The Preparation of 'Monoprecipitin' Antisera to Herpes Virus Specific Antigens

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SUMMARY

Injection of single precipitin bands (from immunodiffusion tests) into the lymph nodes of rabbits produced antisera reacting in immunodiffusion tests with individual specific antigens of herpes simplex virus. Antisera were made to an antigen obtained by preparative electrophoresis of an extract of cells infected with herpes; neutralization of the infectivity of herpes virus was demonstrated. An antiserum was also prepared to the antigen common to extracts of cells infected with herpes and pseudorabies virus; it contained no neutralizing antibody.

INTRODUCTION

Morphological studies on the site of formation of virus-specific antigens by fluorescence microscopy (Ross, Watson & Wildy, 1968) and kinetic studies on their appearance during the growth cycle are hampered by the polyvalency of the antisera (Watson et al. 1966). Accordingly further progress in the studies demands the production of antisera specific for individual antigens. Smith, Gallop & Tozer (1964) showed that rabbits immunized with individual precipitin bands taken from immunodiffusion gels produced antibodies specific for the antigens present in the bands. Goudie, Horne & Wilkinson (1966) produced antibodies by a similar method using far smaller quantities of antigen + antibody precipitate by inoculation of the popliteal lymph nodes of the rabbit. However, since it appears that crude extracts of infected cells contain aggregates of specific antigen (Watson, 1968) we thought that inoculation of bands derived from such extracts might give a response to several antigens. We decided, therefore, to inoculate precipitin bands obtained from fractions obtained by polyacrylamide-gel electrophoresis of the extracts (Watson, 1968). Such fractions contain the antigens in the form of much smaller molecules which were less likely to be aggregated.

Watson et al. (1967) showed that extracts of cells infected with pseudorabies virus give a precipitin band with antisera to herpes-infected cells and a similar antigen is also found in cells infected with herpes B virus. Reciprocal antisera so far prepared against pseudorabies-infected cells do not react with extracts of herpes-infected cells. Since it would be useful to have an antiserum specific for the herpes group-specific antigen in cells infected with herpes simplex virus, we decided to attempt to prepare it by lymph-node inoculation of the precipitin band produced by the reaction of extracts of pseudorabies-infected cells with antisera to herpes-infected cells. Although, in accord with our previous reservations, it seemed likely that such precipitins might contain aggregates of several pseudorabies-specific antigens, we thought that only one of them might be present also in herpes-infected cells.
METHODS

Preparation of antigens. By preparative polyacrylamide electrophoresis we obtained a fraction giving a single precipitin band with antiserum to herpes-infected RK 13 cells (Watson, 1969). The particular fraction selected was one giving the 'Band II' antigenic reaction shown in Pl. 2b of the preceding paper. An extract of RK 13 cells infected with pseudorabies virus was made in an analogous manner to the herpes-infected cell extract (Watson, 1969). This extract gave one precipitin band with antiserum to herpes-infected RK 13 cells (Watson et al. 1967).

Preparation of single precipitin bands. The above antigens were set up in immunodiffusion reactions in 1% (w/v) 'Ionagar' (Oxoid Ltd) in isotonic saline containing 0.1% sodium azide. Antigen was placed in a central well 8 mm. in diameter separated by 3 mm. gel from a hexagonal pattern of six peripheral wells (also 8 mm. in diameter) into which antiserum of allotypic specificity As 1/4 was placed. After allowing the precipitin band to develop for 72 hr in a moist chamber at room temperature the plates were carefully scrutinized and any showing traces of a second line discarded. Several more immunodiffusion plates were then set up for antigen batches giving a satisfactory single precipitin band in the preliminary test. The precipitin bands were allowed to develop as before. If any one plate of a batch showed more than one precipitin band the batch was discarded. Single bands were excised from the plate in the minimum volume of agar using a fine scalpel blade. The gel fragments from a batch were transferred to screw-capped bottles containing 20 ml. phosphate buffered saline (Dulbecco & Vogt, 1954) in which they were gently shaken for at least a week at 4°, the buffer being changed daily. The gel fragments were stored, when necessary, at -20° after removal of excess buffer.

Immunization of rabbits with single precipitin bands. The agar fragments containing single precipitin bands prepared from two or three gel plates were pulverized in an equal volume (about 0.1 ml.) of Freund's incomplete adjuvant by pushing them back and forward between two connected 1 ml. syringes. The pulverized gel extract was drawn back into one syringe and about 0.15 to 0.2 ml. injected into an exposed popliteal lymph node. Rabbits for inoculation were selected to avoid the possibility of an allotypic response to the antibody in the immune aggregate. Two alternative schedules of immunization were employed after the initial inoculation. In the first, a second inoculation was made in the contralateral popliteal lymph node 6 weeks later. The rabbits were bled 10, 11 and 24 days after the second inoculation and sera from the first two bleeds were pooled. In the second method, agar fragments from three plates were pulverized in 1 ml. saline. About 0.5 ml. was inoculated intravenously in an ear vein and 0.25 ml. volumes were inoculated subcutaneously into each front leg. Two or three inoculations of this type were made at fortnightly intervals after the lymph-node inoculation. Rabbits were bled 10 days after the last inoculation. Sera from both methods were sometimes concentrated to about one quarter of their volume by ultrafiltration through 3/8 in. 'Visking' tubing.

Test for neutralizing antibody. A sample of herpes virus containing $5 \times 10^4$ p.f.u./ml. was mixed with an equal volume of antiserum either undiluted or diluted in phosphate buffered saline. After 60 min. at room temperature 1 ml. samples of 1/100 and 1/300 dilutions were assayed for residual infectivity by the suspension-plaque method of Russell (1962). Parallel control samples were incubated with corresponding dilutions
Comparison in immunodiffusion test of monoprecipitin antiserum (no. 226) to Band II with 'poly-
precipitin' antiserum to herpes-infected RK 13 cells diluted 1/5 (A/5) and 1/10 (A/10). The three
sera are tested against an electrophoretic fraction containing Band II (II) and against an unfractio-
ated extract of herpes infected BHK 21 cells (H). There is almost no reaction between A/10 and II and the
reaction of II with A/5 is comparable although weaker than that of monoprecipitin antiserum no. 226.
With unfractioinated cell extract (H) antiserum no. 226 again gives no line, but the dilutions of A
give multiple bands although once again there is little reaction between A/10 and Band II antigen.
This plate shows that the more selective response of the monoprecipitin antiserum cannot be accounted
for by a reduced general response to all the antigens in infected cells.
'Monoprecipitin' antisera to herpes antigens

of pre-immunization sera and similarly assayed after 60 min. at room temperature. All sera were inactivated at 56°C for 30 min. before use. The neutralization constant, \( k \), of an antiserum is defined as

\[
k = \frac{1}{ct} \ln \frac{v_0}{v} = \frac{2.303}{ct} \log \frac{v_0}{v},
\]

where \( c = (\text{volume antiserum in test mixture})/(\text{total volume test mixture}) \), \( t = \text{time (min.) of neutralization test} \), \( v, v_0 = \text{residual infectivities of mixtures with antiserum and pre-immunization serum after 60 min.} \)

RESULTS

Antisera to 'Band II' antigen

Three rabbits were inoculated with single precipitin bands of 'Band II' antigen. Two (serial numbers 172, 226) were immunized according to the first schedule and one (serial number 157) according to the second. Sera from all three rabbits produced a single precipitin line in immunodiffusion tests with extracts of infected cells, although two of the antisera (157, 172) only reacted after concentration. Serum from rabbit no. 226 reacted in the unconcentrated form (Pl. 1). Antiserum from the bleed 24 days after the second inoculation of this rabbit reacted as well as that from the 10 to 11-day bleed. The antisera gave no reaction in immunodiffusion tests with extracts of uninfected cells or with components of the culture media. In tests with fractions from preparative electrophoresis of infected-cell extracts they only reacted with fractions containing the homologous antigen. Thus, in the electrophoresis run illustrated in Fig. 2 of the preceding paper, 'Band II' was detected in fractions 10 to 15 and only these fractions reacted with the antisera.

To describe these antisera as 'monospecific' would imply that they contain antibodies to no antigens other than 'Band II'. Since we could not demonstrate this conclusively we prefer to describe them as 'monoprecipitin' antisera since they gave single precipitin bands in immunodiffusion tests.

The performance of dilutions of the antisera in immunodiffusion tests against electrophoretic fractions containing 'Band II' antigen was compared with that of dilutions of the routine 'polyprecipitin' antiserum to herpes-infected RK 13 cells; all the monoprecipitin antisera had a lower concentration of antibody to 'Band II' than the polyprecipitin antiserum. The \( \times 4 \) concentrates of sera 157 and 172 were roughly equivalent to \( 1/10 \) dilutions of the polyprecipitin antiserum while the unconcentrated serum no. 226 was roughly equivalent to a \( 1/4 \) dilution. That is, serum no. 226 contained about 10 times as much antibody as nos. 157 and 172 before concentration.

A similar single-line response could not be achieved in immunodiffusion tests by corresponding dilutions of the polyprecipitin antiserum. Plate 1 shows an immunodiffusion test in which the performance of monoprecipitin serum no. 226 was compared with that of \( 1/5 \) and \( 1/10 \) dilutions of polyprecipitin antiserum. The reaction of the three antisera with a fraction containing 'Band II' showed that the \( 1/10 \) dilution of polyprecipitin antiserum gave almost no reaction, while the \( 1/5 \) dilution gave a reaction comparable to but weaker than that of monoprecipitin antiserum no. 226. The reaction of the same three antisera with unfractionated cell extract showed that monoprecipitin antiserum gave only one precipitin band, but the dilutions of poly-
precipitin antiserum gave multiple lines. The pattern was consistent with the above conclusion that the 1/10 dilution gave virtually no reaction with ‘Band II’. These results confirmed that the performance of the monoprecipitin antisera could not be ascribed simply to a reduced general response to several antigens. A definite conclusion could not be reached about the comparative merits of the two types of immunization.

**Antiserum to the shared specific antigen**

Two rabbits were inoculated with complexes of the antigen shared between herpes simplex and pseudorabies viruses. The first (no. 165) received one lymph node inoculation and five intravenous + subcutaneous inoculations and the second (no. 234) received two lymph node inoculations. Concentrates of antiserum from no. 165 gave one precipitin line in immunodiffusion tests with extracts of cells infected with herpes virus, while no. 234 gave no reaction even after concentration. Concentrated antiserum from rabbit no. 165 after the first boosting inoculation gave no reaction, so once again no conclusions could be drawn about the relative efficiencies of the two methods. This antiserum reacted in immunodiffusion with an antigen other than ‘Band II’. It gave multiple precipitin bands with extracts of cells infected with pseudorabies virus.

**Table 1. Neutralizing antibody in monoprecipitin antisera**

<table>
<thead>
<tr>
<th>Antiserum no.</th>
<th>With unconcentrated antiserum</th>
<th>With concentrated antiserum</th>
<th>Dilution of antiserum*</th>
<th>Residual infectivity (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>157</td>
<td>None</td>
<td>Anti-Band II</td>
<td>1/2</td>
<td>6</td>
</tr>
<tr>
<td>172</td>
<td>None</td>
<td>Anti-Band II</td>
<td>1/2, 1/4, 1/6</td>
<td>4, 25, 55</td>
</tr>
<tr>
<td>226</td>
<td>Anti-Band II</td>
<td>Not tested</td>
<td>1/6, 1/20, 1/60</td>
<td>0.2, 25, 78</td>
</tr>
<tr>
<td>165</td>
<td>None</td>
<td>Anti-shared-antigen</td>
<td>1/2</td>
<td>103</td>
</tr>
</tbody>
</table>

* Dilution expressed as final dilution of antiserum in virus–antiserum mixture used in neutralization test.
† Residual infectivity of the virus–antiserum mixture is expressed as a percentage of the infectivity of a mixture with pre-immunization serum (at a corresponding dilution) after incubation for the same period (60 min.). The values of the neutralization constant $k$ shown for nos 172 and 226 represent the mean of the values determined for each serum for each of the three dilutions tested.

**Tests for neutralizing antibody to herpes virus**

Table 1 shows the surviving virus infectivity after incubation of herpes simplex virus with various dilutions of monoprecipitin antisera as a percentage of the infectivity found in the control preparation incubated with pre-immune serum. Whereas the antisera to ‘Band II’ antigen plainly contain neutralizing antibody, none can be detected in the antiserum to the shared antigen. Further, the performance of antisera to ‘Band II’ antigen in the neutralization test parallels their performance in immunodiffusion; antiserum no. 226 is once again superior to nos 157 and 172. The mean values of the neutralization constants $k$ of nos 172 and 226 are 0.09 and 0.45; 226 thus
contains 5 times as much antibody, in good agreement with the value of 10 obtained in the immunodiffusion dilution tests. (The nature of the latter was such that a twofold error in the ratio was well within the limits of error of the test.)

**DISCUSSION**

The method described here has been remarkably successful in preparing antisera of increased specificity. The immunodiffusion provides, in effect, an additional stage of purification of the antigen before inoculation. We were successful in all three attempts with 'Band II' antigen by this method but this may, of course, reflect the properties of this particular antigen. Thus Goudie, Horne & Wilkinson (1966) were similarly successful with certain human immunoglobulins but were unsuccessful with horse ferritin. Preliminary experiments suggest less success with precipitin bands from electrophoretic fractions other than 'Band II' and we succeeded with only one rabbit out of two inoculated with complexes containing 'shared' antigen. It is, however, difficult with such small samples to assess the effect of variations in the response of individual animals such as were apparent between two rabbits each inoculated with 'Band II' antigen + antibody complex.

As anticipated in the Introduction, the antiserum prepared against 'shared' antigen gave a multiple precipitin pattern with extracts of cells infected with pseudorabies virus. This observation emphasizes the value of using precipitin bands prepared from polyacrylamide electrophoretic fractions.

It is our intention to re-immunize the rabbits with precipitin bands formed between the appropriate fraction and the 'monoprecipitin' antiserum already produced from the rabbit.

As yet we can say little about the properties of the antigens which react with these antisera. 'Band II' antigen seems to be a structural antigen on the exterior of the virus particles since it is apparently involved in neutralization of virus infectivity. However, it will be necessary to show that antisera to other antigens do not neutralize virus infectivity in order to confirm that the neutralizing power of monoprecipitin antisera to 'Band II' is related to the 'Band II' antigen and not to contamination by another component. Nevertheless, neutralizing activity of the antisera seemed to parallel their anti-'Band II' activity in immunodiffusion, so any contamination is not random. The absence of neutralizing antibody in the antiserum against the shared antigen is consistent with the absence of cross-neutralization between herpes simplex and pseudorabies viruses (Watson *et al.* 1967).

'Band II' antigen has a relative mobility in 7% polyacrylamide of 70 (albumin = 100), which suggests a fairly small protein if this extrapolation is justified. Electron micrographs of negatively stained 'Band II' fractions show 80 to 100 Å spherical particles which are larger than suggested by the above mobility data. However, even if there were no aggregation during specimen preparation, the observed particles may only represent the residue of host protein. None of these particles showed the 40 Å central 'hole' characteristic of the capsomeres of intact virus particles (Wildy, Russell & Horne, 1960).

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REFERENCES


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