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# THE DEVELOPMENT OF A VIRAL ANTIGEN IN THE HEMOCYTES OF *PIERIS BRASSICAE* INOCULATED WITH *TIPULA* IRIDESCENT VIRUS[1]

*De ontwikkeling van virusantigeen in hemocyten van* Pieris brassicae, *geïnoculeerd met het* Tipula-*regenboogvirus*

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Positive reactions in micro-precipitation tests showed the serological relationship between the *Tipula* iridescent virus (TIV) from *Tipula paludosa* and *Pieris brassicae*. In gel diffusion tests, the results were negative. Applying the fluorescent antibody technique the first fluorescent particles in the proleucocytes and amoebocytes of *P. brassicae* were observed on the third day after infection with TIV. On the seventh day the hemocytes having reached their maximum size, were swollen and completely filled with the viral antigen. In the healthy larvae of the same age, the hemocytes appeared to be normal.

## INTRODUCTION

The *Tipula* iridescent virus (TIV) which occurs originally in *Tipula paludosa* Meig., can multiply in the larvae of *Pieris brassicae* L. (SMITH *et al.*, 1961). Serological studies carried out by STOBBART (in SMITH *et al.*, 1961) showed that the TIV, after passage through *P. brassicae*, was still "practically identical" with the TIV multiplying in *T. paludosa* only.

In the present study the application of the fluorescent antibody technique in detecting the viral antigen in the hemocytes of TIV infected *P. brassicae* was investigated.

## MATERIALS AND METHODS

### *Preparation of antisera*

The TIV from *T. paludosa* and that from *P. brassicae* was kindly supplied in a purified form in water by Mr. C. F. RIVERS (Virus Research Unit, Cambridge, England). Both viruses were centrifuged at 5000 *g* for 20 minutes; the pellet was discarded and the supernatant was subjected to centrifugation by 78,000 *g* for one hour. For preparation of antisera, the pellets were suspended in 0.01 M phosphate buffer at pH 7.

With this virus suspension of *P. brassicae*, one rabbit was given eight intravenous injections and a second one received eight intramuscular injections. A third rabbit was given only one intramuscular injection with the antigen emulsified with Freund's incomplete adjuvant. With the TIV suspension of *T. paludosa*, one rabbit was given two intravenous injections.

To determine the antibody titer, samples of blood were taken every ten days after the last injection; for the titer determination, the micro-precipitation test was performed (VAN SLOGTEREN, 1955). To test the diffusion in agar, gels of 1% agar in distilled water were used.

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*Conjugation*

To precipitate the globulins, half saturated ammon iumsulfate pH 7 was added to the antiserum. After staying over night, the material was centrifuged twice at 5000 *g* for 20 minutes and the sediment was dissolved in phosphate-buffered saline (PBS), i.e. 0.85% NaCl in 0.01 M potassium phosphate, pH 7, and dialyzed against several changes of the same buffer for 72 hours with constant stirring by a magnetic stirrer (SINHA & REDDY, 1964).

The globulins were labeled with fluorescein isothiocyanate (FITC) according to the dialysis technique of CLARK & SHEPARD (1963). After conjugation the labeled globulins were placed to dialyze against PBS for 72 hours in order to remove the excess FITC. Anti-TIV conjugates were then centrifuged at 5000 *g* for 20 minutes and the supernatant stored in the deep freeze.

The conjugation procedure was carried out in a cold room at about 4°C.

*Preparation of smears and staining technique*

Larvae of *P. brassicae* were obtained from eggs of the Cambridge strain kindly supplied by Mr. J. MELTZER (Agrobiological Laboratory Boekensteyn, N.V. Philips-Duphar, 's-Graveland). The larvae were fed with cabbage leaves.

In the fifth instar, directly after ecdysis, the larvae were injected with a 0.01 ml suspension of TIV from *P. brassicae*; the virus was diluted in 0.85% NaCl solution to which 200 γ/ml penicillin and terramicin had been added. No attempt was made to standardize the number of virus particles in the suspension ; the suspension was strongly diluted since, according to RIVERS (personal communication), a high virus concentration quickly kills all larvae, as we found within three days.

All rearing and testing was performed at room temperature, which varied between 10° and 20°C.

On successive days after injection, hemolymph was collected from the larvae by cutting a leg; hemolymph from the pupa was obtained by puncturing with a fine needle. The smears for staining with anti-TIV conjugates were made using the direct method of staining (COONS & KAPLAN, 1950) and following the technique according to SINHA & REDDY (1964). The smears from the untreated larvae were handled identically.

The smears were examined under a Wild (Heerbrugg, Suisse) microscope at × 500; the light source was a Osram mercury vapour lamp (HBO 200). The hemocytes were drawn with a Wild drawing tube and measured.

## RESULTS

*Serology*

All titers of the antisera proved to be the same, viz. 2048 in the homologous as well as in the heterologous reactions. Thus the way in which the antigen had been administered did not make a difference in the titers obtained. All the controls with normal serum were negative.

The gel diffusion tests yielded negative results. After conjugation with FITC, the titer of the specific γ-globulin fraction had decreased to 128 in all cases.

*Fluorescent antibody stained smears*

The results which are summarized in Table 1 show that the hemolymph smears of the larvae, made on the first and second day after infection, were

TABLE 1. Diameter of hemocytes in hemolymph smears from fifth instar larvae of *Pieris brassicae* infected with *Tipula* iridescent virus and those from healthy larvae treated with fluorescent antibodies.

*Diameter van bloedcellen in bloed-uitstrijkpreparaten van rupsen van het vijfde stadium van* Pieris brassicae, *geïnfecteerd met het* Tipula-*regenboogvirus en die van gezonde larven, behandeld met fluorescerende antisera.*

| Number of days after infection | Infected larvae | | | Healthy larvae | | |
|---|---|---|---|---|---|---|
| | Number of larvae with antigen/ Total number of larvae | Diameter in μ | | Number of larvae with antigen/ Total number of larvae | Diameter in μ | |
| | | Proleucocytes $\bar{x}$ $s/\sqrt{n}$ n | Amoebocytes $\bar{x}$ $s/\sqrt{n}$ n | | Proleucocytes $\bar{x}$ $s/\sqrt{n}$ n | Amoebocytes $\bar{x}$ $s/\sqrt{n}$ n |
| 1 | 0/15 | | | 0/12 | 9.15 ± 0.14(30)[1] | 12.71 ± 0.22(15)[1] |
| 2 | 0/17 | | | 0/12 | 8.67 ± 0.24(30) | 12.89 ± 0.18(15) |
| 3 | 7/13 | 9.07 ± 0.21(25)[1] | 13.24 ± 0.18(15)[1] | 0/10 | | |
| 4 | 10/13 | 11.52 ± 0.31(25) | 18.57 ± 0.42(15) | 0/10 | 9.65 ± 0.28(25) | 13.64 ± 0.19(15) |
| 5 | 10/10 | 13.49 ± 0.25(25) | 19.47 ± 0.49(15) | 0/10 | 9.81 ± 0.23(25) | 14.67 ± 0.26(15) |
| 6 | 9/10 | 16.00 ± 0.36(25) | 28.91 ± 0.76(25) | 0/10 | | |
| 7 | 15/18 prepupae | 18.56 ± 0.38(25) | 40.23 ± 1.19(30) | 0/10 prepupae | 9.60 ± 0.36(30) | 17.65 ± 0.43(25) |
| 9 | 8/10 pupae | 22.53 ± 0.55(30) | 43.91 ± 1.04(30) | 0/10 pupae | | |
| 12 | 14/20 pupae | 21.16 ± 0.62(30) | 38.03 ± 0.85(25) | 0/18 pupae | 9.87 ± 0.32(25) | 16.69 ± 0.31(25) |
| *Aantal dagen na infectie* | *Aantal larven met antigeen/ Totale aantal rupsen* | *Proleucocyten* | *Amoebocyten* | *Aantal larven met antigeen/ Totale aantal rupsen* | *Proleucocyten* | *Amoebocyten* |
| | | *Diameter in μ* | | | *Diameter in μ* | |
| | *Geïnfecteerde rupsen* | | | *Gezonde rupsen* | | |

[1] Between brackets number of hemocytes measured
*Tussen haakjes aantal gemeten bloedcellen*

indistinguishable from the healthy controls. On the third day the first virus antigen occurred in the cytoplasm of the proleucocytes and amoebocytes. Then the first brilliant green particles were observed in both types of cells after treatment with the fluorescent antibodies (Fig. 1A, B). With the progressive formation of TIV in the cytoplasm of these hemocytes, the total number of fluorescent particles increased until the cells were completely filled (Fig. 1C–H). As a result of the production of the antigen, the hemocytes increased in size from the fourth day and reached their maximum size on the seventh day. At this time the swollen hemocytes, completely filled with virus, were still intact (Fig. 2A, B) or burst; in the second case the contents of the cell had flowed out (Fig. 2C, D). The above described stages of virus multiplication also were observed in smears from pupae.

In all smears, hemocytes occurred which did not contain any fluorescent particles. However, only those hemocytes were measured in which fluorescent particles occurred and which were representative for that date. In the healthy controls, the proleucocytes and amoebocytes appeared to increase somewhat in size from the fourth day.

## DISCUSSION

Homologous and heterologous micro-precipitation tests showed that the TIV from the original host, *T. paludosa*, and that from *P. brassicae* were serologically identical. In the gel diffusion tests, the results were negative. These results were only partly in agreement with those of STOBBART (in SMITH *et al.*, 1961), as he obtained positive reactions not only in precipitation tests but also in gel diffusion tests. An explanation for the fact that we did not get positive results in gel diffusion is difficult to give because SMITH *et al.* (1961) did not mention sufficient data on the procedure they used.

In the hemolymph of *P. brassicae*, LARTSCHENKO (1933) distinguished two types of hemocytes, namely the proleucocytes and amoebocytes; the former occurred in great numbers having relatively large nuclei and small amounts of cytoplasm. The latter were bigger than the proleucocytes and could be distinguished from them by a small nucleus. Under the UV microscope, both types of hemocytes were clearly seen.

XEROS (1954, 1964) and SMITH *et al.* (1961) showed, with the light and electron microscope, that the TIV develops in the cytoplasm of the fat body, muscles and epidermis of several examined insects; as a result of this multiplication, the cells and nucleoli enlarge while the nuclei remain normal. In the present investigation the first small centres of antigen multiplication were observed in the cytoplasm of the proleucocytes and amoebocytes from *P. brassicae* on the third day after infection with TIV at room temperature. KRYWIENCZYK (1963) showed the first fluorescent particles in the cytoplasm of the fat body four days after infection of *Bombyx mori* L. with the nuclear polyhedral virus. The *Sericesthis* iridescent virus in cell cultures from *Antheraea eucalypti* Scott was first seen about two days after infection at 25° or three days at 21 °C (BELLETT & MERCER, 1964).

With the progressive formation of antigen in the cytoplasm, the hemocytes enlarge gradually to reach their maximum size after seven days and then they burst. The number of infected hemocytes increases in the course of the infection process; however even after seven days a number of hemocytes without suffi-

cient antigen for fluorescence could be found. The majority of the 600 infected larvae died between the seventh and ninth day as prepupae; the rest pupated, some of which died. The eight butterflies obtained were not investigated for the occurrence of viral antigen in the hemocytes.

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SAMENVATTING

Homologe en heterologe microprecipitatieproeven hebben aangetoond, dat het *Tipula*-regenboogvirus (TIV) van de originele waard, *Tipula paludosa*, serologisch verwant is met het TIV, dat door *Pieris brassicae* is gepasseerd. In de gel-diffusieproeven waren de resultaten negatief.

Met behulp van de fluorescentie-techniek, waarbij antilichamen tegen het TIV zijn gemerkt met de kleurstof fluoresceïne-isothiocyanaat, kon het verloop van de virusvermeerdering in de bloedcellen van *P. brassicae* worden gevolgd. Daartoe werden rupsen van *P. brassicae* in het vijfde stadium geïnjicieerd met een suspensie van het TIV. Verschillende dagen na de infectie werden van deze dieren bloeduitstrijken gemaakt, die met het geprepareerde antiserum werden behandeld. Onder de UV-microscoop werd op de derde dag na de infectie het eerste virusantigeen in het cytoplasma van de bloedcellen waargenomen (tabel 1, fig. 1A, B). Naar gelang de ziekte voortschreed, nam de hoeveelheid antigeen in het cytoplasma toe als gevolg van de virusvermeerdering en werden de bloedcellen steeds groter (fig. 1C–H). Op de zevende dag hadden de bloedcellen hun maximale grootte bereikt en waren ze nog intact (fig. 2A, B) of gebarsten, waarbij de inhoud naar buiten was gestroomd (fig. 2C, D). Het merendeel van de ongeveer 600 geïnfecteerde rupsen stierf tussen de zevende en negende dag als voorpop. Van de verpopte larven stierven er enkele. De acht uitgekomen vlinders zijn niet onderzocht op de aanwezigheid van virusantigeen in de bloedcellen.

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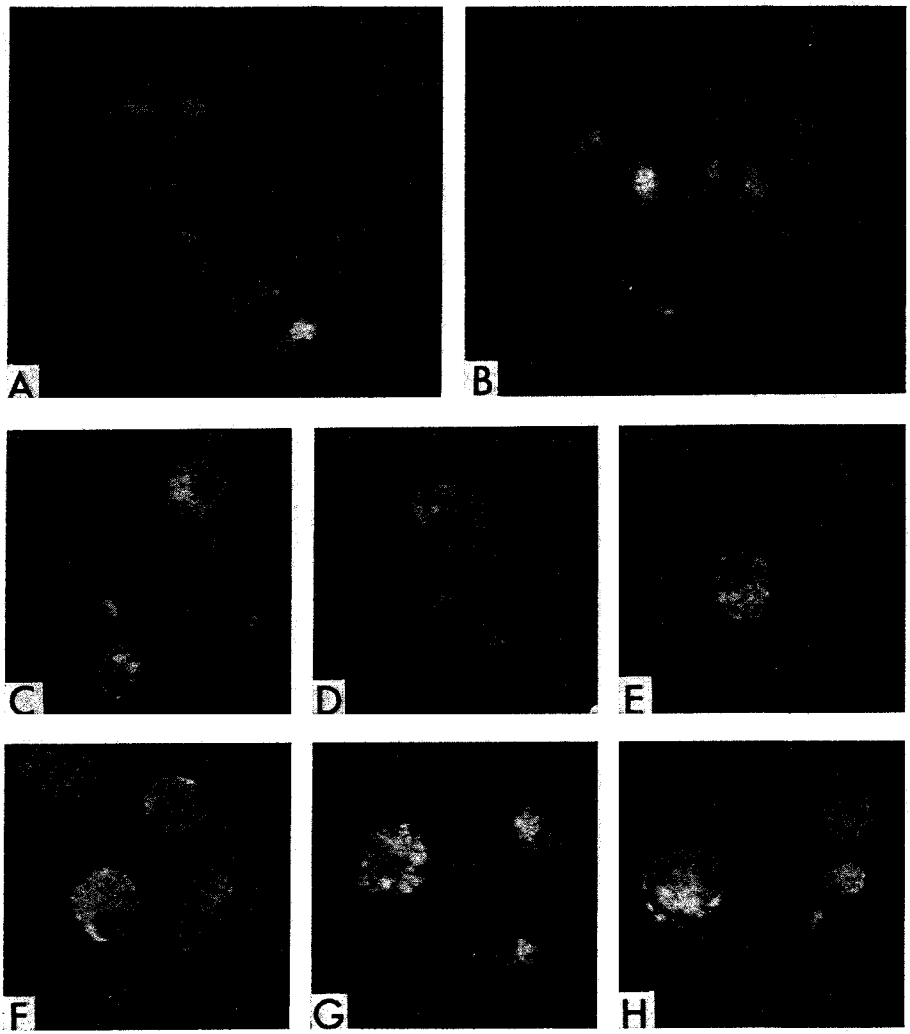


FIG. 1. Fluorescent-antibody stained *Tipula* iridescent virus antigen in hemocytes of *Pieris brassicae*. × 620. A–B. The first centres of fluorescence in the proleucocytes and amoebocytes on the third day after infection. C–E. Four days after infection; some hemocytes are completely filled with the antigen. F–H. Five days after infection; the completely filled hemocytes with the antigen are already bigger than those from the third day.

*Met fluorescerende antilichamen gekleurd* Tipula-*regenboogvirusantigeen in bloedcellen van* Pieris brassicae. *620* ×. A–B. *De eerste centra van fluorescentie in de proleucocyten en amoebocyten op de derde dag na infectie.* C–E. *Vier dagen na infectie; enkele bloedcellen zijn geheel gevuld met het antigeen.* F–H–. *Vijf dagen na infectie; de geheel gevulde bloedcellen met het antigeen zijn reeds groter dan die van de derde dag.*

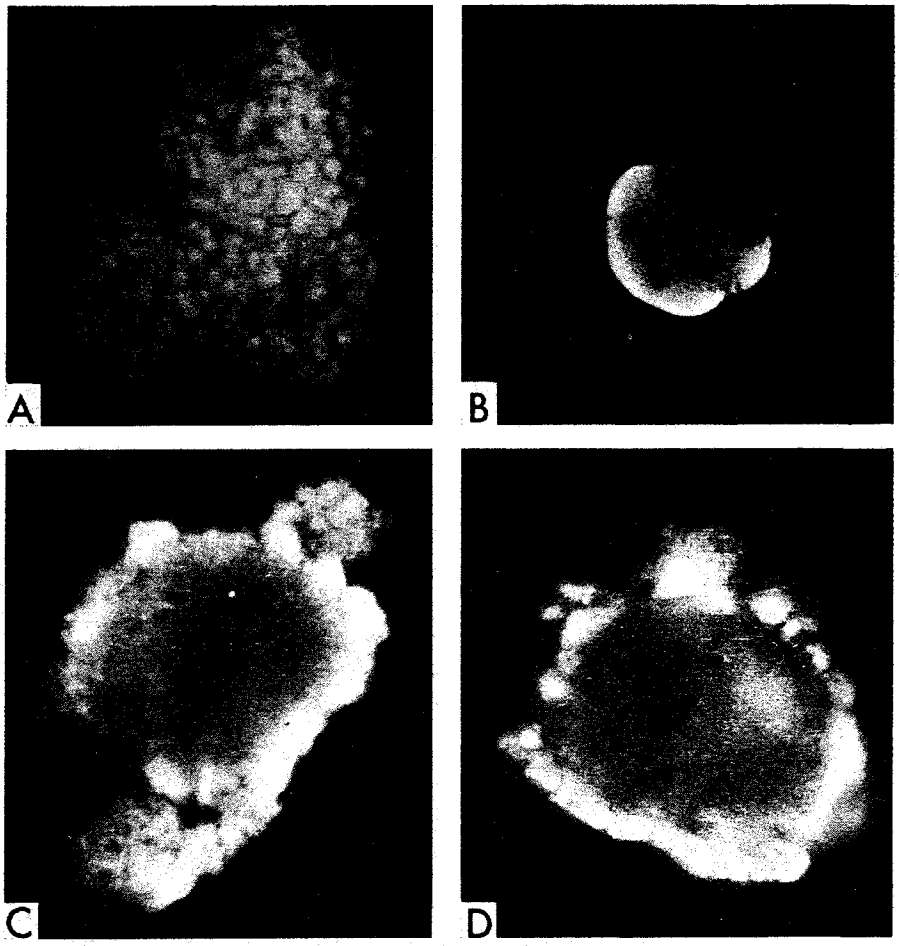


FIG. 2. Fluorescent-antibody stained *Tipula* iridescent virus antigen in the hemocytes of *Pieris brassicae* seven days after infection. × 620. A. Three intact amoebocytes which are swollen by the multiplication of the virus. B. A swollen intact proleucocyte. C–D. Burst amoebocytes of which the contents has flowed out.

*Met fluorescerende antilichamen gekleurd* Tipula-*regenboogvirusantigeen in de bloedcellen van* Pieris brassicae *zeven dagen na infectie. 620 ×. A Drie intacte amoebocyten, die als gevolg van de virusvermeerdering zijn opgezwollen. B. Een opgezwollen, intacte proleucocyt. C–D. Opengebarsten amoebocyten, waarvan de inhoud naar buiten is gelopen.*