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DELAYED HYPERSENSITIVITY IN VACCINIA-INFECTED MICE I. ANTIGEN(S) RESPONSIBLE FOR DEVELOPING DELAYED HYPERSENSITIVITY

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Summary. — The capillary tube cell migration assay was used to identify the antigen(s) responsible for developing delayed hypersensitivity in vaccinia-infected mice. The migration of peritoneal cells obtained from vaccinia-infected mice was most intensely inhibited by LS complex free from virions. The surface components of the virion inhibited the migration of cells obtained from the sensitized mice more intensely than the virus coat proteins or the core. These results suggest that the LS complex plays an important part in delayed hypersensitivity in vaccinia-infected mice.

Introduction

Host defence mechanisms to viral infection may take many forms including the development of humoral and cellular immunity, the production of interferon, etc. The role of these mechanisms in viral infection has been reviewed in several papers (Mims, 1964; Gresser and Lang, 1966; Allison, 1967; Johnson and Mims, 1968). The role of cellular immunity in vaccinia infection was first suggested by von Pirquet (1907). Afterwards the role of delayed hypersensitivity in the development of primary vaccinial lesions in human subjects and experimental animals was studied by Pincus and Flick (1963), and Pincus *et al.* (1963). Allison (1967) suggested that delayed hypersensitivity played an important contributory part in the genesis of rashes. On the contrary, Hirsch *et al.* (1968) demonstrated that rabbit anti-mouse thymocyte serum increased morbidity and mortality induced by vaccinia infection in mice after intravenous virus administration, whereas it did not affect either humoral antibody or interferon production.

Recently it has been shown in several lines of the study on delayed hypersensitivity that the diversity of in vivo manifestation was matched by the diversity of in vitro phenomena, including the capillary tube procedure of George and Vaughan (1962) and the migration inhibiting assay using the normal spleen fragments of Švejcar and Johanovský (1961). It must be stressed that the development of these in vitro assay methods gave a new stimulus to the investigation of delayed hypersensitivity.

We studied what antigen(s) can develop delayed hypersensitivity and how the antigen(s) relates to protective immunity in vaccinia-infected mice by using the inhibition of cell migration technique. In the present paper we

are reporting that the LS complex contained in vaccinia virion and infected tissues is the contributory antigen(s) responsible for developing delayed hypersensitivity in vaccinia-infected mice.

Materials and Methods

Virus. The Lister strain of vaccinia virus, kindly supplied by Dr. C. Nishimura (National Institute of Health, Japan), was multiplied in the dorsal skin of white rabbits weighing 2—3 kg. The bulk obtained aseptically from the infected rabbit skin was purified according to the method of Joklik (1962) with minor modifications. No cellular contaminant was found by immunochemical analysis in the purified virus preparation. The DNA content per mg of protein of the virion was 54 μg.

To obtain inactivated virus preparation, the purified vaccinia virus suspension (275 μg as protein) was irradiated in a layer 1 mm deep at 15 cm from a 15 W Toshiba germicidal lamp for 10 minutes with gentle agitation. Under this condition, no viral infectivity was detected in chick embryo fibroblasts.

Preparation of LS complex from the soluble antigen of vaccinia-infected rabbit dermis was done by the method of Rondle and Williamson (1968) with minor modifications. First the bulk from infected rabbit dermis was centrifuged at 100,000$\times$ g for 30 minutes and filtered through a 220 nm Millipore filter to remove virus particles. Then the filtrate was dialyzed against 0.06 M sodium acetate-acetic acid buffer, pH 4.6, and after centrifuging at 3,000 rev/min for 10 minutes, the precipitate was dialyzed against 0.05 M phosphate buffer, pH 6.3. The dialysate was centrifuged at 3,000 rev/min for 10 minutes and the supernatant was used as LS complex.

Virion fractionation by controlled degradation. The highly purified vaccinia virus was degraded by the method of Holowczak and Joklik (1967). First, vaccinia virions were fractionated into two parts: the mixture of the surface components and the coat proteins released from the virion by treatment with the nonionic detergent NP 40 (Shell Company) and 2-mercaptoethanol followed by alkylation, and the core. Then the virions were fractionated into three parts: the surface components released from the virion by treatment with NP 40 alone, the coat proteins released from the residue by treatment with 2-mercaptoethanol followed by alkylation, and the core. Every fraction was dialyzed against 1 mM tris (hydroxymethyl) amino methane-HCl buffer, pH 8.0.

Sensitization of mice with vaccinia virus. Three- to four-weeks old DD mice of both sexes were injected intraperitoneally with 7×10^4 plaque forming units of highly purified vaccinia virus. From the 3rd day after sensitization, groups of mice were killed daily and the peritoneal exudate cells were harvested.

Capillary tube cell migration assay. The method used was essentially the same as that described by George and Vaughan (1962). The sensitized mice were killed and the peritoneal exudate cells were collected with cold heparinized Hanks' solution followed by three washings with the same solution. The cells were suspended in nutrient medium (Eagle's medium containing 15% normal mouse serum). Each capillary tube 1 by 90 mm was filled with the cell suspension containing $3-4 \times 10^7$ cells/ml and centrifuged. The capillary tube filled with packed cells was placed in a Mackaness chamber. The chamber filled with about 1 ml of the nutrient medium with or without test antigen was incubated for 24 hours at 37° C. The area of cell migration was determined by planimetric measurement of photomicrograph of the chamber taken at 24 hours. The percentage of inhibition of migration was calculated from the following formula:

$$\% \text{ inhibition of migration} = 100 - \frac{\text{area of migration in experimental chamber}}{\text{area of migration in control chamber}} \times 100$$

Analytical methods. Protein was estimated by the method of Lowry *et al.* (1951) and DNA by the method of Burton (1956).

Results

Inhibition of cell migration by three fractions of the virion

First, the inhibiting activity of cell migration was tested with two fractions. The migration of cells obtained from the mice on the 18th day after infection

Table 1. Effect of the mixture containing the surface components and the coat proteins on migration of mouse peritoneal cells taken 5 and 7 days after infection

Day after infection	Dose of test antigen (μg)	% inhibition of migration*
5	0.1	30
	1.3	64
	6.4	98
	12.8	92
7	0.1	43.3
	1.3	73.3
	6.4	96.7
	12.8	90

* Control: migration of the sensitized cells in medium without the mixture.

was inhibited by 6.4 μg of the mixture of the surface components and the coat proteins, but more than 23.3 μg of the core substances was required for the inhibition. It must be added that UV-irradiated virus could inhibit the cell migration. Next, the time after infection required for the development of inhibiting activity was pursued by using the mixture as test antigen. On the 5th and 7th day after infection the cell migration was completely inhibited by 6.4 μg of the mixture as protein, as shown in Table 1 and Fig. 1. This result indicated that at least five days after infection were necessary for full development of hypersensitive activity.

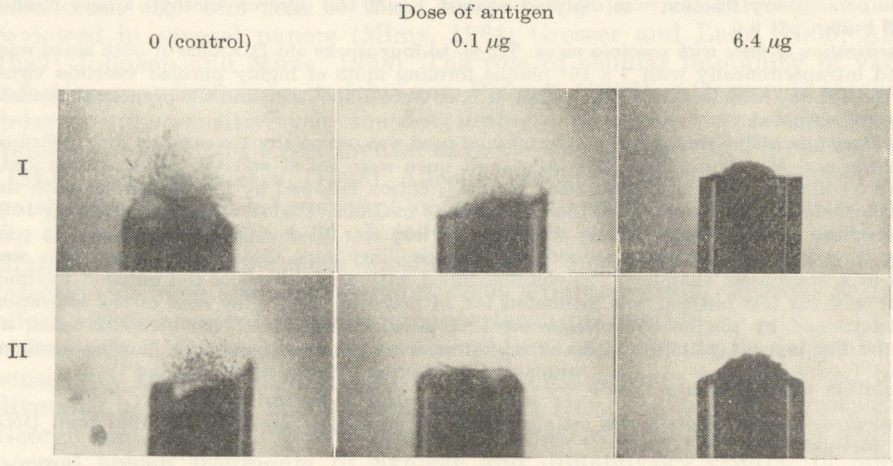

Fig. 1.

Typical examples of the migration of sensitized mouse peritoneal exudate cells from capillary tubes in Mackaness chambers containing no antigen (control), the mixture of surface components and coat proteins (I), or LS complex (II)

In I, peritoneal exudate cells from mice on the 5th day after infection, and in II cells from mice on the 9th day after infection were used. Each chamber was incubated for 24 hours at 37° C.

To gain insight into the part responsible for the strong inhibiting activity of the mixture, the latter was further fractionated into the surface components and the coat proteins as described in Materials and Methods and the respective inhibiting activities were assayed. The surface components in an amount of 9.3 μg as protein inhibited the migration of cells obtained from the mice on the 8th day after infection, whereas 12.4 μg of the coat proteins did not.

Inhibition of cell migration by LS complex

The intense inhibiting activity shown by the surface components led us to test the effect of LS complex on the migration of the cells. The LS complex preparation obtained as indicated in Materials and Methods was subjected to disc electrophoresis on a column of polyacrylamide gel. Two bands were formed in the gel using bromphenol blue as indicator. One tenth microgram (as protein) of LS complex was enough to inhibit the migration of the cells obtained from the mice on the 7th and 9th day after infection (Table 2, Fig. 1), but on the 3rd day after infection the inhibiting activity was rather weak.

Table 2. Effect of LS complex on migration of mouse peritoneal cells

Day after infection	Dose of test antigen (μg)	% inhibition of migration*
3	0.1	40
	1.3	10
	6.5	35
	13	10
7	0.1	96
	1.3	56
	6.5	96
	13	96
9		
	0.1	90.9
	1.3	90.9
	6.5	63.6
	13	54.5

* Control: migration of the sensitized cells in medium containing no LS complex.

It is noticeable that the LS complex exhibited the most intense inhibiting activity among the antigens examined. Considering the fact that LS complex is the constituent of the surface of virion (Cohen and Wilcox, 1968; Williamson, and Rondle 1968), the inhibiting activity manifested by the surface components was suspected to be due to the LS complex contained in them. To separate LS complex from the surface components, the surface components were precipitated with cold ethanol followed by agitation in 0.05 M phosphate buffer, pH 6.3 and the main part of LS complex was dissolved in the supernatant, the other constituents of the components remaining in the residue.

As expected, the inhibition of cell migration by the supernatant was unequivocally positive.

Discussion

The role of delayed hypersensitivity in vaccinia infection has not been satisfactorily explained. Several workers suggested that progressive vaccinia induced by vaccination was closely related to conditions associated with defects of cellular immunity (Kempe, 1960; O'Conell *et al.*, 1964; Gresser and Lang, 1966; Hansson *et al.*, 1966). Also Hirsch *et al.* (1968), using rabbit anti-mouse thymocyte serum, indicated that cell-mediated immune response to vaccinia infection played a protective role. Keeping in mind these facts, studies on delayed hypersensitivity in vaccinia infection may be crucial in considering the problem of protection against the infection or the complications induced by vaccination.

In our investigations we found that LS complex from vaccinia-infected rabbit dermis inhibited most intensely the migration of the cells obtained from vaccinia-infected mice. Regarding the virion, the surface components inhibited more intensely the migration of the cells than the coat proteins or the core did. In addition, PPD tuberculin (Parke, Davis & Company, U.S.A.) did not inhibit the migration of the cells from vaccinia-infected mice.

In our experiment we found no activity of coat proteins in more than 12.4 μg as protein by an in vitro cell migration assay. According to recent investigations, the LS complex occupies an intermediary position on the subsurface and surface of vaccinia virion (Cohen and Wilcox, 1968; Williamson, and Rondle 1968). Thus it may be suggested that the concentration of LS complex contained in the coat proteins is not enought to elicit the inhibiting activity of cell migration. Alternatively, the LS complex may have been lost from our preparation of coat proteins during the fractionating process. Separation of LS complex from the core was not performed.

Our preliminary experiments demonstrated that mice injected subcutaneously with LS complex and incomplete Freund's adjuvant could develop typically delayed hypersensitivity. The migration of peritoneal cells from the sensitized mice was inhibited by LS complex and UV-irradiated vaccinia virus. This coincides with the result that inactivated vaccinia virus can stimulate rabbits to develop delayed hypersensitivity (McNeill, 1966). These results support our suggestion that the LS complex can play an important part responsible for developing delayed hypersensitivity in vaccinia-infected mice.

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