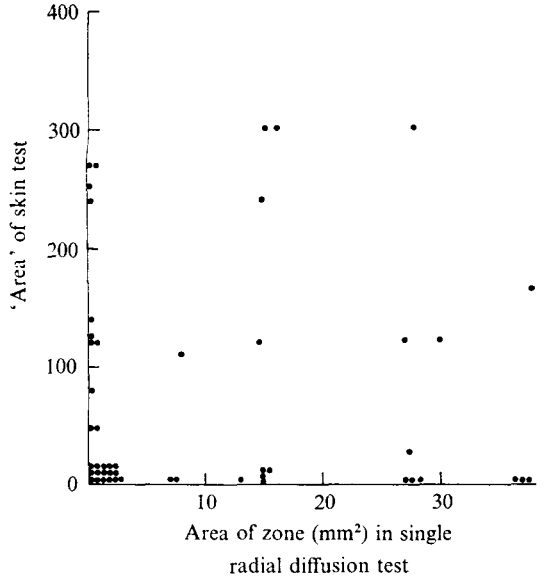

Fig. 3. Relation between 'area' of skin reaction and antibody against both HA and N of H3N2 as measured by radial immunodiffusion. Results in 50 subjects. Relationship with antibody concentration is less obvious.

DISCUSSION

Using purer antigens than earlier workers, we have again produced skin reactions to influenza virus. All the evidence – i.e. the timing, the histological appearances, the lack of relation with circulating antibody and the absence of immunoglobulins or complement in the lesion – points to this being a delayed type reaction due to cell mediated immunity. The specificity of the reactions shows that they are due to a particular viral component, but further work is necessary to determine whether they can be produced by pure haemagglutinin or other peptides extracted from virus particles. In addition, the mechanism of the reaction should be studied; for example, by looking for specific transformation of lymphocytes with virus antigen, as was done in a different context by Denman *et al.* (1970). This approach would make it possible to look for blocking antibodies, the presence of which might account for the existence of at least some of those subjects who have clearly been exposed to virus because they have serum antibody and who resist reinfection but who have negative skin reactions. Waldman & Henney (1971) have shown in animals that there may be separate populations of virus-sensitive lymphocytes, in that those derived from the respiratory tract are more readily transformed after vaccination by the respiratory route than splenic lymphocytes, while the latter are more sensitive after parenteral vaccination. It seems important now to decide whether there is any correlation between the presence of such lymphocytes and resistance to infection and our results suggest that this may be so. If this is confirmed it will become necessary to ascertain the effect of various vaccination procedures on the lymphocyte population as well as on the antibody titres. This may not be straightforward – we think that skin

sensitivity must arise as a result of exposure to natural infection and were therefore surprised not to be able to demonstrate its appearance after artificial or natural infection. Non-specific depression of cell-mediated immunity after influenza infection may well play a part (Reed, Olds & Kisch, 1972) but we think it is unlikely to be the whole explanation.

We would like to thank Mrs Carole Williams for technical assistance and the manufacturers for supplying valuable virus material.

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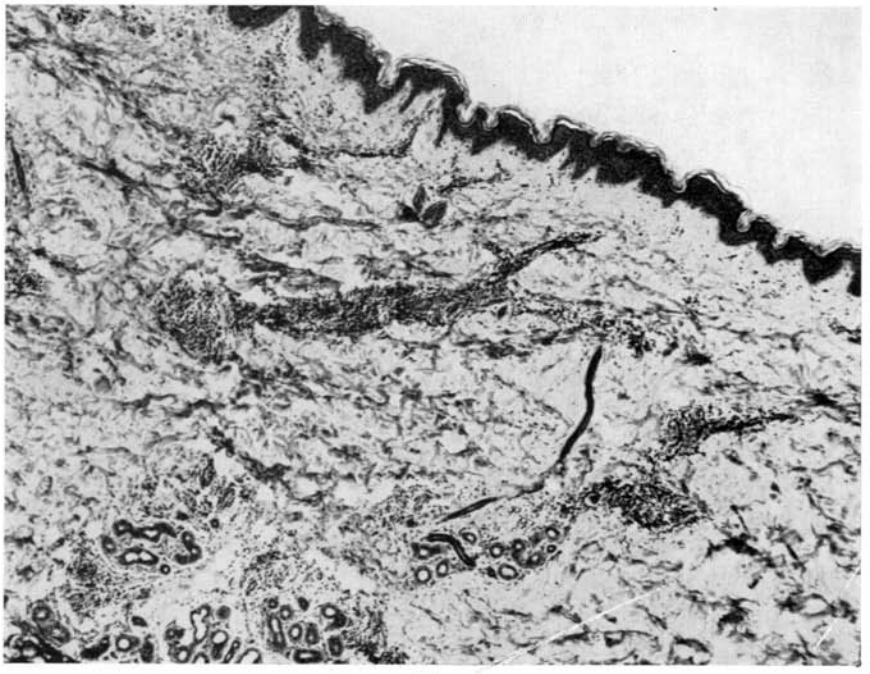
EXPLANATION OF PLATE

PLATE 1

(*a*) Biopsy from subject injected with influenza A material (stained with haematoxylin and eosin, × 32). Blood vessels show well-marked cuffing by lymphocytic and mononuclear infiltrate. A less-intense cell infiltration extends deeply into dermis in region of sweat glands. (*b*) Biopsy from subject injected with influenza B material (haematoxylin and eosin, × 440). Capillary is surrounded by infiltrate consisting largely of lymphocytes with occasional histiocytic type cells.



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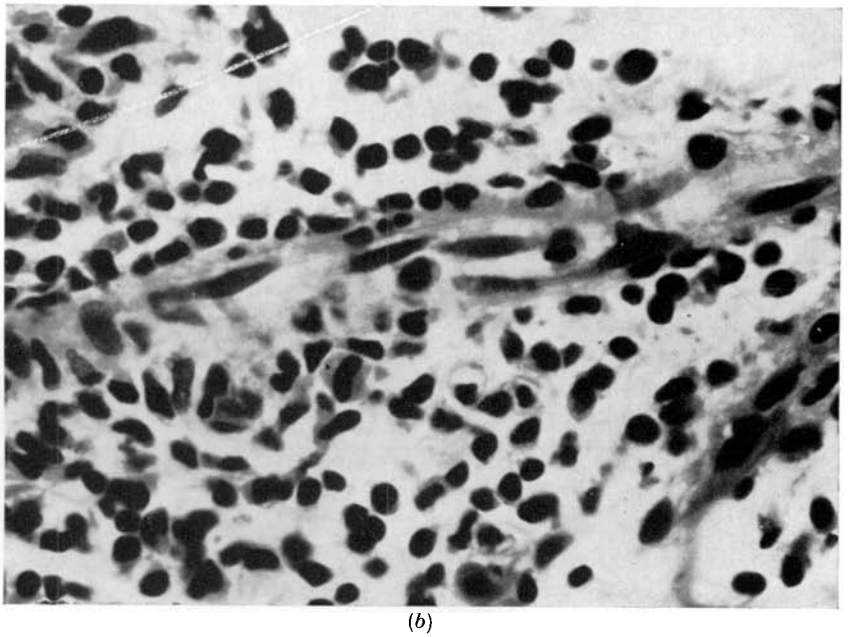

(*b*)

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